Food Safety: ARS National Program 108 Retrospective Review: Accomplishment Report: 2016-2020 Action Plan

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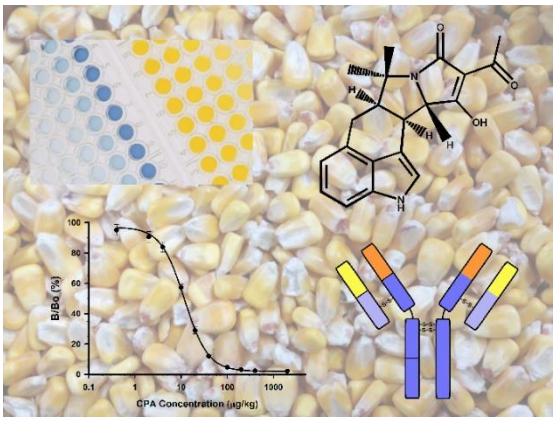

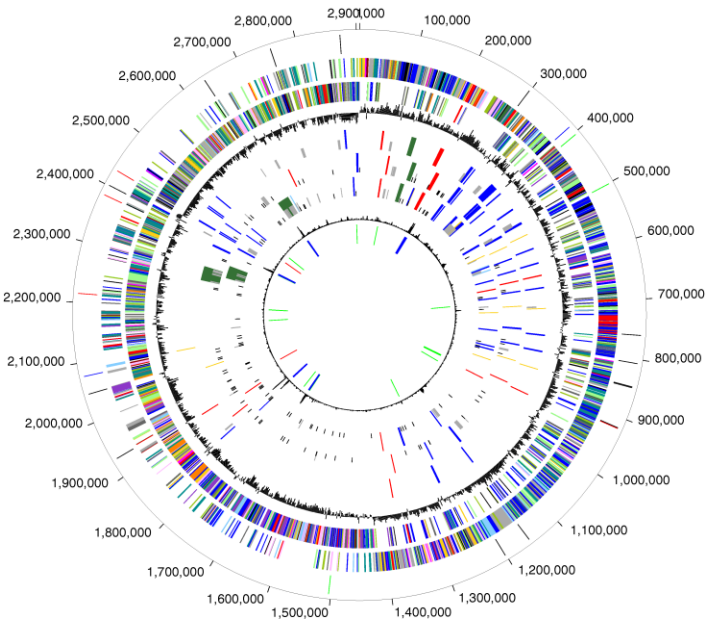

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This Report was compiled in-part from material provided by Program scientists, and from documents within the Office of National Programs including: Action Plans, Project Plans, Annual Reports, and technology transfer and budget office databases. To all those who contributed our graciously expressed thanks. Special thanks go to Nadine Kessler our Program PA, for all her invaluable assistance.

Executive Summary

Background

In the Executive Summary for the 2011-2015 Food Safety Program Retrospective Review and the 2016-2020 Food Safety Program Action Plan, it was noted that the United States (U.S.) Administration had emphasized its commitment to change by overhauling the U.S. food safety system and moving towards more preventative regulatory strategies. The formation of the President's Food Safety Working Group (FSWG) and the development and implementation of the FDA-Food Safety Modernization Act (FSMA) and its various components, were underway, and were concrete examples of initiatives that were expected to be cornerstones of these changes, of which ARS was to play a critical part.

Thus, expectations for the 2016-2020 Agricultural Research Service (ARS) Food Safety Program were high. FSMA was implemented by the Food and Drug Administration (FDA), despite the fact that some components were still under revision, for example, the Produce Safety Rule. Closer relationships were developed between the U.S. Department of Agriculture (USDA) agencies (ARS, Food Safety Inspection Service (FSIS)) and with the Department of Health and Human Services (DHHS) (FDA, Centers for Disease Control (CDC)) at the research level. However, sadly and unfortunately other Administration initiatives fell by the wayside such as the FSWG, whose goal was to coordinate federal food safety efforts. The disbanding of the FSWG was strongly noted with deep concern in the recent U.S. Government Accountability Office (GAO) [GAO-19-157SP High-Risk Series Report].

A positive outcome was the "Formal Agreement between USDA and FDA Relative to Cooperation and Coordination" signed by the USDA Secretary and the FDA Commissioner on Jan 30, 2018. Produce safety is one of the focus areas/issues of shared concern. https://www.fda.gov/food/domestic-interagencyagreements-food/formal-agreement-between-usda-and-fda-relative-cooperation-andcoordination

This loss in coordination efforts, that is the ability *"to participate in a centralized, collaborative mechanism on food safety"* (GAO) was and is difficult to understand. The safety of the food (and feed) supply continues to be of critical concern to certain sectors of government (for example, USDA, DHHS) and yet almost no effort has been made by Administrations to eliminate/or alleviate these concerns. Thus*, "food safety and the safety of the food supply still remain highly visible agriculture and public health priorities, now for more than four decades"*. [pp. 195-197: GAO-19-157SP High-Risk Series].

Rationale

Foodborne outbreaks and consequential illnesses are a major cause of morbidity and mortality, as well as economic devastation (including trade), both nationally and internationally. The data provided by the CDC indicates that in the U.S. as a result of foodborne illness (FBI), about 1 in 6 Americans (~48 million) people get sick annually, ~128,000 are hospitalized and ~3,000 die. Cost estimates (in 2014) by the USDA Economic Research Service (ERS) are in excess of $15.6 billion.

Although high, these data are likely an underestimate since they only account for a limited number and type of microorganisms, and not unknown agents, biological toxins, or chemical contaminants/residues (https://www.cdc.gov/foodborneburden/2011-methods.html.) The CDC data also do not indicate the long-term sequelae of any illnesses and their public health impact, which are likely more extensive. For example, illnesses such as rheumatoid disease, autoimmune thyroid disease, inflammatory bowel disease, autoimmunity, renal disease, neural/ neuromuscular diseases, organ impairment, heart/vascular diseases, nutritional/ gastrointestinal disorders, and personality changes or behavioral disorders. The cost/burden of these illnesses remains unknown, and likely inestimable since most are lifelong.

Contrary to the CDC, other (academic research) data suggests at the national level (the U.S.), there are 76+ million illnesses at a potential cost of $150+ billion. Internationally (world-wide), the incidence of FBI is estimated at 600+ million cases. Thus (1 in 10) people get FBI. Incidents for example are: ~96 million cases of campylobacterosis and ~80 million cases of salmonellosis. The cost (burden) worldwide is incalculable.

Regardless of which data are used for public health policy, these are staggering numbers which cannot be sustained. What can be consistently said, and is of concern both nationally and internationally, is that multi-state and multi-country outbreaks are increasing, and are thus, affecting greater numbers of people. Regrettably, the cause of many of the increased outbreaks remain unresolved. Fortunately however, with the recent implementation and use of culture-independent diagnostic tests (CIDTS) and whole genome sequencing (WGS) by U.S. regulatory and public health agencies, there is now a real potential for faster and greater resolution of foodborne illness events.

Continuing Issues

Food safety is and must continue to be seen as a continuum, not a linear process. It is the intertwining of factors associated with food animals, plants, the environment and humans, that creates opportunities for contamination through infection, intoxication or transmission, by bacterial, viral, and parasitic pathogens; and chemical contaminants, residues, or toxins. Within this continuum there are additional research issues of concern: for example, intensive food production, the globalization of the food supply and resulting international trade, climate change, food integrity (adulteration), changes in pathogenicity, changes in consumption habits, travel, and immigration of people. ARS food safety research must continue to be conducted and evolve, to address many of these issues, especially through the development of improved technologies; being cognizant and pragmatic of the challenges because of the complexity of the production, processing and distribution processes.

Food Safety Program (NP108)

Two decades ago ARS began the Office of Scientific Quality Research (OSQR) and implemented the external Program and project review process. Since that time (year 2000) each of the ARS Food Safety Program's 5-year [Strategic] Action Plans has been considered a progressive step toward the ultimate goal of providing the necessary technological tools (in the broadest sense) to increase the safety of the food [and feed] supply. As technologies advanced, the data improved

and increased in complexity. Thus, each new 5-year cycle provided further opportunity to formulate more complex hypotheses and study new areas of research such as: population and systems biology; development, validation and subsequent implementation of more rapid, sensitive and quantifiable detection methods; development, evaluation and subsequent implementation of better intervention and control strategies; predictive microbiology and modeling; genomics; metagenomics and microbiome analyses; and antimicrobial resistance.

The ARS Food Safety Program continued to strive to provide data for policy change (regulations/guidelines), and to develop and validate new technologies that could be utilized by regulatory, public health, and/or defense agencies, and industry. The ultimate goal is to ensure the safety of the food supply for the American consumer. Nonetheless, to be successfully conducted, scientific research requires a balance between the pursuit of an individual hypothesis and studies on a problem that will have outcomes and impact. This was and still is critically important in times of challenging fiscal and personnel resources, albeit very apparent during the past decade.

Program Funding Status

With regards to ARS, over time there has been a reduction in actual research funds for the Program for a variety of reasons, including budget cut-backs due to sequestration. In 2005 the Food Safety Research Program had 77 appropriated funded projects, compared with 51 projects in 2019. For the next round of OSQR under the 2021-2025 Action Plan we anticipate 43 projects. Regretfully, the Program cannot sustain such reductions and losses, and will be refocused to address issues/needs of high priority.

2016-2020 Action Plan

In developing the 2016-2020 Action Plan; ongoing research activities and technology developments were reviewed, and input was gained from a variety of internal and external sources. Foremost was that the Food Safety Research Program ensured the continued expertise and infrastructure to respond to changing issues in food safety; for agriculture, public health, and the Federal system.

As this Report demonstrates, many of the projected food safety research issues did emerge, and ARS was able to develop new technologies and provide more and better data to answer them. At the same time [we] also recognized that some food safety events would not be foreseen and so one of our goals was to create expertise and flexibility to respond to unforeseen events.

During the first ~4 years of the 2016-2020 research cycle, the Food Safety Program realized many accomplishments. Examples of these accomplishments, their outcomes and impact(s) are reported in the following review document. For a detailed list of accomplishments by year, please refer to the Food Safety Program Annual Reports which are available on the web at: https://www.ars.usda.gov/nutrition-food-safetyquality/food-safety-animal-and-plant-products/docs/annual-reports/

One of the strengths, evident from this report, is the Programs expertise in developing and validating various "technologies". This strength is being 'tapped' to assist in many difficult challenges, that is, the rapid detection and unequivocal characterization of pathogens, chemical residues and biological toxins; the development and validation of intervention technologies; pathogen genomics; pathogen modeling and databases; and antimicrobial resistance. These technologies are not only important to the science of food safety but are critical for agriculture and public health regulatory agencies, producers, and industry.

There were many outstanding accomplishments with major impact, while the impact of others is still being realized. There were also some major disappointments, some-in-part due to mitigating circumstances: for example, loss of critical personnel (mainly retirements/ resignations), lack of continuing and/or new funds (sequestration), changes in research directions which required realignments, or combining project objectives; time expended for retraining, and the Federal Government Shutdown, most recently in 2019 for 35 days.

Scientific Outcome

Despite the external stresses on the Program, so far, the Program has attained/completed 96.1% of its predicted milestones [as officially reported to the Office of Management and Budget (OMB)]. If [we] exclude the mitigating circumstances of the Federal shutdown, loss of staff including retirements, resignations, staff deaths, critical vacancies, insufficient resources, redirections and milestones no longer applicable, realignments; then the milestone accomplishment score increases to 98.3%. Attaining 90% is considered the maximum attainable score by the Office of Management and Budget (OMB) since there is an acceptance that there will always be circumstances that preclude attaining 100%.

In conclusion, the Program expects to complete most of its predicted milestones for the next Program year, even under the current constraints. Scientifically, the future will bring a variety of additional challenges. Some will be new, others will be in response to external influences, such as trade, new food types, new guidelines/regulations, emerging pathogens and antimicrobial resistance, climate change, the implementation of new technologies, or from unexpected events inexplicable at the time they occur. These challenges will have to be addressed and can provide the basis for developing the 2021-2025 Program Action Plan. However, the overarching goal for this National Program is the continued fundamental need for systematic and multidisciplinary [integrated] approaches to food safety research. That is, food safety is truly a continuum; and events (either positive or negative), no matter where they occur have significant consequences that affect agricultural sustainability and public health.

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Program Review

Background

National Program for Food Safety (NP 108) is one of 15 National Programs (NP) within the USDA-Agricultural Research Service (ARS) Office of National Programs (ONP). The National Programs are organized within four broad Program areas: Nutrition, Food Safety and Quality (NFSQ); Animal Production and Protection (APP); Natural Resources and Sustainable Agricultural Systems (NRSAS); and Crop Production and Protection (CPP). http://www.ars.usda.gov/research/programs.htm

The Food Safety National Program is part of NFSQ. Significant collaborations and/or interactions occur between researchers in food safety, both nationally within the Federal Government and academia; internationally with nearly 60 different countries; and between other National Programs, in particular (NP107) Human Nutrition, (NP306) Product Quality and New Uses, (NP103) Animal Health, (NP106) Aquaculture, and (NP216) Sustainable Agricultural Systems. The National Program structure allows ARS scientists to collaborate with researchers regionally, nationally or internationally to address food safety issues, needs or concerns.

Current Relationship of this National Program to the Department and Agency Strategic Plans and Goals:

As of 2018, NP108 is under USDA Strategic Goal 7. Provide All Americans access to a Safe, Nutritious, and Secure Food Supply.
https://www.usda.gov/sites/default/files/documents/usda-strategic-plan-2018-2022.pdf

As of 2018, NP108 is under Research, Education and Economics (REE) Goal 5, Food Safety. Currently the REE Strategic Plan is being reconsidered under five (5) new "Strategic Themes". NP108 Food Safety will be combined with Human Nutrition under a new Theme entitled "Food and Nutrition Translation", and REE focus area "Food Safety and Health Promotion."

Currently, NP108 is under ARS Goal Area 1: Nutrition, Food Safety, and Quality; Goal 1.2 Protect Food from Pathogens, Toxins, and Chemical Contamination during Production, Processing, and Preparation.

Performance Measure 1. Develop new technologies that assist ARS customers in detecting, identifying, and controlling foodborne diseases associated with the consumption of animal products that affect human health.

https://www.ars.usda.gov/ARSUserFiles/00000000/NPS/OAA/Annual%20Report%20on%20Science/ARS%20Annual%20Report%20on%20Science%20FY%202018.pdf

2016-2020 Action Plan for NP108 as Implemented in Fiscal Year 2016

Mission Statement:
To provide through research, the means to ensure that the food supply is safe for consumers; and that food and feed meet foreign and domestic regulatory requirements. Food safety research seeks ways to assess, control or eliminate potentially harmful food contaminants, including both introduced and naturally occurring pathogenic bacteria, viruses and parasites, toxins and non-biological-based chemical contaminants, mycotoxins and plant toxins. Food safety is a global issue; thus, the research program involves both national and international collaborations through formal and informal partnerships. Accomplishments and outcomes are utilized in national and international strategies delivering research results and advances to regulatory agencies, commodity organizations, industry and consumers.

Vision Statement:
To enhance and protect public health and agriculture through the development of technologies, strategies, and data that safeguard food from pathogens, toxins, and chemical contaminants during production, processing, and preparation, thus increasing the safety of the food supply.

Action Plan:

Goal: National Program (NP) 108, Food Safety through research, and in collaboration with regulatory agencies, industry, academia and other stakeholder and partners, provides the means to ensure that the food supply is safe for consumers and that food and feed meet foreign and domestic regulatory requirements. Food safety research seeks ways to assess, control or eliminate potentially harmful food contaminants, including both introduced and naturally occurring pathogenic bacteria, viruses and parasites, toxins and non-biological-based chemical contaminants, mycotoxins and plant toxins. Food safety is a global issue; thus, the research program involves both national and international collaborations through formal and informal partnerships. Accomplishments and outcomes are utilized in national and international strategies delivering research results and advances to regulatory agencies, commodity organizations, industry, academia, research and extension agencies and consumers.

Background
The safety of the food supply continues to be a visible agriculture and public health issue, and a national and international priority for ARS. Outbreaks of foodborne illness are still seen as a significant cause of morbidity, mortality, and chronic sequelae. The cost/burden resulting from these events can result in economic devastation both nationally and internationally. The cause of many outbreaks often remains unresolved, but issues such as intensive food production, international trade, consumption habits, travel and immigration of peoples are still regarded as areas of concern.

Persistent outbreaks of major commodity-specific foods that potentially directly affect public health, regulations, industry, and trade, require our attention. A predominant focus is implementation of the Food and Drug Administration (FDA) Food Safety Modernization Act. Implementation cannot be over-estimated and consequently major changes within several Food Safety regulatory agencies, especially the United States Department of Agriculture (USDA), Food Safety Inspection Service (FSIS); the FDA, Center for Food Safety and Nutrition, and Center for Veterinary Medicine (CFSAN/CVM) have impacted the Agricultural Research Service (ARS) National Food Safety Program (NP108). In the current NP108 Action Plan, research efforts were redirected to problem-solving through technology development, in-line with the Office of Management and Budget (OMB) and their Program Assessment Rating Tool (PART) requirements that existed at that time.

During the past few years it has become more evident that scientists nationally and internationally have differing opinions as to what aspects of food safety research are critically important; and there has been no consensus. For example, recent issues of food adulteration, which included adulterated horsemeat, olive oil, and honey, has placed a greater emphasis on traceability, food integrity, and the assurance of food supply networks. Adulteration is generally not considered a food safety issue per se but a quality issue. Regardless, European food [safety] agencies are stressing a greater focus on food fraud, and consequently are realigning their research and regulatory surveillance programs. In addition, there is national and international concern regarding the increase in antimicrobial resistant (AMR) bacteria. There is also a greater emphasis on assisting emerging nations to develop secure food supplies; however, in most cases this is also not a food safety issue, but food security. Additionally, and perhaps less regarded challenges to ensuring an adequate supply of safe food include: global climate change, water availability, and new and/or re-emerging (more virulent) plant or animal diseases.

In the development of the 2016-2020 NP108 Action Plan, the Food Safety National Program Leaders (NPLs) focused on what we believed were the most critical issues and needs. These are described as Problem Statements (1-7) under one Component, Foodborne Contaminants. The Problem Statements were formulated in part, from interactions with various stakeholder groups, including: the 5-year Retrospective Review reviewers; our Federal partners and stakeholders from USDA, including FSIS, National Institute for Food and Agriculture (NIFA), ERS, AMS; DHHS, including the FDA and CDC; commodity and professional organizations; and academia.

In developing the Action Plan, the NPL's approached the task appreciating that there are many special challenges. To remain responsive to these challenges the Plan was a living document, subject to review, realignment, or revision, when and where required or appropriate. Internally, such issues as balancing stakeholder and partner needs with Program fiscal and personnel resources; ensuring that the research provides accomplishments that have impact and could be translated into practice through technology transfer; collaborating nationally and internationally and focusing on relevant issues to ensure that targeted areas are addressed; and ensuring that the Program has the capability to respond when requested to unexpected needs and/or issues. The Plan is also subject to other external stressors, for example, changes in priorities from the Administration which affect overall Agency budgets, scientist retirement and hiring, research Center, Unit or project closure.

Relationship of this National Program to the ARS Strategic Plan: Outputs of NP108 research support the "Actionable Strategies" associated with the performance measures outlined below from the *USDA Strategic Objective 4.3:* Protect Public Health by Ensuring Food is Safe; *REE Goal 5:* Food Safety; and the ARS Strategic Plan for 2012-2017, *Strategic Goal 1.2* - Protect food from pathogens, toxins and chemical contamination during production, processing, and preparation.

> **Performance Measure 1.2.A:** Develop detection methodologies for foodborne pathogens and technologies for the rapid and sensitive detection of toxins, chemicals, and biologics that can be implemented for improved food safety and food defense. **Targets:** Cumulatively, five new technologies will be developed and transferred.
>
> **Performance Measure** 1.2.B: Conduct and evaluate research that will lead to effective control and intervention strategies for the reduction of microbial, chemical, and other contaminants of the food supply, as well as elucidation of the molecular and physiological mechanisms that allow for persistence, survival, and transmission of foodborne pathogens in the populations and environment. **Targets:** Identify and evaluate potential control and intervention strategies for the reduction and control of foodborne pathogens and contaminants along the food production continuum.

Component 1. Foodborne Contaminants

The production, processing, and distribution system for food in the United States is a diverse, extensive, and easily accessible system. This open system is vulnerable to introduction of contaminants through natural processes and global commerce, and by intentional means. Thus, the food supply must be protected from pathogens, toxins, and chemical contamination that cause disease or injury to humans. The ARS Food Safety Research Program seeks ways to assess and control potentially harmful food contaminants. ARS will conduct research and provide scientific information and technology to producers, manufacturers, regulatory agencies, and consumers to support their efforts to provide a secure, affordable, and safe supply of food, fiber, and industrial products.

Problem Statement 1. Population Systems

The goal of this research area is to identify and characterize the movement, structure, and dynamics of microbial populations within food-animal and plant systems, across the entire food continuum, from production through processing. At a microbial level, the diversity and complexity within environments and food matrices may alter the makeup of the populations, as well as cause change through spatial and temporal influences, or by the competitive or synergistic relationships among pathogens and commensals. Microbial populations can influence the safety of food, and the various environments in which they survive determine the success and impact of the microorganism. In turn, microorganism(s) may influence the conditions prevailing within the environment which also impacts their survival or ability to

thrive. An example of identifiable area of study would include biofilms and the association of quorum sensing.

Components and emphasis for understanding and characterizing microbial populations and their environments must include epidemiology, ecology, and host-pathogen relationships. Epidemiologic studies of microbes within their environment, allows an analysis of the population therein. As such it enables the development of improved detection methods, provides a framework for integration of microbial genomic data with disease, and a mechanism to evaluate risk factors for microbial intervention and/or control. Ecologic studies determine the attributes and changes in various communities, that is, changes to populations in the same space. Such studies allow for a better understanding of the interactions and relationships, and the transmission and dissemination of pathogens and toxins in and among food producing animals and crops. Host-pathogen relationship studies provide an understanding of the acquisition of genetic traits, such as the development and movement of resistance genes; traits connected with colonization and evolution of virulence; and the role of commensals. Where appropriate, a metagenomics approach to selected research areas will be developed to determine the attributes of the ecological communities in which pathogens are found. Knowledge of the attributes, interactions and relationships within the community in which the pathogen lives is critical to the development of control and intervention strategies.

Within this Problem Statement it was critical to differentiate Food Safety from Animal Health. Certainly, there will be some overlap; however, this will be addressed at the Office of National Programs level. There will be an emphasis on how pathogens persist in animals and the related environment, and this will drive mitigation and prevention strategies, as well as guidelines, policy and regulation.

Research Needs

- Improved approaches/designs for microbes based on population-based studies, monitoring of emerging pathogens, and supplying data for identified data gaps.
- Improved sampling collection protocols to maximize the probability of describing the exceedingly large number of diverse organisms that inhabit ecological communities.
- Data on particular ecological niches or reservoirs for specific pathogens.
- Data on factors which enhance or reduce fitness characteristics related to persistent colonization, survival and growth.
- Data on the complex interactions between fungus/crop/environmental factors/production practices.
- Improved methods that allow evaluation of the impact of intervention or management strategies on microbial contamination throughout the entire food chain from field to plate.
- Data on how climate change impacts pathogen growth, persistence, pathogenicity or virulence.

Anticipated Products

- Improved epidemiological methods that allow the collection of quantitative data on the pathogen load within the food safety continuum.
- Capability to predict how environmental, nutritional, and/or biological factors influence or control the attributes and changes in ecological communities and within microbial populations.
- A foundation for developing appropriate intervention strategies based on mechanisms for transmission and dissemination of pathogens and toxins in and among food producing animals and crops.
- A risk-based framework that allows the integration of genomic data with disease outcome
- Descriptions of genetic traits associated with colonization and the evolution of virulence, including the development and movement of resistance genes, and the role of commensalism in resistance gene acquisition.

Potential Benefits/Impact

- Improves and enhances knowledge of how microbial populations in agriculture can potentially affect and impact public health.
- Delineates how microbial pathogens are transmitted and disseminated in and among food producing animals and plant crops (includes mycotoxin related research) allowing for future development of improved/alternate (environmentally compatible) intervention and/or control strategies.
- The critical factors which influence fitness characteristics related to microbial persistence colonization, survival, and growth allowing for future development of improved/alternate (environmentally compatible) intervention and/or control strategies.

Problem Statement 2. Systems Biology

Systems biology involves an integrated, multidisciplinary approach to study the complexities of biological components, a central problem to food safety. Identifying the components and players within the system allows the genetic components of bacterial, viral, and fungal pathogens and food-borne parasites, and their expression and products to be identified and directly related to the microorganisms. In order to study systems, quantitative technologies such as "omics" [genomics, proteomics, transcriptomics, metabolomics and metagenomics] combined with bioinformatics can be applied. There is an increased need for data gained from systems studies to be directly used for both pre- and post-harvest food safety. For example, whole genome sequencing (WGS) efforts using next generation sequencing (NGS) have increased and allowed regulatory agencies to identify and resolve outbreaks of foodborne illness (often for attribution purposes). It is recognized however, that the use of NGS requires extensive collaborations with other researchers.

The main goal of research developed in response to this Problem Statement is to utilize omic-technologies and apply them to the study of foodborne pathogens in complex food systems. For example, research will elucidate how microbes cause disease and assess their prevalence, pathogenicity (ability to infect and cause disease) and virulence (the severity of disease). Understanding pathogenicity and virulence is critical for intervention and control strategies, modeling, and providing data for the development of risk assessments by regulatory agencies. Pathogens have the capacity to readily and rapidly adapt and evolve, so pathogenicity and release of virulence factors is an issue at all stages of the food safety continuum. The prevalence and patterns of contamination in food sectors may vary considerably and needs to be assessed and evaluated carefully. Differences in microbial prevalence, pathogenicity and their virulence are observed across different food production and processing systems, at different sampling times, and by using various methods. Contamination patterns reveal variation in the pathogenicity and virulence and the presence of persistent or sporadic strains and evidence of bacterial transfer from production environments to processing, and from processing environments to food. Continual outbreaks of industry related bacterial contamination emphasize the continued need to examine pathogens in order to avoid public health risks.

Ongoing implemented microbial control strategies may lose their effectiveness, forcing the development of new production processes and products to maintain and improve the safety of foods. This in turn may restart the cycle of pathogen adaptation resulting from the changed environment and its stresses. Risk assessment(s) conducted by our regulatory stakeholders are also predicated on understanding the pathogen, the dose response, the behavior in foods, and any positive or negative influences that may affect virulence. Assessing the virulence of foodborne organisms and differences among serotypes/strains is critical in implementing new surveillance and intervention strategies. A critical issue within this Statement is the need to differentiate between microorganisms that are relevant to agriculture versus food safety and public health.

Research Needs

- WGS of specific pathogens to provide data for developing high resolution genotyping and molecular serotyping methods, for identifying virulence attributes, and elucidating the differences between pathogens and non-pathogens.
- Identification and characterization of pathogen virulence factors and how they interact.
- Data to determine if and/or how virulence is directly related to the infective dose.
- Data on pathogen adaptive responses to intrinsic and extrinsic food stressors such as pH, a_w, temperature, O_2, and determine their role in pathogenicity and/or persistence.
- Data to determine if resistance genes affect virulence, pathogenicity and/or persistence.
- Identification and characterization of virulence attributes and the responses of specific pathogens to their environment relative to changes in immunogenicity in the host.
- A detailed investigation of food production and processing environments for bacterial pathogens, and a determination what genetic and/or environmental factors might determine or allow certain bacterial strains to become persistent.
- Data on the impact of changing management, production and intervention practices on the incidence of parasites as it relates to foodborne risk.

- Identification and characterization of unique fungal genes for specific biological and physiological functions. For example, how mycotoxin synthesis is transcriptionally regulated during the fungal growth cycle.
- Data on the effect of climate change on mycotoxin production in food crops. How environmental stress factors interact to affect plants, fungal growth, and subsequent mycotoxin synthesis.

Anticipated Products

- Identities of the critical/required genetic components that make specific microorganisms pathogenic versus non-pathogenic, or highly versus weakly virulent.
- Principles relating regulatory mechanisms that control or impact gene expression with a microorganism's biology, for example, pathogenicity and virulence.
- Information relating how stress factors such as climate change affect pathogen gene expression.

Potential Benefits/Impact

- Provides knowledge of which genes are required for a microorganism to become a pathogen; generates data on genes that contribute to variations in pathogenicity, and how gene expression is involved in virulence and/or persistence viability in animal, plant and food systems.
- Generates data for the specific development of molecular pathogen phylogenetics, allowing for improved and faster molecular tracking, and determination and characterization (attribution) of outbreaks of foodborne illness by regulatory agencies.
- Supports development of improved risk models, and the revision of risk assessments, e.g., pathogens of low virulence may not be considered as necessary for regulatory control.
- Supports improved mitigation strategies and alternative control measures via identification of genes that code for resistance to antimicrobials and disinfectants, for toxin production; for the ability to grow in specific ecological niches; and for the ability to persist in production and/or processing environments.

Problem Statement 3. Microbial Contaminants: Technologies for Detection and Characterization

The challenge is the unequivocal detection and characterization of pathogenic microorganisms entering the food continuum (both pre- and post-harvest). Detection and characterization are required at the earliest possible stage of the continuum to provide the necessary data for targeted interventions and reducing the need for recall of food products. Where possible, technologies must be developed that allow the most effective and rapid detection and characterization capabilities.

The focus of the research will be on the most promising technologies (depending on the matrix) or point of use, that is, whether the technology will be used for baseline studies, traceability and/or attribution forensics. This requires that decisions be made relative to what should be

detected, and the required level of detection and characterization. It is noted that technologies that have the highest level of detection/characterization capability might not necessarily be the most practical, useful, economically viable, or easily implemented. High-through-put analysis is important, but it may be impractical. Promising technologies will be advanced through technology transfer, and where possible, and appropriate, will undergo validation through national or international bodies from academia, industry, and/or government sectors. Studies that suggest minimal outcome or impact will be terminated, and alternate approaches formulated.

Research Needs

- Sampling protocols to maximize the probability of detecting contaminants especially when combined with innovative approaches to sample processing.
- Sample recovery methods with attention to sample preparation as different matrices may present unique problems.
- Methods that do not have a sample or detection bias.
- Technologies that have applications in surveillance systems, for monitoring the food supply and for food defense.
- Technology development that has uniformity of application in both pre- and post-harvest production and processing system.

Anticipated Products

- Technologies for multiple agents for trace-back and attribution, and where fiscal and personnel resources are also limited.
- Technologies with improved speed, cost effectiveness, and the capability to provide information for the determination and implementation of subsequent actions.
- Validated technologies that allow uniformity of implementation nationally and internationally.

Potential Benefits/Impact

- Provides validated technologies that have public health, regulatory [monitoring, traceability and attribution], trade, industry, and research use and a commonality of interests between stakeholders and partners.
- Allows improved response times to events, and subsequently allows for the development of mechanisms for treating foods taken out of commerce.
- Provides data to identify areas where interventions are most critically needed, thus assisting the implementation of HACCP programs by Federal agencies, and their regulated industries.
- Enables development and validation of predictive microbial models and helps fill identified data gaps.

Problem Statement 4. Chemical and Biological Contaminants: Detection and Characterization Methodology, Toxicology, and Toxinology

Toxicology examines the relationship between dose and its effects on the exposed organism, whereas ***toxinology*** deals specifically with animal, plant, and microbial toxins produced by or accumulated in living organisms, their properties and their biological significance for the organisms involved. Both kinds of studies are required to reduce risks arising from contamination of food by chemical and biological contaminants.

The regulation and control of veterinary drugs, chemical residues, heavy metals, persistent organic pollutants, and biological toxins derived from bacteria, fungi and plants are an integral component of any food safety program. To protect public health and the environment, regulations have been passed and implemented that set limits on contaminants in edible agricultural products. Compliance and enforcement of these regulations is a critical role of the ARS National Program's stakeholders that requires the availability of practical detection and characterization methods for veterinary drugs (antibiotics, beta-agonists), chemical residues (dioxins, pesticides), heavy metals (As, Pb, Cd), and organic pollutants (polybrominated diphenyl ethers). In addition to regulatory monitoring, there is a need to understand the biological effects of any inadvertent contamination by humans or animals. In addition to toxicological and toxinological studies, this Problem Statement also includes research directed towards methods for detection and identification of mycotoxins, toxicity evaluation, and mechanism of action.

Accomplishments and promising technologies within this research area will be quickly advanced through technology transfer and where appropriate, will undergo validation through national or international bodies such as the Association of Official Agricultural Chemists (AOAC). These studies require multidisciplinary approaches to meet the challenge, and accomplishments may have far reaching effects regarding food biosecurity, regulations and trade issues.

Research Needs

- Accurate, rapid, and easily used analytical detection methods: single/multiclass, single/multi-contaminant analytical methods; lab and field-based methods and instruments for analytical screening.
- Mechanism/action-based bioassays for laboratory and field use.
- Multi-task on/in-line inspection technologies that detect contaminants and quality attributes simultaneously functioning in or near real-time.
- Assays for assessing the efficacy of various processing methods to reduce or eliminate the toxicity in contaminated foods for human/animal consumption.
- Assays that have efficacy in toxico/toxinological studies.
- Intervention methods [bioremediation] to reduce bioavailability.
- Data on the fate and transport of contaminants and their derivatives in food systems and the environment for use in risk assessment by regulatory agencies.
- Provide parameters for regulatory agencies on biological residue depletion and withdrawal rates in animals.
- When requested, develop technologies that have a critical use in food defense.

- Data for use by regulatory agencies on the dose-response relationships and tissue specificity of biological toxins.
- Exposure assessment data for regulatory agencies on the relevance of biological toxins with undetermined toxicity through using animal models.
- Biomarker assays as a measure of exposure and disease susceptibility.

Anticipated Products

- New and validated technologies that when implemented provide tangible benefits through a more effective and efficient means of monitoring the food supply and environment where food is grown.
- Improved methods that assist researchers conducting toxico/toxinological studies.
- Toxico/toxinological data providing basic and applied knowledge on the effect of exposure to biological toxins.

Potential Benefits/Impact

- Provides technologies and data for regulatory use, and for better scientific and regulatory decision-making, reducing the likelihood of tolerance limit-errors, protection of consumers, and prevention of economic losses resulting from inappropriate regulatory actions.

Problem Statement 5. Intervention and Control Strategies

Intervention and control strategies will assist in reducing or eliminating pathogens in food animals and their derived products, seafood, and plant crops during production and processing. Reduced shedding of zoonotic pathogens by food producing animals, and contamination of seafood and plant material will subsequently help reduce the pathogen load during slaughter/ harvesting and subsequent processing and storage. Some food processing/storage technologies can have the ability to inactivate microorganisms to varying degrees; however, the intensities required can result in adverse functional and/or sensory properties, combined with a significant reduction in quality. Consequently, there remains a continued need to develop and subsequently combine new and/or innovative processing technologies. Interventions can be additive and/or synergistic, leading to improved control over pathogen growth without potential changes in food quality or reduction in nutrition. Research after an approved period that yields no outcome or requires the purchase of expensive equipment will be terminated, and alternate approaches formulated. If alternate approaches cannot be found, the project will be redirected to another priority. Unintended or unanticipated consequences of processing intervention strategies such as changes in virulence, production of toxins, pathogen resistance, selection of resistant strains, or changes in microbial ecology should be considered for further investigation.

The challenge is that the pathogen load on a product must be significantly reduced by any processing intervention strategy to avoid the consequences in food production resulting from "dirty in, dirty out" processing. There is also the concern that during processing the initial microbial load can be reduced but recontamination occurs with different strains or serotypes present or resident within the processing environment. Such concerns are valid because there

are numerous observations that the pathogens present on product prior to processing are different from those found after processing. This variation in pathogen type has significant public health concerns since those pathogens initially found on the product may not be responsible for any foodborne outbreak and/or clinical outcome.

Research should also address, where possible, the integrated lethality for an intervention process. The purpose of the process lethality determination is to provide processors with science-based validation of the effectiveness of a specific process to destroy any microorganism of concern. For example, a thermal process needs to account for many variables including the initial pathogen load, multiple pathogens, pathogen strain variability, food structure, and the heating and cooling profile of the product. In-plant validation should be conducted to verify the intervention(s). The entire lethality process is incorporated into a systems approach to developing pathogen intervention or control strategies. Problem Statement 5 addresses a wide range of food products including animals, shellfish -seafood, and plant materials. The Problem Statement also includes biocontrol technologies for food crops contaminated by mycotoxins, such a tree nuts, corn and grains.

It is critically important within these studies that for development and validation of any process intervention a common or representative core set of pathogens or surrogates be used. This is critically important in order to make the intervention research results comparable both within and external to the Program. Core sets of strains for different pathogens will be made available through the ARS bacterial culture collection. If a specific strain is not available in the collection, ONP will facilitate researchers obtaining the appropriate isolate.

Research Needs

- Interventions that prevent colonization or modulate pathogens in the gut; target specific metabolic endpoints; decrease shedding of zoonotic pathogens at the time of slaughter.
- Data on the role of transportation and lairage, slaughter/processing methods, and equipment on pathogen survival, transfer, postharvest processing and storage.
- Data on the effect of intrinsic (pH, a_w, Eh, nutrient content, antimicrobials, structure) and extrinsic (temperature, RH, O_2) parameters in the production, processing, handling, preparation, and storage of foods. This need includes food preparation and handling for, or by food service operators and/or consumers.
- Data that elucidate the mechanism(s) of pathogen introduction, persistence/survival in shellfish.
- Production and processing intervention/control strategies for pathogen reduction in shellfish.
- For plant crops (fresh produce), obtaining data on the role of extrinsic and intrinsic factors on pathogen internalization and/or attachment; and pathogen occurrence and movement.
- For plant crops (fresh produce), obtaining data on the role and/or influence of commensals and/or non-pathogens.

- Identification of the critical control points in both production and processing of fresh produce, plant crops (grains/tree nuts) that can be mitigated through the development and implementation of intervention and control strategies.
- Biological control strategies to reduce mycotoxin production and contamination of food and feed crops such as corn/maize, cotton seeds, grains and tree nuts. Any new or modified effective biocontrol organisms and delivery systems must not introduce other toxic factors; for example, for the biocontrol of aflatoxins there should be no introduction or expression of the CPA or Fusarium toxin genes.
- Data that assesses the role of chemicals that might act synergistically to enhance accepted interventions.
- Methods to prevent the growth of pathogenic and spoilage microorganisms in minimally preserved, brined, and fresh-cut foods.
- Data on the effect of single and/or combinations of intervention technologies on pathogen reduction. Validate these data through laboratory, pilot-plant processing and commercial processing facilities.
- Data on whether combinations of non-thermal technologies can be incorporated in the hurdle concept; and determine whether single or combinations of non-thermal technologies are more effective if used in combination with traditional interventions.
- Data that evaluates the outcome/impact of intervention options for small and very-small regulated plants.
- Data in intervention effectiveness to be for use in the development of Quantitative Microbial Risk Assessments (QMRAs)
- Data determining the effect of intervention technologies on sensory/quality deterioration, and accumulation of toxic chemical by-products.

Anticipated Products

- Improved intervention strategies to eliminate and/or control microorganisms in animals and their derived products, seafood and plant production, processing and storage systems. Interventions can have the ability to inactivate microorganisms to varying degrees; therefore, the goal is to maximize intervention effectiveness while minimizing sensory/quality deterioration, and possible accumulation of toxic chemical by-products.
- Improved intervention strategies for various sized operations, utilizing environmentally compatible technologies.
- Improved intervention strategies focusing on the use of combinations of new or innovative technologies for (minimal) processing, thus mitigating the potential for the development of resistance.
- Improved interventions based on an understanding of their modes of action and effects on the microbial ecology of a food product, since inadequate suppression of spoilage could create an opportunity for human pathogen growth and toxin production.

Potential Benefits/Impact

- Provision of critical intervention strategy data to regulatory/action agencies, industry, and commodity organizations that allows for the development, evaluation, and implementation of Good Agricultural Practices (GAPs); Good Manufacturing Practices

(GMPs) or regulations based on sound science.
- Enables methods/strategies for the evaluation of any developed interventions and controls.
- Provides production control interventions that reduce downstream contamination, which subsequently reduces disease risk.

Problem Statement 6. Predictive Microbiology/Modeling; Data Acquisition and Storage; Genomics Database

The tenet of predictive microbiology is that the behavior of any microorganism is deterministic and able to be, within limits, predicted from knowledge of the microorganism itself and the microorganism's immediate environment. However, it has been stressed by stakeholder groups that research should also include a greater emphasis on probabilistic modeling to balance the deterministic approaches. This would benefit predicting the behavior of pathogens under stressed conditions (more relevant to the food industry) where growth/inactivation is stochastic.

Behavioral predictions and models are accepted (globally) as an integral part of microbial risk assessment used to support food safety measures by both food safety regulatory bodies and industry. The Program does not develop or conduct Risk Assessments (RA), where RA is defined as the determination of a quantitative or qualitative value of risk related to a specific situation and a recognized hazard. However; the Program does conduct research and provide data when requested by our regulatory stakeholders (FSIS, FDA) for their use in conducting risk assessments.

The Program develops various modeling programs including; the Pathogen Modeling Program (PMP), a package of models that can be used to predict the growth and inactivation of foodborne bacteria, primarily pathogens, under various environmental conditions. In addition, the Predictive Microbiology Information Portal (PMIP) is geared to assist food companies (large and small) in the use of predictive models, the appropriate application of models, and proper model interpretation. The vision is that the PMIP will be the highway for the most comprehensive websites that bring together large and small food companies in contact with the information needed to aid in the production of the safest foods. The PMIP links users to numerous and diverse resources associated with models (PMP), databases (ComBase), regulatory requirements, and food safety principles.

All predictive models developed must be available for external examination, review and utility. If predictive models are developed for internationally accepted high priority pathogen-food combinations, then they could have a major impact for food companies in the USA and other countries producing and exporting food to the USA. This will require significant interactions with risk assessors and involvement in international initiatives such as National Advisory Committee on Microbial Criteria for Foods (NACMCF), Codex Alimentarius Commission (CODEX), Food and Agriculture Organization (FAO), and the World Health Organization (WHO). Collaborations with stakeholders must be strengthened with regards to what research needs to be conducted so as to effectively utilize the inherent ARS expertise and modeling systems mechanisms.

Data acquisition and storage: ARS and international institutes, including Institute Food Research (IFR-UK) and the University of Tasmania (UTas), as well as associate members University of Querétaro, Mexico; Unilever Research, UK; Agricultural University of Athens, Greece; National Food Research Institute, Japan; Hokkaido University, Japan; and Rutgers University also developed and maintains a publicly available global food safety database, **ComBase - a Combined data Base for predictive microbiology** – which is the number-one web-based resource for quantitative and predictive food microbiology in the world. Its main components include a database of observed microbial responses to a variety of food-related environments and a collection of relevant predictive models. The purpose and goal of ComBase is to provide an electronic repository for food microbiology observations and to make such data and the generated predictive tools freely available and accessible to the entire food safety community. Data acquisition and use is an interdisciplinary research challenge that translates into safer products and improved public health.

Genomics as a functional and critical part of omic-technologies holds great promise for improving the early detection, prevention and control of current and emerging foodborne pathogens, thus contributing to improved food safety and consequently public health. Genomics has the potential as a partner or replacement for culture-based techniques. Therefore, food safety regulatory agencies, USDA and the FDA, have discussed and are planning to implement the increased use of genomics, in particular partial and/or WGS for both regulatory monitoring, attribution and potentially for revising risk assessments. Implementation of such a redirection requires developing a coordinated system of genomic sequencing technology for routine testing. Critical within this issue is the development of an ARS database from our national and international sequencing/annotation efforts. For this work, a common or representative core set of bacterial pathogens or surrogates will be available. Additional data from isolates studies obtained from national and international collaborations will be incorporated. Allied to the sequencing efforts will be meta-data descriptors. This research will also be part of a larger international initiative, the Global Microbial Identifier (GMI), a global, visionary taskforce including more than 30 countries who share an aim of making novel genomic technologies and informatics tools available for improved global infectious disease diagnostics, surveillance and research, by developing needs and end-user based data exchange and analysis tools for characterization of all microbial organisms and microbial communities.

Research Needs

Modeling

- Models that include an emphasis on probabilistic modeling to balance the deterministic approaches. This includes the influence of challenge strain(s); assessment of a model's performance; predictive value on extrapolation; and efficacy especially in complex food matrices where the intrinsic and extrinsic parameters may change.
- Data that examines and determines if growth/no-growth interface models predict the probability of growth occurring when a population faces more than one stressor/constraint.

- Models that have utility for risk assessment from both the producer and consumers perspective. There are distinctly different consequences of conservative (over) vs. non-conservative (under) prediction of growth or risk.
- Data that determines if changes in the microorganism(s) themselves occur, due to up/down regulation of genes; quorum sensing; or transfer of genetic information between species.
- Models that predict pathogen and non-pathogen behavior in complex food systems utilizing inactivation data. These types of studies are fundamental to developing Hazard Analysis Critical Control Point (HACCP) systems and regulations.
- Process risk models for industry that derives predictions for Critical Control Point (CCP) assessment.
- Data that demonstrates how models can be integrated more fully into supply chains, thereby increasing utility to industry and risk assessors.
- Models that determine the effects of food safety interventions, for example carcass and produce sprays; and physical and chemical interventions, for example: radio frequency, heat, cold, irradiation, and Generally Recognized as Safe (GRAS) chemicals.

Database

- Compile modeling data into a shared informational database through national/ international efforts.
- Write program code linking ComBase records to online databases. This feature will collect attributes for individual records, such as journal article title, abstract, authors, and institution.
- Data collection for specific organism-food combinations, enhance the value for the food systems community. Prioritization will be given to data needed to fill current database gaps, as well as records most sought after by ComBase customers.
- Derive and provide relevant data to regulatory agencies for use in HACCP programs, risk assessments, labeling, persistence, and issues relative to international trade.

Genomics

- Conduct sequencing and annotation efforts on pathogens of concern that fall under research efforts in various Problem Statements.
- Development of a genomic database for identification of microorganisms or development of an identifier of microorganisms as a platform for storing data.
- User friendly system to aggregate, maintain, share, mine and translate genomic data for microorganisms, for example: the identification of relevant genes or for the comparison of genomes to detect outbreaks and emerging pathogens.
- Increased focus on bioinformatics (computational biology) as more sequence data becomes available, and the complexity of both the data and questions being asked to become more sophisticated.

Anticipated Products

- Predictive microbiology [models] that have validity and usefulness while addressing the limitations of the predictive ability. Studies leading to development of these models will include "real food systems" not just broth models or model food systems.
- A shared informational database done in-part through the continued development and expansion of the international collaborative project Combase. This will include data from industry/academia that pertains to "real food production/processing systems."
- A computer-based system and database to aggregate, share, mine and translate genomic data for microorganisms in real-time through a direct link using user-friendly platforms.

Potential Benefits/Impact

- Generates data on the responses of microorganisms to both defined and changing environmental conditions and translates these data into mathematical models and user-friendly software tools available on the internet at no cost. These must be readily usable by national and international regulatory and public health agencies, and industry, to assist in ensuring the safety of the food supply.
- An internet-based database ensures that data-mining and acquisition will continue to be coordinated. Genomic database and bioinformatics efforts become increasingly important so that biologists could have the ability to gain information that will foster technological innovation, and an understanding of the genetic basis of foodborne microorganisms.

Problem Statement 7. Antimicrobial Resistance

The discovery of antibiotics transformed human and veterinary medicine and saved millions of lives in the United States and around the world. The rise/increase of antibiotic-resistant bacteria represents a serious threat to both animal and human health and the economy. The concern for the development of antimicrobial resistant (AMR) bacteria has resulted in the development of both national and international strategies to address the issue. In 2014, the President signed an Executive Order, and a strategy was developed by multiple agencies to begin addressing AMR at the National level. Even though the USDA is not the lead regulatory agency for antibiotic use and AMR, USDA-ARS is an important part of the solution.

Areas of concern include detecting, measuring, and assessing the amount of AMR bacteria within the production animal populations with an emphasis on foodborne pathogens. In addition, developing alternative strategies to minimize the use of antibiotics in production animals while maintaining and improving animal health and ensuring safe food for consumers is a critical need. Currently, the role of feeding antibiotics to production animals on the development of AMR bacteria and the impact on public health is not well defined. In addition, there is a critical need for the development of alternative strategies to reduce the level of antibiotic use as well as developing mitigation strategies for foodborne AMR bacteria in food producing animals. These areas are cross-linked with Problem Statements 1 and 2 within the Action Plan

Research Needs

- Multidisciplinary approaches to understand the development, persistence, and transmission of resistant genes, and antimicrobial resistant in foodborne microorganisms.
- Improved detection methods to assess bacteria for antibiotic resistance genetic elements in foodborne pathogens.
- Methods to assist other Federal agencies in measuring and assessing AMR in food animal populations, e.g., assisting FSIS in interpreting National Antimicrobial Resistance Monitoring System (NARMS) results and provide support for USDA's National Animal Health Monitoring System (NAHMS) studies on AMR bacteria.
- Alternatives to antibiotics including management practices, pre-and probiotics, bacteriophage gene products, lytic enzymes, vaccines and other novel products to reduce their levels in food producing animals, thus reducing the need for antibiotics. The development of any practice/product must ensure practicality and potential utilization so that implementation is cost effective to the producer, readily approved by regulatory agencies and industry, and easily incorporated into any management system.
- Elucidating the ecology of foodborne AMR bacteria in terms of gene transfer, the role of the host microbiome in the development and maintenance of AMR, and the role of biofilms in the development of AMR.

Anticipated Products

- Improved detection techniques facilitating the speed, ability, and accuracy of detecting foodborne AMR bacteria in food producing animals and their products.
- Improved strategies to reduce antibiotic use and the number of AMR bacteria in the food supply.

Potential Benefits/Impact

- Provides support for both stakeholders and regulatory agencies in developing strategies to address foodborne AMR bacteria.
- Improves strategies to reduce the use of antibiotics in production animals while maintaining their health and growth efficiency. This is critical for feeding an ever-growing population while also addressing a serious public and animal health concern.

Program Resources

The following ARS locations have research projects addressing the 7 Problem Statements identified under Component 1

Albany, CA; Ames, IA; Athens, GA; Beltsville, MD; Clay Center, NE; College Station, TX; Fargo, ND; Fayetteville, AR; New Orleans, LA; Maricopa/Tucson, AZ; Peoria, IL; Raleigh, NC; Stoneville, MS; West Lafayette (Purdue U.) IN; and Wyndmoor, PA.

Report

Introduction

This Accomplishment Report was prepared by the current NP108 National Program Leaders, Dr. James Lindsay, Senior National Program Leader, and Dr. Kimberly (Kim) Cook, the new National Program Leader for Food Safety, appointed as of October 2018.

The Report provides examples of research accomplishments, their outcomes and impacts, in the fiscal years 2016-2019, with inclusion of predicted accomplishments and their impact for the first quarter of fiscal 2020. Not all accomplishments during the period are listed, since this would be too extensive a document. As noted previously, for a detailed list of accomplishments by year, please refer to the Food Safety Program Annual Reports which are available on the web at: https://www.ars.usda.gov/nutrition-food-safetyquality/food-safety-animal-and-plant-products/docs/annual-reports/

The report is based upon input from the field. Program scientists were requested to provide a Project Report which summarized (so far) their project accomplishments, outcomes, conclusions, and their impact and benefits. In some cases, the scientist acknowledged that the accomplishment had addressed objectives within more than one Problem Statement. So as not to duplicate reporting, we determined and allocated the accomplishment(s) to only one Problem Statement, that we considered the most appropriate. Where a division could not be made or it was determined that a strong cross-cut existed, we have thus indicated that the accomplishment also addressed another Problem Statement.

The accomplishments, their outcomes and impacts should provide a broad picture of the research conducted in the Program. Many of the accomplishments represent a summary of several research projects conducted by multiple scientists. Some research described was conducted with partners from academia: however, individual laboratories and scientists are not identified since the purpose of the review is to assess the overall National Program. The Accomplishment Report is organized by Problem Statement with a brief introduction followed by accomplishments and discussion.

We asked the scientists to address the impact of the research noting that any assessment of an accomplishment's impact and benefit is inherently a qualitative and imprecise science. A series of criteria were identified in order to impartially conduct the evaluation, these were:

- Did the research advance the knowledge of food safety?
- Was the research innovative?
- Was there technology transfer?
- Was there regulation and policy development?
- Was there academic, industry and/or consumer relevance?

Return on Investment

As noted previously; the Action Plan and the Projects are dynamic and flexible and could be modified at any time due to changing priorities. An example of a major change that occurred during this research cycle is described below, and some changing priorities:

Major Changes:

- Projects "Investigation of Immunoregulation in Reducing Foodborne Pathogen Colonization in Poultry" and "Identification of the Ecological Niches and Development of Intervention Strategies to Reduce Pathogenic Foodborne Pathogens in Poultry" at College Station, Texas were combined due to retirement/resignation.
- Projects "Genomic and Proteomic Approaches for the Characterization of Foodborne Pathogens in Poultry" and "Novel Pre-harvest Interventions and Alternatives to Antibiotics to Reduce Foodborne Pathogens" at the National Poultry Research Center, Athens, Georgia were realigned and combined due to retirement/resignation.
- Project "Health Risks Posed by the Consumption of Cooked Foods Prepared from Naturally Contaminated Corn" at the National Poultry Research Center, Athens, Georgia was eliminated due to retirement/resignation.
- Project "Identification of Plant-derived Bioactive Compounds for their Potential as Alternatives to Conventional Antimicrobials" at the Beltsville Regional Research Center, Beltsville, Maryland was eliminated due to retirement/resignation.

Changing Priorities: The following are examples of changing priorities during the cycle which required program/project realignment.

- Assist FDA and industry regarding manure and growing conditions (soil type and location) for use in fresh produce production.
- Assist FSIS investigating chemical residues of concern in food animal contamination.
- Assist FSIS investigating pathogen contamination and processing system for chicken livers.
- Assist FSIS developing, validating and implementing recovery medium for Salmonella in poultry processing.
- Decision by the Administration to stop all Toxoplasma related research.

Scientist loss from Program: From FY2016 to the end of FY2019: 37 scientists left the Program due to retirement, resignation, promotion, or health issues.

Collaborations

The Program has numerous international collaborations. Listed below are [> 60] countries where collaborative research with NP108 are conducted: Algeria; Argentina; Australia; Belgium; Brazil; Burkina-Faso; Burundi; Canada; Chile; PR-China; Colombia; Costa Rica; Czech Republic; Denmark; Egypt; Finland; France; Germany; Ghana; Greece; Guatemala; Haiti; Hungary; India; Indonesia; Israel; Italy; Japan; Kenya; Luxembourg; Malawi; Mali; Mexico; Mozambique; Nepal; Netherlands; New Zealand; Nigeria; Norway; Pakistan; Poland; Republic of Ireland; Romania; Russia; Rwanda; Saudi Arabia; Senegal; Serbia, Singapore; South Africa; South Korea; Spain; Switzerland; Taiwan; Tanzania; Turkey; Uganda; United Kingdom; Uruguay, and Zambia.

The following are examples of official [research] Memorandum of Understanding (MoU) implemented/renewed/current during the 2016-2020 Program cycle.

- University College, Dublin, Republic of Ireland
- University of Stirling, Scotland, United Kingdom
- Lincoln University, Christchurch, New Zealand
- National Agricultural Products Quality Management Service, Experiment and Research Institute, Republic of Korea
- Chungnam National University, Daejeon, Republic of Korea
- Ghent University, Ghent, Belgium
- Gwangju Institute of Science and Technology, Gwangju, Republic of Korea
- RIKLT, University of Wageningen, Wageningen, The Netherlands

<u>Examples of existing collaborations that were expanded and new collaborations</u>

Shanghai Jai Tong University, Shanghai, PR-China

- Biosensor research. The initiative now included the Center for Food Safety Engineering (funded in part by NP 108) at Purdue University. This initiative falls under the Ministry of Science and Technology (MoST) Agreement, Annex V111.

NAPQMS-Research Institute, Gyeongsangbuk-do, Republic (South) Korea

- Development and validation of sensing technologies used to determine chemical and biological contamination of the food supply. Research also funded by South Korean grant.

Lincoln University, Christchurch, New Zealand

- Detection technology for bacterial pathogens using BEAM technology. Collaborations also funded by a New Zealand Government grant.

University of Tasmania, Hobart, Australia

- Continued development of Combase, the international food safety modeling database to assist regulatory agencies, industry for developing regulations & risk assessments to make foods safer for consumers.

University of Ghent, Ghent, Belgium

- Research to determine the form and function of metabolites produced by uncharacterized Aspergillus (A.) flavus gene clusters of maize that play a role in host resistance during the A. flavus-maize interaction.

University College, Dublin, Republic of Ireland

- Continued studies on E. coli STEC's in cattle, genomics, and the behavior of E. coli STEC's in foods.

University of Southampton, Southampton, UK (under development)

- Effect of processing interventions on mutation rates, virulence and pathogenicity.

Africa: Various countries in collaboration with the International Institute of Tropical Agriculture *[Burkina Faso, Burundi, Costa Rica, Ghana, Guatemala, Haiti, Kenya, Malawi, Mali, Mozambique, Nigeria, Pakistan, Rwanda, Senegal, South Africa, Tanzania, Uganda, Zambia]*

- Development, validation and use of atoxigenic strains of Aspergillus flavus for biocontrol of alfatoxins. Significant progress has been made across Africa with the launch of competitive cultures registered as AflaSafe.

Publications

As of Fall 2019 research conducted by NP108 during the 2016-2020 cycle had produced an extensive list of peer reviewed research publications (> 1500). The list is available in a Publication Appendix which accompanies this report. Due to the extensive nature of the data, information on Conference Proceedings, Abstracts and Presentations etc., was not collated or included.

Extramural (Incoming) Funds/ Tech Transfer

There are several mechanisms for funding Agreements [CRADA, Reimbursable, Trust and Grants]. The funding information in the Table below was provided by the ARS Office of Technology Transfer. The Food Safety Program and NPL's were aggressive in encouraging scientists to obtain extramural funds. This was in-part the result of increased collaborations and the need for scientists in the Program to understand and appreciate that collaborations both nationally and internationally [where appropriate], enhance the capability to deliver accomplishments through creative science and innovative solutions.

Extramural Funds/Technical Transfer Through Fiscal Years 2016- 2019 (as of 9/9/2019)

- ***Research Agreements*** ***187***
- ***Invention Disclosures*** ***59***
- ***Licenses/Biological Materials (see following for examples)*** ***43+***
  (+ pending)
  - Detection system for the surveillance of multiple bacterial & viral food-borne pathogens: (Arrayit Corporation)
  - STX1A Recombinant Toxoid, (List Biologics)
  - High affinity antibody specific to C. botulinum serotype A. (Allergan, Inc.)
  - Mycotoxin Deoxynivalenol Test Kits, (Europroxima BV)
  - Antimicrobial Wash, (Natureseal, Inc)
  - Hybridoma Cell Line BOE9-15 & BOE66-29, (List Biologics)
  - Hybridoma cell lines producing monoclonal antibodies against MCR-1 (Abraxis Inc.)
  - Hybridoma cell lines producing monoclonal antibodies against botulinum neurotoxin serotypes A and B (Biosentinel Inc.)
  - Hybridoma cell line producing monoclonal antibody Stx2-5. (Tetracore, Inc.)
  - Hybridoma cell line(s) producing Stx1, Stx1-2, Stx2e-1, Stx2e-2, Stx2e-3, and Stx2e-4 monoclonal antibodies (EMD Millipore Corporation).

- ***Patents (official) (see following list)*** ***18+***
  (+ 10 applications pending)
  - 9,174,850 Gaseous ammonia removal system
  - 9,176,110 Method of determining histamine concentration in fish
  - 9,220,261 Volatile blends and effects thereof on the Navel Orange-worm moth
  - 9,241,497 Method and apparatus for treatment of food products
  - 9,310,368 High affinity monoclonal antibodies for Stx2
  - 9,512,461 Detection of aflatoxins and aflatoxigenic aspergilli
  - 9,513,287 High affinity monoclonal antibodies for detection of Shiga toxin 2
  - 9,568,438 Single-camera angled conveyance imaging method and apparatus For whole-surface inspection of rotating objects
  - 9,655,366 Volatile blends and effects thereof on the Navel Orange-worm
  - 9,770,041 Antimicrobial wash
  - 9,863,882 Variable thermodynamic Raman spectroscopy system and method
  - 9,868,769 Mutated salmonella enterica
  - 9,883,682 Method and apparatus for pasteurizing shell eggs using radio Frequency heating
  - 9,927,364 Line-scan Raman imaging method and system for sample evaluation
  - 9,995,735 A method to introduce salmonella into ground meat and poultry
  - 10,233,482 Micro-fluidic mixer and method of determining pathogen inactivation via antimicrobial solutions
  - 10,264,808 Use of phyllosphere associated lactic acid bacteria as biocontrol agents to reduce bacterial growth on fresh produce
  - 10,352,311 Cryogenic trap

NP108 Food Safety OSQR Projects in FY2016 [2016-2020 ActionPlan]

First name is Projects Lead Scientist; (name) major contributor who left ARS during project

Title: Umbrella Project for Food Safety (pass through funding)
Project: 0500-00031-001-00D: Location: MSU, Stoneville, MS.
Investigators: Mississippi Center for Food Safety and Postharvest Technology, MSU

Title: Improved Environmental and Crop Safety by Modification of the Aspergillus Flavus Population Structure.
Project: 2020-42000-022-00D: Location: Maricopa, AZ
Investigators: Naranjo, Callicott, (Cotty)

Title: Biocontrol Interventions for High-Value Agricultural Commodities
Project: 2030-42000-039-00D: Location: Albany, CA
Investigators: McGarvey, Palumbo, Cheng, Haff, Kim, (Beck, Hua, Light,)

Title: Advance the Development of Technologies for Detecting and Determining the Stability and Bioavailability of Toxins that Impact Food Safety and Food Defense
Project: 2030-42000-049-00D: Location: Albany, CA
Investigators: Cheng, He, Hernlem, Rasooly, (Brandon, Stanker)

Title: Ecology and Detection of Human Pathogens in the Produce Production Continuum
Project: 2030-42000-050-00D; Location: Albany, CA
Investigators: Gorski, Cooley, Wu, Carter, Brandl, Tian, Hnasko, Silva

Title: Molecular Identification and Characterization of Bacterial and Viral Pathogens Associated with Foods
Project: 2030-42000-051-00D; Location: Albany, CA
Investigators: Parker, Miller, Fagerquist, Quinones, Wu, Silva (Ravva)

Title: Assessment of Genotypic and Phenotypic Factors for Foodborne Pathogen Transmission and Development of Intervention Strategies
Project: 3040-32000-032-00D; Location: Clay Center, NE
Investigators: Berry, Wells

Title: Genomic and Metagenomic Differences in Foodborne Pathogens and Determination of Ecological Niches and Reservoirs
Project: 3040-42000-017-00D: Location: Clay Center, NE
Investigators: Bono, Harhay, Schmidt, Wang

Title: Mitigation Approaches for Foodborne Pathogens in Cattle and Swine for use During Production and Processing
Project: 3040-42000-018-00D; Location: Clay Center, NE
Investigators: Bosilevac, Arthur, Kalchayanand, Schmidt, Shackelford, Wang, Wheeler

Title: Detection and Fate of Chemical and Biological Residues in Food and Environmental Systems
Project: 3060-32420-001-00D: Location: Fargo. ND
Investigators: Smith, Shelver, (Shappell)

Title: Environmental Chemical Residues and their Impact in the Food Supply
Project: 3060-32420-002-00D; Location: Fargo, ND
Investigators: Lupton, Shelver, (Hakk)

Title: Ecological Reservoirs and Intervention Strategies to Reduce Foodborne Pathogens in Cattle and Swine
Project: 3091-32000-033-00D: Location: College Station, TX
Investigators: Anderson, Poole, Harvey, Nisbet, Hume, Bier, Crippen, (Callaway, Edrington)

Test: Identification of the Ecological Niches and Development of Intervention Strategies to Reduce Pathogenic Foodborne Pathogens in Poultry
Project: 3091-32000-035-00D; Location: College Station, TX
Investigators: Kogut, Hume, Poole, Genovese, He, Swaggerty

Title: Novel Methods for Controlling Trichothecene Contamination of Grain and Improving the Climate Resilience of Food Safety and Security Programs
Project: 5010-42000-048-00D; Location: Peoria, IL
Investigators: McCormick, Vaughan, Ward, Proctor, Hao, (Bakker)

Title: Improved Analytical Technologies for Detection of Foodborne Toxins and their Metabolites
Project: 5010-42000-049-00D; Location: Peoria, IL
Investigators: Maragos, Appell, Busman

Title: Genomic and Metabolomic Approaches for Detection and Control of Fusarium, Fumonisins and Other Mycotoxins on Corn
Project: 5010-42000-050-00D; Location: Peoria, IL
Investigators: Proctor, Brown, Busman, Naumann, O'Donnell, Ward (Kurtzman, Peterson)

Title: Intestinal Microbial Ecology and Metagenomic Strategies to Reduce Antibiotic Resistance and Foodborne Pathogens
Project: 5030-31320-004-00D; Location: Ames, IA
Investigators: Allen, Looft, Loving, Sylte

Title: Characterization of Colonization of Shiga Toxin-Producing Escherichia coli (STEC) in Cattle and Strategies for Effective Preharvest Control
Project: 5030-32000-112-00D; Location: Ames, IA
Investigators: Kudva, Sharma

Title: Analysis of Virulence and Antibiotic Resistance Mechanisms of Salmonella and Development of Intervention Strategies
Project: 5030-32000-113-00D; Location: Ames, IA
Investigators: Bearson

Title: Antibiotic Alternatives for Controlling Foodborne Pathogens and Disease in Poultry
Project: 6022-31230-001-00D; Location: Fayetteville, AR
Investigators: Donoghue, Rath, (Huff)

Title: Monitoring and Molecular Characterization of Antimicrobial Resistance in Foodborne Bacteria
Project: 6040-32000-009-00D; Location: Athens, GA
Investigators: Jackson, Frye, Meinersmann, Berrang

Title: Characterizing Antimicrobial Resistance in Poultry Production Environments
Project: 6040-32000-010-00D: Location: Athens, GA
Investigators: Oladeinde, Meinersmann, (Cook)

Title: Reduction of Invasive Salmonella Enterica in Poultry Through Genomics, Phenomics and Field Investigations of Small Multi-Species Farm Environments
Project: 6040-32000-011-00D; Location: Athens, GA
Investigators: Guard, Rothrock

Title: Production and Processing Intervention Strategies for Poultry Associated Foodborne Pathogens
Project: 6040-32000-069-00D; Location: Athens, GA
Investigators: Buhr, Cox, Hinton, Cosby

Title: Novel Pre-Harvest Interventions and Alternatives to Antibiotics to Reduce Foodborne Pathogens Project: 6040-32000-071-00D; Location: Athens, GA
Investigators: Line, Yeh, Hinton, (Hiett)

Title: Evaluation of Management of Laying Hens and Housing Systems to Control Salmonella and Other Pathogenic Infections, Egg Contamination, and Product Quality
Project: 6040-32420-002-00D; Location: Athens, GA
Investigators: Jones, Gast

Title: Eliminating Fusarium Mycotoxin Contamination of Corn by Targeting Fungal Mechanisms and Adaptations Conferring Fitness in Corn and Toxicology and Toxinology Studies of Mycotoxins
Project: 6040-42000-043-00D; Location: Athens, GA
Investigators: Glenn, Gold, (Bacon, Riley, Voss)

Title: Develop Rapid Optical Detection Methods for Food Hazards
Project: 6040-42000-044-00D; Location: Athens, GA
Investigators: Park, Yoon, Gamble, Lawrence

Title: Genetic and Environmental Factors Controlling Aflatoxin Biosynthesis
Project: 6054-41420-008-00D; Location: New Orleans, LA
Investigators: Cary, Chang, Moore, Rajasekaran, Lebar, Gilbert (Bhatnagar)

Title: Use of Classical and Molecular Technologies for Developing Aflatoxin Resistance in Crops
Project: 6054-42000-025-00D; Location: New Orleans, LA
Investigators: Rajasekaran, Cary, Gilbert, (Brown)

Title: Molecular and Environmental Factors Controlling Aflatoxin Reduction by Non-Toxigenic Aspergillus Strains
Project: 6054-42000-026-00D; Location: New Orleans, LA
Investigators: Moore, Cary, Chang, Lebar, Gilbert, (Brown)

Title: Intervention Strategies for Controlling Human Pathogens Associated with Fermented and Acidified Vegetables
Project: 6070-41420-008-00D; Location: Raleigh, NC
Investigators: Breidt, Johanningsmeier, Perez-Diaz

Title: Design and Implementation of Monitoring and Modeling Methods to Evaluate Microbial Quality of Surface Water Sources used for Irrigation
Project: 8042-12630-011-00D; Location: Beltsville, MD
Investigators: Pachepsky, (Shelton)

Title: Zoonotic Parasites Affecting Food Animals, Food Safety, and Public Health
Project: 8042-32000-100-00D; Location: Beltsville, MD
Investigators: Santin-Duran, (Fayer)

Title: Antimicrobial Resistance and Ecology of Zoonotic Foodborne Pathogens in Dairy Cattle
Project: 8042-32000-110-00D; Location: Beltsville, MD
Investigators: Van Kessel, Haley, (Karns)

Title: Characterization and Mitigation of Bacterial Pathogens in the Fresh Produce Production and Processing Continuum
Project: 8042-32420-006-00D; Location: Beltsville, MD
Investigators: Patel, Sharma, Luo, Nou, Millner

Title: Detection and Control of Foodborne Parasites for Food Safety
Project: 8042-32420-007-00D; Location: Beltsville, MD
Investigators: Rosenthal, Dubey, (Hill)

Title: Sensing Technologies for the Detection and Characterization of Microbial, Chemical, and Biological Contaminants in Foods
Project: 8042-42000-020-00D; Location: Beltsville, MD
Investigators: Kim, Chao, Schmidt, (Lefcourt)

Title: Identification of Plant-derived Bioactive Compounds for their Potential as Alternatives to Conventional Antimicrobials
Project: 8042-42000-007-00D; Location: Beltsville, MD
Investigators: (Bhagwat)

Title Environmental and Plant Factors that Influence Trace Element Bioavailability in Food Crops
Project: 8042-42430-001-00D; Location: Beltsville, MD
Investigators: Codling, (Chaney)

Title: Integration of Multiple Interventions to Enhance Microbial Safety, Quality and Shelf-Life of Foods
Project: 8072-41000-101-00D; Location: Wyndmoor, PA
Investigators: Fan, Jin, Mukhopadhyay

Title: Bacterial Pathogens in Regulated Foods and Processing Technologies for their Elimination
Project: 8072-41420-019-00D; Location: Wyndmoor, PA
Investigators: Luchansky, Porto-Fett

Title: Development of Alternative Intervention Technologies for Fresh or Minimally Processed Foods Project: 8072-41420-020-00D; Location: Wyndmoor, PA
Investigators: Niemira, Sites, Olanya, Annous

Title: Development and Validation of Innovative Food Processing Interventions
Project: 8072-41420-021-00D; Location: Wyndmoor, PA
Investigators: Ukuku, Gurtler, (Geveke)

Title: Shiga Toxin-Producing Escherichia coli in Biofilms and Within Microbial Communities in Food Project: 8072-42000-076-00D; Location: Wyndmoor, PA
Investigators: Paoli, Chen, Uhlich (Irwin)

Title: Advanced Development of Innovative Technologies and Systematic Approaches to Foodborne Hazard Detection and Characterization for Improving Food Safety
Project: 8072-42000-077-00D; Location: Purdue U./Wyndmoor, PA
Investigators: Center for Food Safety Engineering, Purdue U./Paoli*

Title: The Role of Genotype in the Development and Validation of Growth Models and Intervention Technologies for Pathogenic Non-Shiga Toxigenic Escherichia coli Found in Foods
Project: 8072-42000-078-00D; Location: Wyndmoor, PA
Investigators: Sheen (Sommers, Rajkowski)

Title: Data Acquisition, Development of Predictive Models for Food Safety and their Associated use in International Pathogen Modeling and Microbial Databases
Project: 8072-42000-079-00D; Location: Wyndmoor, PA
Investigators: Juneja, Oscar

Title: Development, Evaluation, and Validation of Technologies for the Detection and Characterization of Chemical Contaminants in Foods
Project: 8072-42000-080-00D; Location: Wyndmoor, PA
Investigators: Lehotay, Sapozhnikova, Chen, (Perez)

Title: Development of Detection and Intervention Technologies for Bacterial and Viral Pathogens Affecting Shellfish
Project: 8072-42000-081-00D; Location: Wyndmoor, PA
Investigators: Richards, Kingsley

Title: Molecular Characterization of Foodborne Pathogen Responses to Stress
Project: 8072-42000-082-00D; Location: Wyndmoor, PA
Investigators: Paoli, Gunther, Liu, (Bhaduri, Fratamico)

Title: Development of Predictive Microbial Models for Food Safety using Alternate Approaches
Project: 8072-42000-083-00D; Location: Wyndmoor, PA
Investigators: Huang, Hwang

Title: Development of Portable Detection and Quantification Technologies for Foodborne Pathogens Project: 8072-42000-084-00D; Location: Wyndmoor, PA
Investigators: Gehring, He, Paoli, Armstrong, Capobianco, (Brewster)

Title: Food Safety Research Office
Project: 8250-88888-002-00D; Location: Beltsville, MD
Investigators: Shaw

Examples of Accomplishments and their Impact by Problem Statement

Note: Projects are listed by the Lead Scientists name, however, in all cases more than one scientist was responsible for conducting and/directing the research. Projects may have several or many objectives, and each objective is coded to address a specific Problem Statement. Therefore, it is possible that the same Lead Scientist's name and Project might be found in several sections within the review document. Accomplishments, Outcomes and Impacts, and Publications are associated with that objective.

Problem Statement 1. Population Systems

Goal

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
The goal of this research area is to identify and characterize the movement, structure, and dynamics of microbial populations (bacteria, viruses and parasites) within food-animal and plant systems, across the entire food continuum, from production through processing. At a microbial level, the diversity and complexity within environments and food matrices may alter the makeup of the populations, as well as cause change through spatial and temporal influences, or by the competitive or synergistic relationships among pathogens and commensals. Microbial populations can influence the safety of food, and the various environments in which they survive determine the success and impact of the microorganism. In turn, microorganism(s) may influence the conditions prevailing within the environment which also impacts their survival or ability to thrive. An example of identifiable area of study would include biofilms and the association of quorum sensing.

Components and emphasis for understanding and characterizing microbial populations and their environments must include epidemiology, ecology, and host-pathogen relationships. Epidemiologic studies of microbes within their environment, allows an analysis of the population therein. As such, it enables the development of improved detection methods, provides a framework for integration of microbial genomic data with disease, and a mechanism to evaluate risk factors for microbial intervention and/or control. Ecologic studies determine the attributes and changes in various communities, that is, changes to populations in the same space. Such studies allow for a better understanding of the interactions and relationships, and the transmission and dissemination of pathogens and toxins in and among food producing animals and crops. Host-pathogen relationship studies provide an understanding of the acquisition of genetic traits, such as the development and movement of resistance genes; traits connected with colonization and evolution of virulence; and the role of commensals. Where appropriate, a metagenomics approach to selected research areas will be developed to determine the attributes of the ecological communities in which pathogens are found. Knowledge of the attributes, interactions and relationships within the community in which the pathogen lives is critical to the development of control and intervention strategies.

It is critical to understand the particular ecological niches or reservoirs where pathogens may exist; and to understand the complex factors and interactions that may impact their survival and competitiveness within the gut ecosystem and the production environment. The focus of the research was to identify ecological niches or reservoirs for pathogenic and antimicrobial resistant bacteria, and the nutritional, biological, and environmental factors affecting their ability to colonize, survive, and persist within the gut of food-producing animals and their production environment.

Identification of bacterial factors and elucidation of mechanisms promoting intestinal colonization and adherence of, for example, E. coli O157:H7 (O157) and other Shiga-toxin-producing E. coli (STEC's) in beef cattle are important prerequisites for reducing fecal shedding.

In this situation, the long-term goal was to develop intervention strategies to reduce or eliminate O157 and STEC's of public health significance from the bovine intestine using a coordinated and multipronged approach. Additionally, the ability of some bacteria to form biofilms in order to colonize surfaces, a risk for cross contamination, was studied.

Research that identifies interventions that prevent or mitigate colonization of the gut of food-producing animals (particularly the lower GI tract before slaughter) or that reduce pathogenic or antimicrobial resistant bacteria in the production environment were performed. This provided an improved understanding of microbial adaption to intrinsic and extrinsic stressors on the acquisition, exchange, and expression of incompatibility plasmids and antimicrobial resistance elements in foodborne pathogens in the production and processing environments.

Population Systems: Animals

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Animal agriculture has consistently been considered a primary source of the zoonotic food-borne pathogens, Escherichia coli, Salmonella enterica, and Listeria monocytogenes that can contaminate the food chain and lead to illness in humans. The focus of this research was to elucidate the mechanisms by which bacterial pathogens enter and persist in preharvest production systems. The successful reduction of foodborne pathogens depends in part upon an understanding of the microbial communities present in settings that allow them to gain entry to the food supply, as well as their ability to survive in these settings. Biofilm formation also plays a profound role in bacterial survival and persistence in adverse conditions and thus strongly influences a pathogen's ability to cause contamination. Studies aimed at understanding the environmental signals that stimulate biofilm formation (or dissolution), as well as the molecular mechanisms underlying a pathogen's' ability to form strong biofilms, will provide valuable information, possibly identifying new targets for the successful control and reduction of foodborne pathogens in pre- and post-harvest environments.

Studies examined the symbiotic bacterial communities (microbiota) that live in the animal gastrointestinal tract and how they interact with the host immune system. This work is aimed to define the microbiota and gut mucosal immunity under both normal and disrupted conditions, and basic research to test several modulation strategies, including alternatives to traditional antibiotics.

Studies on dairy animals continued where the focus of this research was to elucidate the mechanisms by which bacterial pathogens enter and persist on dairy farms. Specifically understanding the identity, prevalence, and persistence of pathogens in dairy cows and the dairy environment, as well as the distribution of antibiotic resistance within the pathogen population will improve both meat and milk safety and lead to the development of strategies to reduce or eliminate these zoonotic bacteria in products before they leave the farm.

Research studies is this area were conducted at MARC, Clay Center, Nebraska; College Station, Texas; NADC, Ames Iowa; U.S. NPRC, Athens, Georgia; and BARC, Beltsville, Maryland.

Studies at MARC, Clay Center, NE were conducted by three projects (Bono, Bosilevac and Berry)

The (Bono) project objectives were to: (1) Molecular characterization including WGS and transcriptomic characterization of foodborne bacteria, including pathogens and commensals, exposed to various physiologically relevant conditions reflective of the production continuum; and (2) Characterize the ecological niches and reservoirs to identify mechanisms of foodborne pathogen, especially biofilms, for their ability to colonize and persist leading to the development of intervention strategies.

Examples of Accomplishments

- **Global transmission of Escherichia O157:H7 was likely facilitated by animal movement.** Shiga toxin–producing Escherichia coli (STEC) O157:H7 is a zoonotic pathogen that causes numerous food and waterborne disease outbreaks. It is globally distributed, but its origin and the temporal sequence of its geographical spread are unknown. WGS of isolates from 4 continents and subsequent analysis identified a common ancestor that originated from the Netherlands around 1890. Further analysis identified 34 major transmission events that were predominantly intercontinental, moving from Europe to Australia, the United States and Canada, and from Australia to New Zealand. Inter- and intra-continental transmission events have resulted in the current international distribution of E. coli O157:H7, and it is likely that these events were facilitated by animal movements (e.g., Holstein Friesian cattle).

- **Genomic relatedness of two strains of STEC O157:H7 that caused food-borne outbreaks**. WGS with short-read technology is being used as a method for determination of strain relatedness in investigating foodborne outbreaks. In this study, the genomes of two strains of STEC O157:H7 that caused two food-borne outbreaks associated with the same restaurant that were only 8 weeks apart were sequenced using short-read technology. Short-read sequencing of the genomes divided the outbreak strains into two sub-clusters separated by only three single-nucleotide polymorphisms in the core genome. However, traditional typing identified them as separate strains. Combined long-read sequencing approaches and optical mapping revealed that over an estimated 1-year period the two outbreak strains had undergone significant microevolution in the accessory genome by acquiring a multi-drug resistant plasmid, a prophage and genomic rearrangement. These genomic changes may account for the higher number of cases associated with the second outbreak that included multiple cases of human to human transmission.

- **Comparative genomics of Salmonella to reveal differences in virulence potential.** Salmonella Montevideo is on the CDC's list of top 10 Salmonella causing foodborne illness in the US. It is also a type of Salmonella that is frequently associated with cattle and can be found in beef products. However, S. Montevideo outbreaks are more often attributed to contaminated nuts, seeds or spices. To determine if there was a genetic basis for this distinction, 13 strains of S. Montevideo from live cattle, fresh beef and humans were sequenced and a comparative analysis of these genomes along with 72 S. Montevideo genomes from the Genbank was performed. The results showed that S. Montevideo strains fall into four distinct groups, and that while human isolates could be found in all four groups, cattle strains were restricted to one group, and were observed to lack several genes needed for making humans sick. Overall, the data suggest that the success of S. Montevideo strains associated with outbreaks may be attributed to a combination of fitness advantages for surviving in food types that are not normally cooked, and traits that make them better able to cause disease. The markers identified may be used to screen for strains of S. Montevideo that are more commonly associated with foodborne illness outbreaks, resulting in a safer food supply for consumers.

Outcomes and Impacts

- Determined how zoonotic pathogens can be spread in a global market and the potential to reduce the further spread of E. coli O157:H7 and other (emerging) STEC strains globally.
- Highlighted the value of combining different sequencing and in vitro approaches to assist investigations into the epidemiology of outbreaks.
- Provide markers for S. Montevideo that can be used to detect strains associated with foodborne illness outbreaks, resulting in a safer food supply for consumers.

Examples of Relevant Publications

- Shaaban S, Cowley LA, McAteer SP, Jenkins C, Dallman TJ, Bono JL, Gally DL. 2016. Evolution of a zoonotic pathogen: investigating prophage diversity in enterohaemorrhagic *Escherichia coli* O157 by long-read sequencing. Microb Genom 2:e000096. 10.1099/mgen.0.000096.
- Cowley LA, Dallman TJ, Fitzgerald S, Irvine N, Rooney PJ, McAteer SP, Day M, Perry NT, Bono JL, Jenkins C, Gally DL. 2016. Short-term evolution of Shiga toxin-producing *Escherichia coli* O157:H7 between two food-borne outbreaks. Microbial Genomics 2:e0.000084. https://doi:10.1099/mgen.0.000084.
- Franz E, Rotariu O, Lopes BS, MacRae M, Bono JL, et al. 2018. Phylogeographic analysis reveals multiple international transmission events have driven the global emergence of *Escherichia coli* O157:H7. Clin Infect Dis 69:428-437. 10.1093/cid/ciy919.
- Nguyen SV, Harhay DM, Bono JL, Smith TPL, Fields PI, Dinsmore BA, Santovina M, Wang R, Bosilevac JM, and Harhay GP. 2018. Comparative genomics of *Salmonella enterica* serovar Montevideo reveals lineage-specific gene differences that may influence ecological niche association. Microbial Genomics. 4:1-17. https://doi: 10.1099/mgen.0.000202)

- Fitzgerald, S., Beckett, A.E., Palarea-Albaladejo, J., McAteer, S., Shaaban, S., Morgan, J., Ahmad, N.I., Young, R., Mabbott, N., Morrison, L., Bono, J.L., Gally, D.L., McNeilly, T.N. 2019. Shiga toxin sub-type 2a increases the efficiency of *Escherichia coli O157* transmission between animals and restricts epithelial regeneration in bovine enteroids. PLOS Pathogens. Accepted 7-25-2019.

The (Bosilevac) project objective was: (1) Identify environmental and management practices that influence antimicrobial resistance, colonization of lymph nodes, and colonization rates of cattle, veal, and swine;

Examples of Accomplishments

- **High Event Period (HEP) and Escherichia coli O157:H7.** HEP is defined as a time period when beef processors experience an increased occurrence of product contamination by E. coli O157:H7. The contamination mechanism and source of the pathogen responsible for HEPs is unknown. E. coli O157:H7 strains isolated during HEPs were characterized for their biofilm forming ability, sanitizer resistance, and the genetic basis for these traits. Compared to control strains, the HEP strains had a higher biofilm-forming ability and lower sanitizer susceptibility. Moreover, the HEP strains retained higher copy numbers of the pO157 plasmid, suggesting that this plasmid may possess the genetic locus for the HEP strains' enhanced ability to survive in processing plants and cause contamination.

- **Salmonella are present in peripheral lymph nodes of healthy cattle at slaughter.** Beef carcass lymph nodes have been identified as a potential source of human exposure to Salmonella when fat trim containing these nodes is incorporated into ground beef formulations. Peripheral lymph nodes were collected at slaughter from healthy feedlot cattle and healthy cattle culled from breeding herds. Salmonella was recovered from 5.6% of all cattle lymph nodes across all cattle sources, with 2.9% of all lymph nodes having quantifiable (more than 100 CFU) Salmonella present. The majority (80.6%) of the Salmonella were neither resistant to any antimicrobial agents, nor of serotypes commonly reported by the CDC in human disease.

- **Shiga toxin-producing E. coli (STEC) and Salmonella contamination problems in veal processing.** The USDA FSIS has increased scrutiny of bob veal (calves less than 3 weeks old) and formula-fed veal (calves 20 weeks in age) because a higher percentage of positive tests for adulterant STEC were found in veal compared to beef. FSIS has also reported that Salmonella was more often found in bob veal than formula fed veal. The levels and prevalence of E. coli O157:H7, non-O157 STEC, and Salmonella on veal hides and carcasses were measured at five veal processors. A year later, follow-up samples were collected at three of the processors. Significantly more non-O157 STEC were found on all veal hides and carcasses than E. coli O157:H7, as compared to beef where the opposite is observed. The follow-up samples showed that processing practices had improved and less STEC and Salmonella were detected on carcasses. A greater proportion of bob veal was found to be contaminated by STEC and

Salmonella compared to formula-fed veal. However, the strains of Salmonella found on bob veal were types rarely seen in human illness, whereas formula-fed veal had strains more often linked to severe human illnesses.

Outcomes and Impacts

- Biofilm-forming ability and sanitizer resistance of HEP O157:H7 strains highlights the potential role of biofilm formation and sanitizer resistance in HEP contamination of beef by E. coli O157:H7 and reveals a potential molecular mechanism for HEP strain's enhanced survival.
- FSIS has begun studies to determine the potential risk of Salmonella in lymph nodes of cattle. The USDA-Agricultural Marketing Service (AMS) has implemented policies to remove the major lymph nodes from beef cuts before they can be used by the School Lunch Program. The studies increase our understanding of the sources of Salmonella contamination and sheds light on transmission dynamics that may be useful in targeting interventions to prevent foodborne illness resulting from contaminated beef.
- Validated that efforts made by veal processors have improved the safety of veal, however the results indicate further efforts to control Salmonella are necessary from both bob and formula-fed veal processors.

Examples of Relevant Publications

- Wang, R., Luedtke, B.E., Bosilevac, J.M., Schmidt, J.W., Kalchayanand, N., Arthur, T.M. 2016. Escherichia coli O157:H7 strains isolated from High Event Period beef contamination have strong biofilm-forming ability and low sanitizer susceptibility, which are associated with high pO157 plasmid copy number. Journal of Food Protection. 79(11):1875-1883. https://doi:10.4315/0362-028X.JFP-16-113.
- Webb, H.E., Harhay, D.M., Brashers, M.M., Nightengale, K.K., Arthur, T.M., Bosilevac, J.M., Kalchayanand, N., Schmidt, J.W., Wang, R., Granier, S.A., Brown, T.R., Edrington, T.S., Shackelford, S.D., Wheeler, T.L., Loneragan, G.H. 2017. Salmonella in peripheral lymph nodes of healthy cattle at slaughter. Frontiers in Microbiology. 8:2214. https://doi.org/10.3389/fmicb.2017.02214.
- Bosilevac, J.M., Wang, R., Luedtke, B. E., Hinkley, S., Wheeler, T.L., Koohmaraie, M. 2017. Characterization of enterohemorrhagic Escherichia coli on veal hides and carcasses. Journal of Food Protection. 80(1):136-145. https://doi:10.4315/0362.028X.JFP-16-247.
- Bosilevac, J.M., Zhilyaev, S., Wang, R., Luedtke, B.E., Wheeler, T.L., Koohmaraie, M. 2019. Prevalence and Characterization of Salmonella Present during Veal Harvest. J Food Prot. May;82(5):775-784. https://doi: 10.4315/0362-028X.JFP-18-478.

The (Berry) project addressed the objective to: (1) Determine the genotypic and/or phenotypic factors associated with the levels and persistence of pathogens and antibiotic resistance in the host animal and the livestock production environment.

Examples of Accomplishments

- **Identification of gastrointestinal Escherichia coli O157:H7 colonization differences between low shedding and super-shedding cattle.** Both the presence and levels of E. coli O157:H7 throughout the GIT of low-shedding and super-shedding cattle were determined at harvest. All animals had similar levels of E. coli O157:H7 in samples collected from the mouth. The digesta from the rumen, small intestine, cecum, and large intestine locations was rarely enumerable, regardless if the animal was a low- or super-shedder. However, animals that were low shedders had lower prevalence for E. coli O157:H7 in the digesta samples from the rumen and from all locations of the intestinal tract compared to animals that were super-shedders. These latter results suggest that low-shedding animals might exhibit intrinsic factors that reduce E. coli O157:H7 passage and colonization, whereas super-shedders appear to exhibit colonization throughout the lower intestinal tract. Super-shedders were always highly colonized at the recto-anal junction, but high enumerable levels of E. coli O157:H7 were also observed in the digesta of the rectum indicating that mucosa of the terminal colon and rectum may also be major colonization sites.

- **Bacterial diversity in the bovine colon and impact on Escherichia coli O157:H7 shedding by cattle.** Much research has described the bacteria in the bovine rumen, but until recently, little research has described the bacteria in the bovine colon. Rumen bacterial populations are more complex and robust than previously reported, and diet was shown to have a significant impact on the structure and composition of the bacterial microbiota. In addition, previously unknown ruminal bacterial populations that directly impact animal performance and health were identified. Colonic bacterial populations were significantly different than the ruminal bacterial populations, and very few ruminal bacteria were observed in the lower gastrointestinal tract and the colon. When the microbiome composition of bovine feces was analyzed, the effect of the fecal microbiome on E. coli O157:H7 shedding appears to be limited to a small set of bacterial types, and these populations are strongly influenced by diet. The colonic microbiota is recognized to be important for animal health and well-being, and this work provides a foundation for understanding why the bacterial composition of this gastrointestinal location in cattle is unique, and how it changes with diet, antibiotic use, and can potentially be manipulated to reduce E. coli O157:H7 in cattle.

- **Escherichia coli O157:H7 on feedlot pen surfaces of cattle fed distillers grains**. Feeding wet distillers' grains with solubles (WDGS) to cattle can increase the load of the foodborne pathogen E. coli O157:H7 in feces and on hides, but the reasons are not fully understood. Research was conducted to explore a role for environmental persistence of E. coli O157:H7 in increasing this pathogen in cattle fed WDGS. Greater persistence of E. coli O157:H7 on the pen surfaces of animals fed 40% WDGS was not demonstrated, however these pens had higher prevalence of E. coli O157:H7 in feedlot

surface manure after cattle were removed. Hence, attention to factors affecting both shedding and environmental contamination may be needed to reduce E. coli O157:H7 in cattle, and to reduce the risk of human foodborne illness.

- **Beef cattle dietary supplements and Escherichia coli O157:H7 shedding.** Previous research has indicated that both monensin and beta-agonists might impact fecal shedding of E. coli O157:H7. A series of multi-year studies was conducted with these dietary supplements in which E. coli O157:H7 in bovine feces was evaluated using both enrichment (presence/absence) and enumeration testing. Neither dietary monensin nor beta-agonists appeared to impact fecal presence or absence for E. coli O157:H7 in the studies and feeding beta-agonists had no significant impact on fecal concentrations for E. coli O157:H7. However, feeding monensin at the highest allowable level increased the percentage of animals that were shedding E. coli O157:H7 at high levels, although the average concentration per animal was not significantly affected.

- **Source of dietary protein affects fecal shedding of Escherichia coli O157:H7 in cattle.** Previous studies determined that cattle diets with high levels of distillers' grains led to increased fecal shedding of E. coli O157:H7, but low levels did not have any effect. To determine if increased shedding was due to high protein or to distillers' grains in the diet, a series of studies was designed to evaluate this question. Supplementation of even moderate levels of wet distillers' grain with solubles appear to increase E. coli O157:H7 shedding from cattle, whereas cattle fed diets supplemented with soybean meal appeared to have reduced shedding for E. coli O157:H7. Dietary protein does not appear to be a factor in fecal shedding of E. coli O157:H7, but supplementation with soybean meal may be beneficial to reducing fecal shedding of this pathogen by cattle.

- **Escherichia coli O157:H7 transmission by cattle pest flies found in leafy greens.** Delicate fresh produce items like leafy greens are normally consumed raw, making it especially critical to protect fresh produce from pathogen contamination in the preharvest environment. E. coli O157:H7-positive pest flies of several species (house, face, blow, and flesh flies) were common in leafy greens planted up to 600 feet from a beef cattle feedlot. The isolation of closely-related E. coli O157:H7 isolates from the feedlot pen surface, flies, and leafy greens suggests that flies can transport this pathogen from cattle production to nearby produce crops. This research provided a detailed study of the occurrence of E. coli O157:H7-positive flies and their potential risk to transmit this pathogen to leafy greens grown near a cattle feedlot, as well as a starting point for determining set-back distances to protect leafy greens from this possible contamination route.

Outcomes and Impacts

- Determined the presence and levels of E. coli O157:H7 throughout the GIT at harvest. Low-shedding animals exhibit intrinsic factors that reduce passage and colonization, whereas super-shedders exhibit colonization throughout the lower GIT. Super-shedders were highly colonized at the RAJ, although E. coli O157:H7 were found in the digesta of the rectum indicating the terminal colon and rectum maybe major colonization sites.

- Determined that rumen microbiota is very complex than previously recognized, with diet having a significant impact. Identified previously unknown ruminal bacterial populations that impacted animal performance and health. Colonic bacterial populations were significantly different than the ruminal bacterial populations.
- Determined that the effect of fecal microbiome on E. coli O157:H7 shedding appears to be limited but strongly influenced by diet.
- Explored a role for environmental persistence. Greater persistence on the pen surfaces of animals fed 40% WDGS was not demonstrated, however these pens had higher prevalence of E. coli O157:H7 in feedlot surface manure after cattle were removed.
- Determined that neither dietary monensin nor beta-agonists impacted fecal presence or absence of E. coli O157:H7. Feeding beta-agonists had no significant impact on fecal concentrations of E. coli O157:H7. Feeding high levels of monensin increased the percentage of animals shedding although the average concentration per animal was not significantly affected.
- Determined that dietary protein was not a factor in E. coli O157:H7 shedding, however, supplementation with soybean meal may be beneficial to reducing fecal shedding.
- Determined that E. coli O157:H7-positive pest flies could contaminate leafy greens planted up to 600 feet from a beef cattle feedlot. The study detailed the potential risk of produce contamination which will affect FDA produce set-back distance guidelines under the Food Modernization Act.

Examples of Relevant Publications

- Myer, P.R., Wells, J.E., Smith, T.P.L., Kuehn, L.A., Freetly, H.C. 2016. Microbial community profiles of the jejunum from steers differing in feed efficiency. Journal of Animal Science. 94(1):327-338. https://doi.org/10.2527/jas.2015-9839.
- Myer, P. R., Kim, M., Freetly, H. C., Smith, T.P.L. 2016. Evaluation of 16S rRNA amplicon sequencing using two next-generation sequencing technologies for phylogenetic analysis of the rumen bacterial community in steers. Journal of Microbiological Methods. 127(1):132-140. https://doi.org/10.1016/j.mimet.2016.06.004.
- Kim, M., Kuehn, L.A., Bono, J.L., Berry, E.D., Kalchayanand, N., Freetly, H.C., Benson, A.K., Wells, J. 2017. The impact of the bovine faecal microbiome on Escherichia coli O157:H7 prevalence and enumeration in naturally infected cattle. Journal of Applied Microbiology. 123:1027-1042. https://doi.org/10.1111/jam.13545.
- Myer, P. R., Freetly, H. C., Wells, J. E., Smith, T.P.L., Kuehn, L. A. 2017. Analysis of the gut bacterial communities in beef cattle and their association with feed intake, growth, and efficiency. Journal of Animal Science. 95(7):3215-3224. https://doi.org/10.2527/jas.2016.1059.
- Wells, J.E., Berry, E.D., Kim, M., Shackelford, S.D., Hales, K.E. 2017. Evaluation of commercial ß-agonists, dietary protein, and shade on fecal shedding of Escherichia coli O157:H7 from feedlot cattle. Foodborne Pathogens and Disease. 14(11):649-655. https://doi.org/10.1089/fpd.2017.2313.

- Berry, E.D., Wells, J.E., Varel, V.H., Hales, K.E., Kalchayanand, N. 2017. Persistence of Escherichia coli O157:H7 and total Escherichia coli in feces and feedlot surface manure from cattle fed diets with or without corn or sorghum wet distillers grains with solubles. Journal of Food Protection. 80(8):1317-1327. https://doi.org/10.4315/0362-028X.JFP-17-018.
- Hales, K.E., Wells, J., Berry, E.D., Kalchayanand, N., Bono, J.L., Kim, M.S. 2017. The effects of monensin in diets fed to finishing beef steers and heifers on growth performance and fecal shedding of Escherichia coli O157:H7. Journal of Animal Science. 95(8):3738-3744. https://doi.org/10.2527/jas2017.1528.
- Melchior, E.A., Hales, K.E., Lindholm-Perry, A.K., Freetly, H.C., Wells, J.E, Hemphill, C.A., Wickersham, T.A., Sawyer, J.E., Myer, P.R. 2018. The effects of feeding monensin on rumen microbial communities and methanogenesis in bred heifers fed in a drylot. Livestock Science. 212:131-136. https://doi.org/10.1016/j.livsci.2018.03.019.
- Paz, H.A., Hales, K.E., Wells, J.E., Kuehn, L.A., Freetly, H.C., Berry, E.D., Flythe, M.D., Spangler, M.L., Fernando, S.C. 2018. Rumen bacterial community structure impacts feed efficiency in beef cattle. Journal of Animal Science. 96(3):1045-1058. https://doi.org/10.1093/jas/skx081.
- Berry, E.D., Wells, J.E., Durso, L.M., Friesen K.M., Bono, J.L., Suslow, T.V. 2019. Occurrence of Escherichia coli O157:H7 in pest flies captured in leafy greens plots grown near a beef cattle feedlot. Journal of Food Protection. 82(8):1300-1307. https://doi.org/10.4315/0362-028X.JFP-18-601.

Poultry studies by two sister projects (Kogut/Byrd) at College Station, TX were combined (Kogut) (due to several scientist retirements/resignations from ARS). The objectives were to: (1) Investigate the interaction between yeast and fungi and foodborne bacteria to determine their role as commensals and inhibitors or their use as alternatives to antibiotics as pre-and probiotics; and (2) Identify ecological reservoirs of pathogens and the potential role of dispersal of animal waste that enable the retention of foodborne pathogens within animal production facilities and the surrounding environments.

Examples of Accomplishments

- **Identification of bacterial and fungal of populations poultry digestive tract.** Current poultry management programs are standardized on single production areas, called poultry production complexes. However, poultry production parameters and food safety parameters can vary dramatically between the individual farms within a complex. Studies identified 14 bacterial and 28 fungal populations that occur in high-producing versus low-producing farms or in Salmonella-positive versus Salmonella-negative farms.

- **Ecology of the poultry gastrointestinal (GI) microbiome**. Studies compared GI bacterial communities by sample type (fecal or cecal), time (1, 3, and 6 weeks post-hatch), and experimental pen (1, 2, 3, or 4), and measured cecal mRNA transcription of the cytokines IL18, IL1β, and IL6, IL10, and TGF-β4. Significant differences were observed in the microbiome by GI location (fecal versus cecal) and bird age. Increasing

community complexity through time were observed in increased taxonomic richness and diversity. IL18 and IL1β significantly increased to maximum mean expression levels 1.5-fold greater at week 3 than 1, while IL6 significantly decreased to 0.8- and 0.5-fold expression at 3- and 6-week post-hatch, respectively relative to week 1. Transcription of pro-inflammatory cytokines (IL-18, IL1β, IL6) were negatively correlated with the relative abundance of various members of the phylum Firmicutes and positively correlated with Proteobacteria.

Outcomes and Impacts

- Showed that fungal and bacterial populations differ between poultry houses exhibiting high or low Salmonella loads. Stresses the importance of management factors and population characterization to understand pathogen persistence
- Determined correlations of the microbiome with specific cytokine mRNA transcription highlighting the importance of the GI microbiome for bird health and productivity. This may be a successful high-throughput strategy to identify bacterial taxa with specific immune-modulatory properties.

Examples of Relevant Publications

- Oakley, B.B., Kogut, M.H. 2016. Spatial and temporal changes in the broiler chicken cecal and fecal microbiomes and correlations of bacterial taxa with cytokine gene expression. Frontiers in Veterinary Infectious Diseases. https://doi: 10.3389/fvets.2016.00011.
- Broom, L.J., Kogut, M.H. 2018. The role of the gut microbiome in shaping the immune system of chickens. Veterinary Immunology and Immunopathology. 204:44-51. https://doi.org/10.1016/j.vetimm.2018.10.002.
- Kogut, M.H. 2018. The Effect of Microbiome Modulation on the Intestinal Health of Poultry. Animal Feed and Technology 250:32-40. https://doi: 10.1016/anifeedsci.2018.10.008.

Studies at NADC. Ames, IA (Allen) addressed the objective to: (1) Evaluate environmental and host influences on gut bacterial ecological niches and foodborne pathogen control strategies, including vaccines, on phenotypic and genotypic characteristics of foodborne pathogens.

Examples of Accomplishments

- **Campylobacter jejuni responds to animal mucus for environment specific adaptations.** Campylobacter jejuni (C. jejuni) is the leading cause of bacterial foodborne illness in the US and typically occurs after ingesting C. jejuni contaminated poultry products. Mucus lines the intestinal tract where C. jejuni grows, and mucus can serve as an energy source for the bacteria. C. jejuni responded differently when grown on chicken or turkey mucus, compared to cow, pig, or sheep mucus. Changes were associated with factors essential for survival in the poultry gut. Binding of C. jejuni to intestinal cells was also altered by the source of mucus, suggesting that mucus may be an environmental cue for C. jejuni growth and attachment to intestinal epithelial cells. C.

jejuni usage of mucus is a target to modulate C. jejuni colonization in poultry food animals.

- **Experimental model of Campylobacter jejuni intestinal colonization in commercial turkeys.** Campylobacter jejuni is the main bacterial foodborne disease in humans and ingesting contaminated poultry products is the most common route of human exposure. Lack of a reliable challenge model limits Campylobacter food-safety studies in turkeys. A research model was developed using contemporary strains of C. jejuni in commercial turkeys, which highly colonized the cecum. Campy-Line agar with sulfamethoxazole and ChromeAgar Campylobacter were equally reliable to enumerate the number of Campylobacter from turkey intestinal samples. The turkey-Campylobacter colonization model is necessary to evaluate pre- and post-harvest interventions for the reduction of Campylobacter in turkeys to promote food safety.

Photo: Dr. Looft and Dr. Sylte, with a Beltsville White Turkey used in the modeling study

Outcomes and Impacts

- Discovered that mucus can serve as an energy source for Campylobacter jejuni. Binding of C. jejuni to intestinal cells was altered by the source of mucus, suggesting that mucus may be an environmental cue for C. jejuni growth and attachment to intestinal epithelial cells, and therefore a target for colonization intervention.

- Developed a research colonization model using strains of C. jejuni in commercial turkeys, which highly colonized the cecum. The model is necessary to evaluate pre- and post-harvest interventions for the reduction of Campylobacter.

Examples of Relevant Publications

- Looft, T.P., Cai, G., Choudhury, B., Lai, L.X., Lippolis, J.D., Reinhardt, T.A., Sylte, M.J., Casey, T.A. 2019. Avian intestinal mucus modulates *Campylobacter jejuni* gene expression in a host-specific manner. *Frontiers in Microbiology*. 9:3215. https://doi.org/10.3389/fmicb.2018.03215.
- Sylte, M.J., Inbody, M.H., Johnson, T., Looft, T.P., Line, J.E. 2018. Evaluation of different *Campylobacter jejuni* isolates to colonize the intestinal tract of commercial turkey poults and selective media for enumeration. *Poultry Science*. 97(5):1689-1698. https://doi.org/10.3382/ps/pex384

Studies at U.S. NPRC, Athens, GA were addressed by two projects (Guard and Line).

The (Guard) project objectives were to: (1) Identify the environmental drivers impacting the presence and variability of Salmonella enterica serotypes and other common food borne pathogens within local, natural, multi-use poultry production systems; (2) Determine the linkage between phenotypes and genotypes of Salmonella enterica to find markers associated with colonization or invasion in chickens, as well as patterns of antibiotic resistances present in the poultry production environment; (3) Test mixtures of Salmonella enterica serotypes that vary in their ability to invade and colonize hens to determine the ability of commensal-like serotypes reduce the ability of pathogenic serotypes to colonize and persist. This information will be used to assess and improve vaccination strategies and reduce the use of antibiotics; and (4) Determine the impact of infectious dosage of the various Salmonella enterica isolates on their ability to colonize and persist in egg-laying hens to facilitate their detection and reduction in poultry.

Examples of Accomplishments

- **Poultry management systems were associated with a low incidence of foodborne Salmonella serotypes on the final product.** Alternative poultry management systems are increasing in prevalence, and now account for about 20% of the U.S. poultry market. Studies followed 42 pastured poultry flocks over 4 years using a farm-to-fork approach to isolate and characterize Salmonella serotypes from live production, processing, and final retail product samples. Serotypes more commonly associated with foodborne illness accounted for 15% of the >2100 samples collected, and only 0.4% of these were isolated from the final retail product; however, serotype Kentucky, which is not associated with foodborne illness, was the most dominant serotype recovered overall and on the final retail product (73.6% and 95.9 % of recovered Salmonella, respectively).

- **Sequence-based serotyping of Salmonella enterica identifies fundamental biology.** Only 30 out of 1500 serotypes of Salmonella enterica are frequently associated with causing foodborne illness. To better understand how Salmonella serotype diversity impacts foodborne illness, dkgB-linked Intergenic Sequence Ribotyping (ISR) was applied and used to determine that i) the house mouse is a historical carrier of at least 7 major foodborne Salmonella serotypes on-farm, and ii) serotypes Enteritidis and Typhimurium undergo chromosomal exchange, or homologous recombination, more often than do other serotypes.

- **Protein source for pasture-raised broiler impacts gut microbiome.** The elimination of soy-based protein from alternatively grown broiler diets is increasing, but the animal and public health effects of this diet shift is relatively unknown. While changes were observed in the general gut microbiomes between pastured broilers fed soy and soy-free diets during live production, most of these changes were attributable to maturation of the broilers during their time on pasture, and not the protein source in the feed. However, when observing the postharvest processing (ceca, whole carcass rinses) and final product (whole carcass rinses) samples, significant microbiome changes were observed between diets. Specifically, Campylobacter prevalence was significantly lower on the final product samples from the soy-free flocks.

- **Salmonella enterica phenotypes to prevent resistance to food preservatives and to make better vaccines.** Phenotypic microarray (PM) analysis of the complex metabolism of organisms is a relatively new approach that was applied to Salmonella enterica. Two biological factors evaluated were i) the protein SefD, which undergoes naturally-occurring mutation in a manner that alters the ability of serotype Enteritidis to cause disease, and ii) acid resistance because of the use of acids as food preservatives. Results were: i) expression of SefD drastically altered metabolism, and ii) several pathogenic Salmonella serotypes were found that appeared to have coordinated resistance to acid (pH 4.5) and salts (sodium lactate and sodium chloride).

- **Salmonella enterica serovar Enteritidis genes associated with survival and growth in eggs and in the poultry environment.** Contamination of the internal contents of eggs is a substantial food safety problem. In collaboration with the FDA, ARS performed WGS of a historical collection of 91 isolates of Salmonella enterica serotype Enteritidis obtained from mice caught on-farm during the 1990s. Primary findings were that strains that had i) no antibiotic resistances and ii) a complete sefD gene had, respectively, uniform survival in albumen and better growth in yolk. Additionally, experiments conducted in egg-laying hens found that strains that efficiently colonize the cecum of chickens were more likely to have mutations in the sulfur assimilation and degradation genes tcyP, dsdA and cysN.

- **Predictive algorithms for predicting Listeria spp. prevalence.** In order to predict the prevalence of Listeria species during pastured poultry production, studies were conducted using random forest modeling in combination with questionnaire-based farm management data and meteorological data for the origin farms. Modeling showed that time of year the flock was on pasture and the age of the broiler flock were major farm management drivers for Listeria prevalence in preharvest samples, while brood feed and chlorination of the processing rinse water were the most relevant drivers of Listeria prevalence in postharvest samples. Average minimum temperature and average humidity over the 3-4 days prior to sampling were the variables that most closely correlated to Listeria prevalence in preharvest samples.

- **Antibiotic resistances of foodborne bacterial pathogens varied by farm.** Fifteen (15) all-natural, antibiotic-free broiler poultry flocks raised on pasture from 6 different small farms throughout the 2014 growing season were sampled. Isolates examined were Salmonella, Campylobacter, Listeria, and E. coli, and isolates from a variety of environmental samples were collected. The antibiotic resistance patterns were found to be diverse among the different isolates, with resistance profiles being farm-specific under certain circumstances (e.g. Salmonella).

Outcomes and Impacts

- A unique distribution of Salmonella serotypes was found within pastured poultry management systems; however, pasture management produces a final product as safe as that produced from larger systems.
- Intergenic Sequence Ribotyping (ISR) has been adopted by diagnosticians and service providers both nationally and internationally (Columbia, Brazil, Peru).
- There is a need for further research into the effects of removing soy-based proteins from pasture-raised broiler diets; noting how management decisions can have unexpected food safety consequences downstream.
- Resistance of Salmonella to commonly used preservatives could alter the way food is handled and stored. Producers now have knowledge that preventing resistance to food preservatives is on par with avoiding resistance to antibiotics.
- Analysis of SefD led to the development of a better killed vaccine, and a new hypothesis that it was a “burr-like” fimbrial protein was involved in the transfer of serotype Enteritidis from the environment to adherence in the oral mucosa of hens.
- It appears important for serotype Enteritidis to be able to balance sulfurous intermediates that can be toxic to the bacterial cell while it is within the GI tract of the chicken.
- Models will help pastured poultry farmers understand the variables that impact the safety of their products, with the ultimate goal of providing them with management targets that can be easily controlled to reduce food safety issues within their flocks.
- Antibiotic resistance genes and adherence-associated genes undergo natural variation in SE, which indicates that evolution of the pathogen on-farm can occur rapidly and continuously.

- Stress the importance of including background antibiotic resistance profiling and also multi-drug resistance, can be found on farms that have never used antibiotics during production.

Examples of Relevant Publications

- Guard, J., Abdo, Z., Byers, S.O., Kriebel, P., Rothrock, M.J., Jr. 2016a. Subtyping of *Salmonella enterica* subspecies I, using single-nucleotide polymorphisms in Adenylate Cyclase. Foodborne Pathogens and Disease 13, 350-362. https://www.ncbi.nlm.nih.gov/pubmed/27035032.
- Guard, J., Rothrock, M.J., Shah, D.H., Jones, D.R., Gast, R.K., Sanchez-Ingunza, R., Madsen, M., El-Attrache, J., Lungu, B. 2016b. Metabolic parameters linked by phenotype microarray to acid resistance profiles of poultry-associated *Salmonella enterica*. Research in Microbiology 167, 745-756. https://www.ncbi.nlm.nih.gov/pubmed/27418207.
- Locatelli, A., Lewis, M.A., Rothrock, M.J., Jr. 2017. The Distribution of Listeria in Pasture-Raised Broiler Farm Soils Is Potentially Related to University of Vermont Medium Enrichment Bias toward *Listeria innocua* over *Listeria monocytogenes*. Front Vet Sci 4, 227. https://www.ncbi.nlm.nih.gov/pubmed/29312967.
- Guard, J., Henzler, D.J., Ramadan, H., Jones, D.R., Gast, R.K., Davison, S., Allard, M. 2018. Serotyping of *Salmonella enterica* isolated from mice caught on US poultry farms 1995 through 1998. Food Safety 6, 44-50. https://doi.org/10.14252/foodsafetyfscj.2017022.
- Lourenco, J.M., Rothrock Jr, M.J., Sanad, Y.M., Callaway, T.R. 2019b. The effects of feeding a soybean-based or a soy-free diet on the gut microbiome of pasture-raised chickens throughout their lifecycle. Frontiers in Sustainable Food Systems 3. https://doi.org/10.3389.fsufs.2019.00036.
- Rothrock Jr, M.J., Locatelli, A., Feye, K.M., Caudill, A.C., Guard, J., Hiett, K.L., Ricke, S.C. 2019. A Microbiomic Analysis of a Pasture-Raised Broiler Flock Elucidates Foodborne Pathogen Ecology Along the Farm-To-Fork Continuum. Frontiers in Veterinary Science 6. https://doi.org/10.3389/fvets.2019.00260.
- Golden, C.E., Rothrock, M.J., Jr., Mishra, A. 2019b. Comparison between random forest and gradient boosting machine methods for predicting *Listeria spp*. prevalence in the environment of pastured poultry farms. Food Res Int 122, 47-55. https://www.ncbi.nlm.nih.gov/pubmed/31229101.
- Guard, J., Cao, G., Luo, Y., Baugher, J.D., Davison, S., Yao, K., Hoffmann, M., Zhang, G., Likens, N., Bell, R.L., Zheng, J., Brown, E., Allard, M. 2019. Genome sequence analysis of 91 *Salmonella* Enteritidis isolates from mice caught on poultry farms in the mid-1990s. Genomics. https://www.ncbi.nlm.nih.gov/pubmed/30974149.

Two sister projects (Hiett/Line) were combined (Line) due to several retirements/resignations. The objective was to: (1) Establish changes in the microbial ecology along the "local" farm to fork continuum, including performing microbiome analyses on samples to establish changes in the microbial ecology along the "local" farm to fork continuum.

Examples of Accomplishments

- **Determining the bacterial communities present in broiler ceca.** Poultry production is a major agricultural output worldwide. It is known that the gut health of broilers is essential for their growth and for providing wholesome products for human consumption. Previously, the microbial diversity of broiler ceca was studied at the genetic level. However, the functional diversity and metabolic activity of broiler cecal bacterial communities are not fully investigated. Recently, EcoPlates™ from Biolog, Inc. have been used for characterizing bacterial communities from various environments. In this study, we utilized these plates to physiologically profile cecal bacterial communities in broilers. The results show sigmoidal growth curves with three phases in all 12 cecal samples. Cecal bacterial communities could not use 11 carbon substrates for carbon sources; instead, they used pyruvic acid methyl ester, glycogen, glucose-1-phosphate and *N*-acetyl-*D*-glucosamine most frequently.

Outcomes and Impacts

- Determined that each bacterial community metabolized various numbers of the substrates at different rates among broilers. Future studies on modification of culture conditions to mimic the gut environment are needed to determine the effects of nutrients; on Salmonella or Campylobacter on physiological functions of cecal bacterial communities.

Examples of Relevant Publications

- Yeh, H., Line, J.E., Hinton, A. 2019. Community-level physiological profiling for microbial community function in broiler ceca. Current Microbiology. 76: 173-177.

Population Systems: Produce

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR); https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf *Most of fresh produce eaten is safe, and while there has reputedly been a reduction in cases of foodborne illnesses linked to produce, the numbers are still unacceptably high, and even more alarming in their impact. For example, even in 2018/2019 with the implementation of the Produce Safety Rule (PSR) as part of the FDA-FSMA, fresh produce (romaine lettuce) was linked to two multistate outbreaks of STEC O157:H7 with more than 150 cases and several deaths; several other major outbreaks occurred (mixed salad: 511 confirmed cases/15 states; vegetable trays 250 confirmed cases/4 states) due to the Cyclospora parasite; Salmonella spp. in melon and sprouts; STEC E. coli O121 in sprouts 5 states; and in 2019 Salmonella in pre-cut melon and fruit medley ~120 people, 32 hospitalized from 10 states.*

One of the roles of the Program has been to provide the scientific-basis for practices/criteria in existing GAPs (Good Agricultural Practices) within PSR/FSMA, and other industry commodity -specific guidance documents. The goal is to reduce the risk of pathogen contamination from various sources, which requires specific evaluation to support development of national standards by action agencies.

There were several studies within this objective, both pre- and post-harvest. Studies investigated sources of contamination of fresh produce at the farm level namely, irrigation water, compost, soil amendments, insect as vectors, and other issues. The transfer of pathogens from these sources to foliar surfaces, and the effect of contamination level. Intervention strategies to reduce contamination at the farm level were also developed and evaluated at the field level (refer to Problem Statement 5).

Studies were further conducted to develop plant-microbe model systems: for example to identify and characterize the microbial genes that are involved in the attachment, colonization and survival of enteric pathogens on produce; to determine the genetic and biochemical factors in plants that effect the attachment, growth and survival of human pathogens in/on plants; to assess the role of other microflora and aerosols in survival and transmission of enteric pathogens in agricultural environments; and to develop methods for the detection of enteric viral and bacterial pathogens from produce and soil.

Studies at WRRC, Albany, CA were conducted through a large multidisciplinary project (Gorski) whose objectives were to: (1) Identify environmental factors that affect the persistence and transmission of enteric pathogens in the produce production environment for risk assessment.; and (2) Study the ecology of Shiga toxin-producing E. coli (STEC) bacteriophages and its association with bacterial hosts.

Examples of Accomplishments

- **Prevalence of enteric pathogens in public access watersheds near leafy green growing regions of California.** An extensive effort in the Salinas, CA region provided five years of STEC, Salmonella, Listeria monocytogenes, norovirus and phage prevalence data during a collaborative project funded by FDA/CFSAN for a predictive geospatial risk assessment model (PGRAM) to estimate the spread of pathogens in 5-watersheds in this important produce production environment. Samples were taken weekly and more than 3,000 were processed from 30 locations. Results indicated considerable pathogen prevalence, especially with Salmonella and L. monocytogenes at 57% and 44% respectively. In contrast, the other pathogens were recovered in 7-13% of the samples. Also, the prevalence varied considerably with respect to pathogen type, sample location and season. In addition, cattle presence and number were provided in the vicinity of the watershed sample sites.

- **Model to predict E. coli O157:H7 persister cells on lettuce**. Contamination of lettuce with pathogenic E. coli causes human illness and death, and impacts lettuce production in the US, which is valued at $2 billion per year. Little is understood about the survival of this pathogen on lettuce despite its poor fitness on plants in the field. In collaboration with scientists at Cleveland State University, Ohio, scientists at ARS, developed a predictive model for the prevalence of E. coli O157:H7 persister cells on lettuce. Application of the model to field studies revealed that the rate of formation of persister cells on lettuce matched that observed in their laboratory studies.

- **Persistence of STEC in food production environments.** Persister cells are a subpopulation of bacteria that are metabolically inactive, thus have high tolerance to antibiotics and other stresses. Biofilm-associated cells are often protected by extracellular matrix, thus have higher tolerance to antibiotics and stresses than planktonic cells. Persistence in STEC Escherichia coli contributed by biofilm-associated cells or persister cells under conditions related to produce production was investigated. STEC strains expressing the Curli fimbriae exhibited increased surface attachment and formation of mixed biofilm by STEC and spinach microflora, implying a critical role of curli fimbriae in persistence of STEC in food production environments.

Outcomes and Impacts

- The prevalence of enteric pathogens data has enabled the generation of time-dependent incidence maps to inform FDA, growers and exporters of indicators of elevated pathogen risk. A preliminary, report of the first 2 years of data was published. (Cooley et al. 2014)
- The predictive model developed for the prevalence of E. coli O157:H7 persister cells provides a tool for industry and public health agencies to assess the survival of this important pathogen on lettuce in the field following an accidental contamination event, and also supports the value of laboratory-acquired information to improve produce safety in agricultural environments. (Munther et al. 2019, submitted)

- Enhanced formation of STEC persisters in spinach wash water and in field surface water suggests that STEC persisters are likely common at diverse points throughout agricultural production. Mitigation approaches aimed at lowering subpopulation of persister cells or disrupting biofilm by targeting curli fimbriae may be an important strategy to minimize microbial contamination of produce. (Carter et al. 2016a; Carter et al. 2019a; Thao et al. 2019)

Examples of Relevant Publications

- Cooley MB, Quiñones B, Oryang D, Mandrell RE, Gorski L (2014) Prevalence of shiga toxin producing *Escherichia coli*, *Salmonella enterica*, and *Listeria monocytogenes* at public access watershed sites in a California Central Coast agricultural region. *Front Cell Infect Microbiol* 4:30. https://doi:10.3389/fcimb.2014.00030
- Carter MQ, Louis JW, Feng D, Zhong W, Brandl MT (2016a) Curli fimbriae are conditionally required in *Escherichia coli* O157:H7 for initial attachment and biofilm formation. *Food Microbiol* 57:81-89.
- Carter MQ, Feng D, Li HH (2019a) Curli fimbriae confer shiga toxin-producing *Escherichia coli* a competitive trait in mixed biofilms. *Food Microbiol* 82:482-488
- Thao S, Brandl MT, Carter MQ (2019) Enhanced formation of Shiga toxin-producing *Escherichia coli* persister variants in the environments relevant to leafy greens production. *Food Microbiol* 84:103241. https://doi:10.1016/j.fm.2019.103241.
- Munther DS, Carter MQ, Palaganas JC, Ivanek R, Brandl MT (2019) Formation of *E. coli* O157:H7 persister cells in the lettuce phyllosphere and application of differential equation models to predict their prevalence on lettuce plants in the field. (submitted)

Population Systems: Parasites

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR); https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf *There is no required reporting for illnesses attributed to parasites, thus the full extent of the burden and cost is currently unknown. Many of these parasites are zoonotic, spread by animals through direct contact, or by contaminated water and food. Research was conducted on the foodborne pathogens Toxoplasma gondii and Trichinella spiralis, which pose significant public health risks due to their presence in fresh pork, and in the case of Toxoplasma, in water, and on fresh fruits, vegetables, and low moisture foods (LMF). Once hogs are infected with Toxoplasma or Trichinella, there are no available treatments to eliminate these pathogens from muscle tissues, nor are their validated procedures for removing infectious Toxoplasma oocysts from the surface of contaminated fruits, vegetables, and LMF.*

Studies at BARC, Beltsville, MD were addressed by two projects Rosenthal and Santin.

The (Rosenthal) project [previously Hill] objectives were to: Elucidate the molecular epidemiology and molecular genetics of environmental Toxoplasma oocyst contamination and define virulence and persistence of particular genotypes in food animals; and (2) Determine and validate methods for improved inactivation and surveillance of meat-borne exposure to Toxoplasma gondii and Trichinella sprialis

Examples of Accomplishments

- **Industry practices have reduced toxoplasmosis in U.S pork.** ARS researchers in Beltsville, Maryland conducted a national serological survey to determine the seroprevalence of T. gondii in the national swine herd using a statistically valid sampling of market weight pigs and sows at slaughter, covered 95% of slaughter production in the U.S. and providing the most current national dataset for Toxoplasma seroprevalence in market pigs destined for the fresh meat case and in sows destined for processed meat products.

- **Tracking a parasite's dissemination in swine.** Improved biosecurity has reduced the prevalence of zoonotic parasites in swine, but reliable risk assessment requires an understanding of how rapidly parasites disseminate through the body and how many parasites establish themselves in meat tissues. Research tracked the development of infections, learning that parasites encysted within a week.

- **Unusual foodborne parasite strains.** Two decades of research has suggested that American livestock typically harbor only a handful of frequently encountered lineages of a parasite important to food safety and women's reproductive health. Other parasite lineages, by contrast, have been discovered in wildlife, raising concerns as to their proclivity to cause human disease. Collaborative studies with Stanford University; University of Tennessee; Palo Alto Medical Foundation; and with partners in France and Romania characterized the parasites responsible for severe human infections. Unexpectedly they discovered that nearly half of the severe human cases came from unusual parasite strains.

- **Free-range chickens do not pose appreciable toxoplasmosis risk.** The consumption of undercooked meat is suspected as an important mode of this parasite's transmission, and free-range chickens have been found to be infected. In order to determine whether chickens available for purchase pose a significant risk, a large sample of chicken hearts were obtained from local markets and tested for T. gondii infection. Viable T. gondii was not isolated from any hearts by bioassays.

- **Detecting Toxoplasma gondii oocyst in ready-to-eat salad.** T. gondii are environmentally hardy and highly infectious to people if ingested in contaminated food, for example, in vegetables. Pre-washed ready-to-eat salads may pose a growing risk for consumers, so ARS developed and validated a sensitive and robust method capable of

detecting as few as 25 parasites in 50g of ready-to-eat baby lettuce. The procedure was also adapted for a faster visualization of positive results using a lateral flow dipstick chromatographic detection method.

- **Treating and vaccinating parasitic infections and understanding the basis of their pathogenesis.** ARS collaborated with several national and international teams to make major breakthroughs in understanding how foodborne parasitic infections cause disease, how to protect against infection through vaccination, and how to treat people who have become infected.

Outcomes and Impacts

- Demonstrated the impact of industry-led changes in swine management on the reduction of this zoonotic pathogen in the U.S. commercial pork supply.
- Demonstrated the extent to which the level of Toxoplasma has been reduced in commercial pork as compared to previous surveys.
- Demonstrated the reduction of risk from Toxoplasma transmission from pork to humans in the U.S.
- Demonstrated that even small helpings of meat were found to harbor infectious tissue cysts, arguing that precautions should be used while handing pork, that all pork should be cooked thoroughly before human consumption, and that uncooked pork should never be fed to cats, who naturally amplify such infections.
- Placed the medical community on notice that this disease often derives from poorly-characterized, sometimes especially virulent forms, and focuses attention on environmental and food sources, other than pork, which may be responsible for many such infections.
- Demonstrated that unlike free-range back yard chickens, chickens from grocery stores do not pose an appreciable risk for consumers.
- Demonstrated and validated an improve detection for T. gondii as an aid in efforts to prevent future human infections.

Examples of Relevant Publications

- Guo M, Mishra A, Buchanan RL, Dubey JP, Hill DE, Gamble HR, Jones JL, Pradhan AK. 2016.A Systematic Meta-Analysis of Toxoplasma gondii Prevalence in Food Animals in the United States. Foodborne Pathogens Disease. 2016 Mar;13(3):109-18. https://doi: 10.1089/fpd.2015.2070.
- Mcphillie, M., Zhou, Y., Dubey, J.P., et al. 2016. New paradigms for understanding and step changes in treating active and chronic, persistent apicomplexan infections. Nature Scientific Reports. 6:29179.
- Bissati, K., Chentoufi, A., Krishank, P., Zhou, X.N., Woods, S., Dubey, J.P., Vang, L., Lykins, J., Broderick, K., Mui, E., Suzuki, Y., Bi, S., Cardona, N. 2016. Adjuvanted multi-epitope vaccines protect HLA-A*1101 transgenic mice against Toxoplasma gondii. Journal of Clinical Immunology Insights (JCI Insights). 1(15):e85955.

- Guo M, Lambertinie e., Buchanan, R. Dubey JP, Hill DE, Gamble HR, Jones JL, Pradhan AK. 2017. Quantifying the risk of human Toxoplasma gondii infection due to consumption of fresh pork in the United States. Food Control 73: 1210-1222. https://doi.org/10.1016/j.foodcont.2016.10.038
- Ying, Y., Verma, S.K., Kwok, O.C., Alibana, F., Mcleod, R., Su, C., Dubey, J.P., Pradhan, A.K. 2017. Prevalence and genetic characterization of Toxoplasma gondii in free-range chickens from grocery stores and farms. Parasitology Research. 116:1591-1595.
- Pomares, C., Devillard, S., Holmes, T.H., Olariu, T.R., Press, C.J., Ramirez, R., Talucod, J., Estran, R., Su, C., Dubey, J.P., Aizenberg, D., Montoya, J.G. 2018. Genetic characterization of Toxoplasma gondii DNA samples isolated from humans living in North America: An unexpected high prevalence of atypical genotypes. Journal of Infectious Diseases. 218(11):1783-1791. https://doi.org/10.1093/infdis/jiy375
- Lalle, M., Possenti, A., Dubey, J.P., Pozio, E. 2018. Loop-Mediated Isothermal Amplification-Lateral-Flow Dipstick (LAMP-LFD) to detect Toxoplasma gondii oocyst in ready to eat salad. Food Microbiology. 70:137-142. https://doi.org/10.1016/j.fm.2017.10.001
- Rani, S., Cerqueira-Cézar, C.K., Murata, F.H., Sadler, M., Kwok, O.C., Pradhan, K., Urban Jr, J.F., Hill, D.E., Dubey, J.P. 2019. Toxoplasma gondii tissue cyst formation and quantitative density of tissue cysts in shoulders of pigs 7 and 14 days after feeding infected mice tissues. Veterinary Parasitology. 269:13-15. https://doi.org/10.1016/j.vetpar.2019.04.004.

The (Santin) project objective was to: (1) Characterize the differences between zoonotic/non-zoonotic and pathogenic/non-pathogenic Cryptosporidium, Giardia, Blastocystis, and Enterocytozoon bieneusi.

Examples of Accomplishments

- **Molecular characterization of Blastocystis in dairy calves in the United States.** Blastocystis is an enteric parasite commonly found in humans and many animals worldwide. Despite its high prevalence and ubiquitous presence, limited data exist on the occurrence and genetic diversity of Blastocystis in the United States and in food animals. ARS in collaboration with the National Animal Health Monitoring Service of Animal and Plant Health Inspection Service (APHIS) conducted the largest and most comprehensive Blastocystis study carried out in food animals worldwide. A total of 2,539 fecal samples from dairy heifer calves from 13 states were examined, and Blastocystis was detected in 73 (2.9%) of the dairy calves. Molecular characterization showed a wide diversity of subtypes. Eleven subtypes were identified, seven previously reported (ST3, ST4, ST5, ST10, ST14, ST17, and ST21) and four novel subtypes (named ST23 to ST26). Three zoonotic subtypes (ST3, ST4, and ST5) were found in 49/73 of the positive calves.

- **Identification of zoonotic species and subtypes of Cryptosporidium in poultry.** Cryptosporidium is recognized as a significant cause of diarrhea in both immunocompetent and immunocompromised patients worldwide. Although Cryptosporidium is an important parasite in poultry, information regarding Cryptosporidium in poultry species including molecular characterization is still scarce. To fill this gap, a study to determine the occurrence and genetic diversity of Cryptosporidium species in several poultry species was conducted by ARS in collaboration with the Universidade Federal de Uberlândia (Brazil). Cryptosporidium was found to be a common parasite in poultry suggesting they could contribute to environmental contamination. The most frequently identified species was Cryptosporidium meleagridis, a zoonotic species with potential to infect mammals and birds, that is the third most common species reported in humans. Molecular characterization identified two novel genotypes and three subtypes previously identified in humans (IIIgA22G3R1, IIIbA24G1R1 and IIIbA23G1R1) indicating zoonotic potential. This represents an advance for understanding the epidemiology of this parasite by expanding the host and geographic range of Cryptosporidium spp. in poultry.

- **Widespread presence of human-pathogenic Enterocytozoon bieneusi genotypes in wild and domestic animals.** E. bieneusi is the most frequently diagnosed Microsporidia species in humans worldwide, mainly associated with chronic diarrhea and wasting syndrome. It has been frequently reported in multiple animal hosts, including wild and domestic, raising concerns of zoonotic transmission. However, there is still little information available on the presence and diversity of E. bieneusi genotypes in livestock, companion animals, and wildlife. ARS in collaboration with multiple international institutions conducted identification and molecular characterization of E. bieneusi in cattle, sheep, birds, dogs, cats, and wild carnivores. E. bieneusi was frequently identified and molecular analysis revealed the presence of human-pathogenic E. bieneusi genotypes as well as novel genotypes (with unknown zoonotic potential). Finding zoonotic genotypes raise the concern of the role of animals in the zoonotic transmission of E. bieneusi and points to those animals as potential sources of E. bieneusi transmission to humans through direct human contact with infected animals or by contamination of water and food.

Outcomes and Impacts

- Conducted in collaboration with the National Animal Health Monitoring Service of Animal and Plant Health Inspection Service (APHIS) a comprehensive Blastocystis study. Molecular characterization of samples from 13 states showed a wide diversity of subtypes. Three zoonotic subtypes were found in the positive calves suggesting a potential role of cattle in transmission to human infections and environmental contamination.

- Conducted a study in collaboration with the Universidade Federal de Uberlândia, Brazil to determine the occurrence and genetic diversity of Cryptosporidium species. The most frequently identified species was Cryptosporidium meleagridis, a zoonotic species that is the third most common species reported in humans.
- Conducted a study in collaboration with multiple international partners to identify and characterize of Enterocytozoon bieneusi in cattle, sheep, birds, dogs, cats, and wild carnivores. Analysis revealed the presence of human-pathogenic genotypes which points to those animals as potential sources of E. bieneusi transmission.

Examples of Relevant Publications

- Fiuza, V.R.S., Lopes, C.W.G., de Oliveira, F.C.R., Fayer, R., Santin, M. 2016. New findings of *Enterocytozoon bieneusi* in beef and dairy cattle in Brazil. Veterinary Parasitology 216:46-51.
- da Cunha, M.J.R., Cury, M. C., Santin, M. 2016. Widespread presence of human-pathogenic *Enterocytozoon bieneusi* genotypes in chickens. Veterinary Parasitology 217:108-112.
- Fiuza, V.R., Lopes, C.W., Cosendey, R.I., de Oliveira, F.C., Fayer, R., Santín, M. Zoonotic *Enterocytozoon bieneusi* genotypes found in Brazilian sheep. Res. Vet. Sci. 107:196-201. 2016.
- da Cunha, M.J.R., Cury, M.C., Santín, M. 2017. Molecular identification of *Enterocytozoon bieneusi*, *Cryptosporidium*, and *Giardia* in Brazilian captive birds. Parasitology Research 116: 487-493.
- da Cunha, MJR, Cury, MC, Santín, M. 2018. Molecular characterization of *Cryptosporidium* spp. in poultry from Brazil. Research Veterinary Science 118:331-335.
- Santín, M, Calero-Bernal, R., Carmena, D., Mateo, M., Balseiro, A., Barral, M., Lima Barbero, J.F., Habela, M.Á. 2018. Molecular Characterization of *Enterocytozoon bieneusi* in wild carnivores in Spain. Journal of Eukaryotic Microbiology 65: 468-474.
- Maloney, J.G., Lombard, J.E., Urie, N.J., Shivley, CB, Santín, M. 2019. Zoonotic and genetically diverse subtypes of Blastocystis in US pre-weaned dairy heifer calves. Parasitology Research 118(2): 575-582.
- Li, W., Feng, Y., Santin, M. 2019. Host specificity of *Enterocytozoon bieneusi* and public health implications. Trends in Parasitology 35(6): 436-451.
- Dashti, A., Santín, M., Cano, L, de Lucio, A, Bailo, B, de Mingo, MH, Köster, PC, Fernández-Basterra, J.A., Aramburu-Aguirre, J., López-Molina, N., Fernández-Crespo, J.C., Calero-Bernal, R, Carmena, D. 2019. Occurrence and genetic diversity of *Enterocytozoon bieneusi* (Microsporidia) in owned and sheltered dogs and cats in Northern Spain. Parasitology Research. 2019 https://doi: 10.1007/s00436-019-06428-1].

Problem Statement 2. Systems Biology

Goal

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Systems biology involves an integrated, multidisciplinary approach to study the complexities of biological components, a central problem to food safety. Identifying the components and players within the system allows the genetic components of bacterial, viral, fungal and parasitic pathogens, and their expression and products to be identified and directly related to these microorganisms.

There is an increased need for data gained from systems studies to be used for both pre- and post-harvest food safety. Data obtained using omic-technologies combined with bioinformatic analysis can be directly applied. For example, genome sequencing efforts worldwide have significantly increased and allowed regulatory and public health agencies to identify and resolve outbreaks of foodborne illness (often for attribution purposes) more rapidly. Whereas 5-10-years ago sequencing efforts often required extensive collaborations both nationally and internationally, they are now routine as exemplified by the international Global Microbial Indentifier (GMI) and U.S. Federal Interagency Collaboration on Genomics for Food and Feed Safety (Gen-FS) initiatives.

Continual outbreaks of bacterial contamination emphasize the continued need to examine pathogens in order to avoid public health risks. The main goal of this area of research was to utilize omic-technologies and apply them to the study of foodborne pathogens found in complex food systems. For example, research would elucidate how microbes cause disease and assess their prevalence, pathogenicity and virulence. Understanding pathogenicity and virulence is critical for development of intervention and control strategies, modeling, and providing data for the development of risk assessments by regulatory agencies. Pathogens have the capacity to readily and rapidly adapt and evolve, so pathogenicity and release of virulence factors is an issue at all stages of the food safety continuum. The prevalence and patterns of contamination in food sectors may vary considerably and needs to be assessed and evaluated carefully. Differences in microbial prevalence, pathogenicity and virulence have been observed across different food production and processing systems, at different sampling times, and by using various methods. Contamination patterns reveal variation in the pathogenicity and virulence and the presence of persistent or sporadic strains and evidence of bacterial transfer from production environments to processing, and from processing environments to food.

While any group of pathogens may exhibit a variety of uncharacterized responses to foods and to conditions under which foods are processed and stored, more importantly there may be a subset of strains demonstrating higher virulence in humans, as noted by differences in infective dose, likely due to the presence of specific virulence genes and genes that allow a survival advantage in food and in the human GI tract. These specific pathogen genetic profiles have not yet been elucidated. Determining the physiological responses that are initiated by pathogens under the conditions particular to foods and food processing conditions is an essential prerequisite for the development of directed intervention methods. Considering existing and pending regulatory

policies and the paucity of available literature, research is needed to better quantify the fate of select food-borne pathogens, for example, different serotypes of L. monocytogenes in high risk foods, such as ready-to-eat foods.

Ongoing implemented microbial control strategies may lose their effectiveness, forcing the development of new production processes and products to maintain and improve the safety of foods. This in turn may restart the cycle of pathogen adaptation resulting from a changed environment and stresses therein. Risk assessment(s) conducted by our regulatory stakeholders /partners are also predicated on understanding the pathogen, the dose response, the behavior in foods, and any positive or negative influences that may affect virulence. Assessing the virulence of foodborne organisms and differences among serotypes/strains is critical in implementing new surveillance and intervention strategies.

Systems Biology: Animals

Introduction
As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
The prevention and control of pathogens entering the food-chain remains an elusive goal, despite intensive research efforts. While most of the research has focused on intervention and prevalence, little is known regarding the genetic variation among these pathogens in terms of the virulence and metabolic genes present, nucleotide polymorphisms, and differences in the transcriptional response and control mechanisms employed when they are exposed to adverse environmental stimuli.

Studies in this area was conducted through several projects at four different research locations; WRRC, Albany, California; MARC, Clay Center, Nebraska; College Station, Texas; U.S. NPRC, Athens, Georgia; BARC, Beltsville, Maryland; and ERRC, Wyndmoor, Pennsylvania.

Studies at WRRC, Albany, CA were conducted project (Parker) whose objective was to: (1) Identify and characterize genetic factors associated with virulence and/or environmental adaptation of human bacterial pathogens using genomic, transcriptional and proteomic analyses.

Examples of Accomplishments

- **Characterization of Campylobacter jejuni response to environmental cues.** Campylobacter jejuni is a leading cause of foodborne illness in the United States and a commensal bacterium residing in the intestinal tracts of livestock. To understand the responses of C. jejuni to environmental cues and understand its physiology, we examined C. jejuni grown under a variety of environmental conditions. Among our results, we identified metabolism-dependent repression of certain carbon source utilization genes when preferred carbon sources are available, and we demonstrated that gradients of microbiota-generated short-chain fatty acids within chicken intestines provide cues for

spatial discrimination so that the C. jejuni can locate niches in the lower intestinal tract for optimal growth.

Outcomes and Impacts

- Comparisons of transcriptomic, proteomic and metabolomic changes of C. jejuni grown under varying conditions have revealed C. jejuni responses to various environmental cues and stresses. The results provide valuable insights into the physiology of C. jejuni and can provide mechanisms to control this pathogen within the poultry industry.

Examples of Relevant Publications

- Luethy, P.M., Huynh, S., Ribardo, D.A., Winter, S.E., Parker, C.T., Hendrixson, D.R. 2017. Microbiota-Derived Short-Chain Fatty Acids Modulate Expression of *Campylobacter* jejuni Determinants Required for Commensalism and Virulence. MBio. 8(3): e00407-17.
- Van der Stel, A.X., Boogerd, F.C., Huynh, S., Parker, C.T., van Dijk, L., van Putten, J.P.M., Wösten, M.M.S.M. 2017. Generation of the membrane potential and its impact on the motility, ATP production and growth in *Campylobacter jejuni*. Mol Microbiol. 105(4): 637-651.
- Van der Stel, A.X., van de Lest, C.H.A., Huynh, S., Parker, C.T., van Putten, J.P.M., Wösten, M.M.S.M. 2018. Catabolite repression in *Campylobacter jejuni* correlates with intracellular succinate levels. Environ Microbiol. 20(4): 1374-1388.
- Negretti, N.M., Clair, G., Talukdar, P.K., Gourley, C.R., Huynh, S., Adkins, J.N., Parker, C.T., Corneau, C.M., Konkel, M.E. 2019. *Campylobacter jejuni* Demonstrates Conserved Proteomic and Transcriptomic Responses When Co-cultured With Human INT 407 and Caco-2 Epithelial Cells. Front Microbiol. 10: 755.

Studies at MARC were conducted by two projects (Bosilevac and Bono) as asides to the larger projects.

This (Bosilevac) study was part of an international collaboration developed during the research cycle to characterize virulence potential of serogroup O91, and O113 STEC isolated from meat, food, and environmental samples.

Examples of Accomplishments

- **Virulence potential of serogroup O91, and O113 STEC.** STEC of serogroups O91 and O113 are commonly found in foods, animals and the environment. Disease reports for these STEC are rare, but can be severe and life threatening, especially in the case of STEC O113 in other countries. STEC O91 isolated from foods, the environment and human disease, in the United States and Europe were examined to determine how they are related to one another and cause disease. Four different serogroups of STEC O91 were identified. Of these, the STEC O91 serotype O91:H21 isolated from foods and the environment were like those from human disease. STEC O113 isolated from beef and cattle in the U.S. were compared to the disease-causing strains from other countries. U.S.

strains of STEC O113 were of two related groups with a small number of STEC O113 of one of the groups overlapping with the disease causing STEC O113, but these came from imported beef products and were not found in U.S. beef and cattle.

Outcomes and Impacts

- The studies were a collaboration between ARS, FDA-Center for Food Safety and Applied Nutrition; Food, Environmental and Occupational Health & Safety in Maisons, Alfort, France, and the Federal Institute for Risk Assessment and the Free University of Berlin. They determined that the STEC O91 serotype O91:H21 isolated from foods and the environment were like those from human disease, suggesting these strains have the potential to cause severe illness. This genetic characterization provided a greater understanding of the diversity of STEC O91. Imported ground beef manufacturing components may require monitoring for STEC-O113 while domestic components do not.

Examples of Relevant Publications

- Feng, P., Delannoy, S., Lacher, D., Bosilevac, J.M., Fach, P. 2017. Characterization and virulence potential of serogroup O113 Shiga toxin-producing Escherichia coli strains isolated from beef and cattle in the United States. Journal of Food Protection. 80(3):383-391. https://doi:10.4315/0362-028X.JFP-16-325.
- Feng, P.C.H., Delannoy, S., Lacher, D.W., Bosilevac, J.M., Fach, P., Beutin, L. 2017. Shiga toxin-producing serogroup O91 *Escherichia coli* strains isolated from food and environmental samples. Appl Environ Microbiol. 83(18). pii: e01231-17. https://doi: 10.1128/AEM.01231-17.

The (Bono) study was a new initiative based on previous observations and requested by ONP during the Program cycle.

- **Understanding extreme heat resistance in Salmonella.** Thermal interventions are commonly used by the food industry to control for bacterial contaminants. The emergence of heat resistant bacteria within beef patties grilled to well done has been a cause for recent concern. Genomic sequencing revealed the presence of a conserved gene sequence that confers heat resistance and appears to have been horizontally transferred between members of some bacteria. A historical isolate of Salmonella originally characterized by ARS in 1968, has been used in numerous heat resistance studies by food microbiologists over the last several decades. The genetic determinants of this isolate's unusually high heat resistance have not been previously characterized. WGS of the Salmonella isolate revealed the presence of two gene clusters strongly related to gene clusters previously identified in other heat resistant foodborne pathogens. This discovery marks the first report of the presence of these two gene clusters in Salmonella and offers clues to the unusually high heat resistance of this strain. Finally, the presence of the two gene clusters on a horizontally transmissible genetic element underscores the potential for transfer of this heat resistance phenotype to other foodborne pathogens.

Outcomes and Impacts

- Identified the potential for pathogens to resist interventions and enter the food chain. A test to screen for the presence in food products of these resistance genes would indicate an intervention failure and keep contaminated food products from reaching consumers.

Examples of Relevant Publications

- Nguyen SV, Harhay GP, Bono JL, Smith TP, Harhay DM. 2017. Genome Sequence of the Thermotolerant Foodborne Pathogen *Salmonella enterica* Serovar Senftenberg ATCC 43845 and Phylogenetic Analysis of Loci Encoding Increased Protein Quality Control Mechanisms. mSystems. 2(1). pii: e00190-16. doi: 10.1128/mSystems.00190-16. eCollection 2017 Jan-Feb.

Studies at College Station, Texas were conducted by the (Kogut) project. The objectives were to: (1) Analyze and characterize both host and Salmonella proteins that are modulated in expression during infection using quantitative proteomic; and (2) Develop strategies to reduce foodborne pathogens by targeting host immune-metabolic signaling pathways affected by Salmonella and Campylobacter virulence factors.

Examples of Accomplishments

- Studies showed that upon infection with Salmonella, the regulation of immune and metabolic pathways in the chicken cecum are altered. During an early (4-48 h) and late (4-14 days) infection with S. Enteritidis, studies observed three separate immune-metabolic phases associated with different times post-infection.

    - First, an inflammatory phase 1-3 days post-infection, characterized by the up-regulation of pro-inflammatory cytokine mRNA transcription and the induction of mTOR-mediated anabolic metabolism.
    - Second, a post-infection phase characterized by profound changes in cecal immunity and metabolism. The local immune micro-environment changes from pro-inflammatory to anti-inflammatory exemplified by a decrease in pro-inflammatory cytokine mRNA transcription to control levels; a dramatic increase in anti-inflammatory cytokine mRNA expression accompanied by a significant increase in the number and function of T regulatory cells (Tregs) in the cecum; and a metabolic reprogramming in the cecal tissue with a shift from anabolic to catabolic reactions mediated by the significant phosphorylation of AMPK.
    - Third, after 4-day post-infection a gradual return to homeostasis. The number of Tregs in the cecum remains elevated over non-infected tissue and IL-10 and TGF-β appear to regulate a more tolerant microenvironment; and a final metabolic reprogramming from catabolic to homeostasis with no difference in metabolism between infected and non-infected birds.

Outcomes and Impacts

- Determined that Salmonella establishes itself as part of the normal microbiome of the cecum through a three-phase process and is no longer recognized by the host immune system.

Examples of Relevant Publications

- Kogut, M.H., Swaggerty, C.L., Byrd, J.A., Selvaraj, R., Arsenault, R.J. 2016. Chicken-Specific Kinome Array Reveals that Salmonella enterica serovar Enteritidis Modulates Host Immune Signaling Pathways in the Cecum to Establish a Persistence Infection. International Journal of Molecular Sciences 17:1207 https://doi: 10.3390/ijms17081207.
- Kogut, M.H., Genovese, K.J., He, H., Arsenault, R.J. 2016. MPK and mTOR: Sensors and Regulators of Immunometabolic Changes During Salmonella Infection in the Chicken. Poultry Science 95:345-353. Doi:10.3382/ps/pev349.

Studies at BARC, Beltsville, MD were conducted by two projects (Santin and Rosenthal)

The (Santin) project objective was to: (1) Conduct WGS to characterize the differences between zoonotic/non-zoonotic and pathogenic/non-pathogenic Cryptosporidium, Giardia, Blastocystis, and Enterocytozoon bieneusi.

Examples of Accomplishments

- **Next generation sequencing improves detection of Blastocystis mixed subtype infections.** Blastocystis has emerged as an important zoonotic parasite that is of public health concern because of its wide geographic distribution and host range. Accurate assessment of Blastocystis subtype diversity is crucial to understand epidemiology and sources of infection and to characterize subtype level differences in host specificity, transmission, public health significance, and pathogenicity. However, the extent of within-host subtype diversity remains largely unexplored due to the limitations of current detection methods. ARS developed a method applying next generation sequencing (NGS) to detect and characterize Blastocystis subtypes as well as to investigate intra-host Blastocystis diversity. This method is more sensitive than conventional sequencing and demonstrated that mixed Blastocystis infections may be far more common than previously thought. This NGS strategy improves detection of mixed subtype infections and low abundance subtypes and represents a valuable resource for future Blastocystis studies to improve understanding of its epidemiology and pathology.

- **A method for generating culture free Giardia genomes.** The protozoan Giardia duodenalis is extremely common and is responsible for ~280 million human cases of diarrhea every year. Giardia duodenalis also infects a wide range of animals and is considered a species complex consisting of eight assemblages. Assemblage and strain level differences in G. duodenalis influence the host specificity and pathogenicity of this intestinal parasite. WGS and comparative genomics are tools that can be used to identify traits that are responsible for these differences. However, these types of analyses require

data from the genomes of many Giardia isolates obtained from multiple hosts. The ability to produce this kind of data is not possible using currently available methods which require pure cultures that are difficult or impossible to obtain. ARS developed a method using a combination of cleaning techniques and short and long read sequencing platforms to produce intact whole genomes directly from fecal isolates of Giardia. This method can now be utilized to produce the data needed to determine the genetic basis of Giardia's pathogenicity and host specificity as well as genes that may be drug or vaccine targets to aid in the treatment and prevention of this common pathogen.

Outcomes and Impacts

- Developed an NGS method to detect and characterize Blastocystis subtypes as well as to investigate intra-host Blastocystis diversity. This method is more sensitive than conventional sequencing and demonstrated that mixed Blastocystis infections may be far more common than previously thought.
- Developed a method to produce intact whole genomes directly from fecal isolates of Giardia. The method can now be utilized to determine the genetic basis of Giardia's pathogenicity; host specificity; as well as genes that may be drug or vaccine targets.

Examples of Relevant Publications

- Maloney, J.G., Molokin, A., Santin M. 2019. Next generation amplicon sequencing improves detection of *Blastocystis* mixed subtype infections. Infection, Genetics and Evolution 73:119-125.

The (Rosenthal) project [previously Hill] on Trichinella and/or Toxoplasma, where the objective was to: (1) Elucidate the molecular epidemiology and molecular genetics of environmental Toxoplasma oocyst contamination and define virulence and persistence of particular genotypes in food animals.

Examples of Accomplishments

- **Agricultural impact on the parasite diversity and virulence.** Most emerging infectious diseases come from animals, but our activities can strongly influence where they occur, how they prosper, and how much harm they can cause us. ARS worked with partners at the University of Tennessee and in Germany, China, France, and South Africa to determine how agriculture has influenced the epidemiology and the virulence an important parasitic threat to food safety and public health.

Outcomes and Impacts

- Linking landscape ecology to parasite virulence, their novel framework contributes fundamental insights on the ecology and evolution of infectious disease and suggest control strategies that would benefit veterinary and human health.

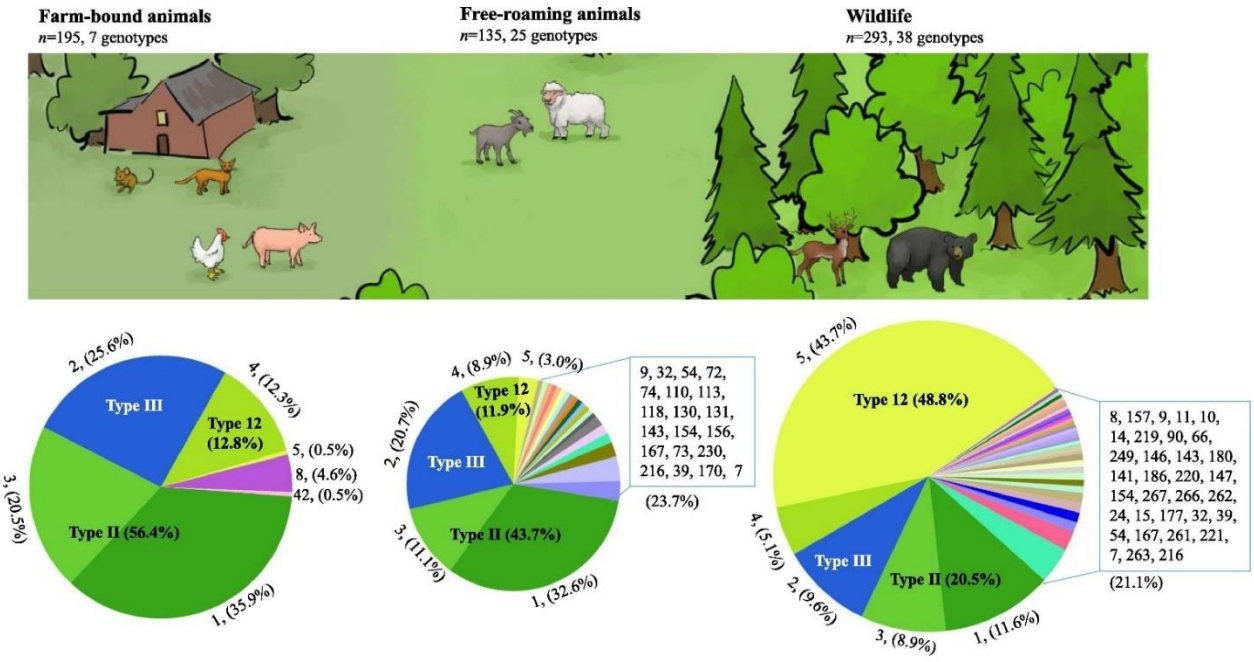

Figure: Toxoplasma gondii genotype distribution along the spatial gradient from farm to the wild. The cartoon illustrates the spatial gradient and animal populations with varied proximities to human habitat. Representatives of animal species in each population are depicted. The numbers around pie chart edges indicate ToxoDB PCR-RFLP genotypes. The archetypal lineages (Type II, III and 12) are overlaid on pie charts in bold lettering. The sizes of pie charts correlate with the total number (and percentage) of isolates; colors of the slices represent different genotypes. (From Rosenthal)

Examples of Relevant Publications

- Keats, S., Saraf, P., Zhu, X., Zhou, D., Mcferrin, B., Ajzenberg, D., Schares, G., Hammond-Aryee, K., Higgins, S., Gerhold, R., Rosenthal, B.M., Zhao, X., Dubey, J.P., Su, C. 2018. Human impact on the diversity and virulence of the ubiquitous zoonotic parasite 2 Toxoplasma gondii. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.1722202115.
- Jiang, T., Shwab, K., Martin, R., Gerhold, R., Rosenthal, B.M., Dubey, J.P., Su, C. 2018. A partition of Toxoplasma gondii genotypes across spatial gradients and among host species, and decreased parasite diversity towards areas of human settlement in North America. International Journal for Parasitology. 48(8):611-619. https://doi.org/10.1016/j.ijpara.2018.01.008.

Studies at ERRC, Wyndmoor, PA were conducted by two projects (Paoli, previously Fratamico) and Paoli*

The (Paoli) project objectives were: (1) Molecular characterization of Shiga-toxin producing Escherichia coli (STEC) and extra-intestinal pathogenic E. coli (ExPEC) with specific emphasis elucidating the responses to food-related stresses, and genomic and proteomic studies to assess virulence and to identify genetic markers for detection and typing; (2) Genomic and proteomic analysis of Campylobacter with emphasis on virulence and the molecular characterization of the effects of acidification and other food-processing related stresses on survival Campylobacter in poultry products; As part of objective 2, During the project FSIS requested studies on interventions for chicken livers, as this was considered a priority need; and (3) Functional and molecular characterization of L. monocytogenes serotypes with emphasis on elucidating responses to food-related stresses through functional genomics; and determining virulence differences among L. monocytogenes strains and serotypes through comparative genomics.*

Examples of Accomplishments

- **A method to identify E. coli strains based on specific genetic sequences.** Traditionally, serotyping has been used to distinguish among the >180 different E. coli O-serogroups and 53 H-types (O-polysaccharide antigen and H-flagellar antigen are E. coli cell surface structures); however, this procedure can only be performed in specialized laboratories, and it is laborious and often inaccurate. To develop more rapid and simple methods for detection, typing, and identification of E. coli belonging to all of the different types and to identify the specific virulence genes (genes associated with causing disease) the strains carry, ARS sequenced the genomes of E. coli reference O-group strains, determined the DNA sequence of the cluster of genes involved in production of cell surface polysaccharides that define the different E. coli O-serogroups. Sequences were deposited in the GenBank DNA sequence public database. Working with CRADA partners, unique genetic regions that can be targeted in methods to identify the different O- and H-groups have been determined. Based on this genetic information, a molecular DNA sequencing-based platform known as AgriSeq was developed to test for the presence of O- and H-group genes, as well as virulence genes in E. coli strains. The accuracy of the method was tested with all reference strains, as well as with other strains isolated from humans, animals, and the environment. This new molecular method is inexpensive, will greatly enhance the ability to identify, detect, and type E. coli, and will eliminate the use of the labor-intensive and inaccurate traditional serotyping procedure.

- **Understanding the nature and behavior of Listeria monocytogenes strains through WGS.** L. monocytogenes is an important foodborne pathogen that causes listeriosis associated with high mortality rates. L. monocytogenes is very difficult to control in the food industry since it can survive under very harsh conditions such as high salt, low pH, and low temperature. ARS conducted WGS of seven (7) strains of L. monocytogenes that varied in their ability to cause disease and response to stresses, to gain a better understanding about how to control this pathogen. The sequences were

also compared to determine the genes involved in virulence (disease causing ability) and stress responses. The genome sequences were deposited in a GenBank database that can be publicly accessed. Information obtained from this research helps in the design of strategies to control L. monocytogenes in food, and potentially in the development of more effective therapeutic approaches.

- **Olive leaf extract is a natural compound that has antimicrobial properties.** There is a need for novel methods to control pathogenic bacteria in the food supply. Olive leaf extract (OLE) is an herbal supplement that is beneficial to human health, having both antioxidant and antimicrobial properties. Listeria monocytogenes, Escherichia coli O157:H7, Salmonella Enteritidis, and Staphylococcus aureus are major foodborne pathogens that cause serious human illness. Studies showed that OLE inhibited growth of these foodborne pathogens, as well as the formation of biofilms (aggregates of bacteria attached to a surface). Therefore, OLE has the potential to be used as a natural antimicrobial to control foodborne pathogens in food and the food environment. Oleuropein is the key compound in OLE that has antimicrobial activity. A modeling approach based on genomic information from S. aureus was developed to predict the metabolic changes in S. aureus triggered by oleuropein. The genome scale modeling approach has the potential to be used for oleuropein dose optimization to control foodborne pathogens.

- **Understanding the survival mechanism of Listeria monocytogenes with exposure to organic acids.** L. monocytogenes is an important foodborne pathogen that causes listeriosis associated with high mortality rates, and furthermore, this pathogen can survive antimicrobial treatments that are normally used during food processing. Organic acids such as lactic acid and diacetic acid have been applied to control L. monocytogenes in ready-to-eat (RTE) meat; however, the mechanism used by L. monocytogenes to adapt to exposure to organic acids remains unclear. Studies used RNA sequencing to determine the genes that are affected in L. monocytogenes with exposure to levels of sodium lactate, and intervention commonly used during food processing. Genes involved in transport of nutrients into the cell and bacterial movement and attachment were affected. These results reflect the pathogen's response to the environmental conditions and provide information essential to determine specific mechanisms required for growth or survival in the selected food-related stress conditions.

- **Swine as a reservoir for Shiga toxin-producing E. coli (STEC) that cause human illness.** Although cattle are considered an important reservoir for STEC, food products from other animal species, including swine have been linked to foodborne illness. However, little is known of the prevalence and fecal shedding of STEC in clinically healthy swine over time. ARS conducted longitudinal studies to fill this gap by investigating fecal shedding of STEC in swine raised on conventional farms during the finishing period. Most of the fecal samples tested were positive for the presence of the STEC Shiga toxin genes, and STEC strains of various types were recovered from a large portion of the pigs. The virulence (disease-causing potential) genes carried by the strains

differed, while some STEC's carried by the pigs were like those associated with human illness and strains from cattle.

- **Antimicrobials known as biscationic quaternary ammonium compounds (QACs) are effective at inactivating Campylobacter.** Monocationic QACs have been commercially used as disinfectants for many years and represent a significant portion of the cleaning products market. However, resistance to the QACs has steadily developed over the years. To address the developing resistance, new QACs were constructed with unique structural changes from currently commercially available QACs. Together with collaborators, studies showed that a subgroup of these new QACs proved to be more successful at inactivating Campylobacter jejuni, when compared to currently commercially available QACs. The chemical structure of the commercially available QACs has only one negative charge, and they are classified as monocationic. The more successful sub-group of the experimental QACs have two negative charges, and these are known as biscationic. Additionally, the remaining experimental QACs, a mix of tricationic (three negative charges) and tetracationic (four negative charges) structures, were less successful at inactivating C. jejuni cells compared to the monocationic and biscationic QACs. This research has resulted in the development of a new class of disinfectants with effectiveness higher than commercially available QACs, which will help to reduce contamination by Campylobacter and potentially other foodborne pathogens.

- **The effectiveness of gamma irradiation to inactivate Campylobacter jejuni in chicken liver**. Recent outbreaks linked to undercooked chicken liver contaminated with C. jejuni necessitated the development of a safer liver product. Intervention methods to reduce the numbers of C. jejuni present on or in uncooked chicken liver would reduce the number of disease cases resulting from a contaminated product that may be consumed undercooked. Studies showed that application of gamma irradiation was successful in reducing Campylobacter numbers on or in the liver without any visual or undesirable changes to the liver product itself. When irradiation was followed by cold storage, the level of irradiation needed to decrease C. jejuni numbers to undetectable levels was significantly reduced. This work represents the first time that irradiation has been used to inactivate C. jejuni present in chicken liver and therefore has produced novel information with regards to treatment dose and bacterial reductions, as well as the interaction this treatment has with typical food storage conditions. This work is of considerable interest and value to poultry producers, as well as consumers. Since the current methods for processing chicken liver is likely to continue to result in disease outbreaks, this new inactivation method is essential for producing a liver product that is safe for consumers.

- **The effectiveness of high hydrostatic pressure to inactivate Campylobacter jejuni in chicken liver.** Recent outbreaks of disease caused by C. jejuni contamination in undercooked chicken liver has necessitated the development of methods to reduce C. jejuni numbers in processed chicken liver. High hydrostatic pressure processing has been used successfully for reducing bacterial counts in a variety

of different food products. Studies showed that a range of high-pressure treatments of chicken liver produced only modest reductions in C. jejuni levels. Additionally, the pressure treatments produced undesired changes in the appearance of the liver at the highest pressures tested; therefore, high pressure treatments were also performed in conjunction with reduced temperature storage. With this combination treatment, the reductions in C. jejuni numbers increased; however, they still did not reach the desired levels as required. The work demonstrated that high pressure treatment in conjunction with cold storage is more effective than high pressure treatment alone; however, this combination treatment will not be enough to significantly reduce the number of illnesses or outbreaks. This work is of considerable interest and value to poultry producers and food safety researchers who will need to include additional technologies in conjunction with high pressure/cold storage treatment if this method is to be utilized as a primary intervention for increasing the safety of chicken liver.

Outcomes and Impacts

- Developed and validated a molecular DNA sequencing-based platform known as AgriSeq to test for the presence of O- and H-group genes, as well as virulence genes in E. coli strains. This new method is accurate, inexpensive and will eliminate the use of inaccurate traditional serotyping procedure.
- Conducted WGS seven strains of L. monocytogenes that varied in their ability to cause disease and response to stresses to gain a better understanding about how to control this pathogen.
- Determined that olive leaf extract (OLE) inhibited growth of several foodborne pathogens, as well as the formation of biofilms. OLE has the potential to be used as a natural antimicrobial to control foodborne pathogens in food and the food environment.
- Determined the genes that are affected in L. monocytogenes after exposure to sodium lactate, a common preservative. Information provides insight on the adaptation mechanism of L. monocytogenes aids in developing more effective control strategies.
- Conducted longitudinal studies to determine fecal shedding of STEC in swine raised on conventional farms during the finishing period. The virulence genes carried by the pigs were like those associated with human illness and strains from cattle.
- New biscationic quaternary ammonium compounds (QAC's) were constructed with unique structural changes from currently commercially available QACs. This new class of disinfectants has effectiveness higher than commercially available QACs.
- Determined that gamma irradiation was successful in reducing Campylobacter levels on or in chicken livers without any visual or undesirable changes to the product. When irradiation was followed by cold storage, the level of irradiation needed to decrease C. jejuni numbers to undetectable levels was significantly reduced.
- Examined high-pressure treatments of chicken livers. Demonstrated that treatment in conjunction with cold storage is more effective than high pressure treatment alone; however, this combination treatment was not enough to significantly reduce Campylobacter levels.

Examples of Relevant Publications

- Baranzoni, G., Fratamico, P.M., Reichenberger, E.R., Kim, G., Breidt, F., Kay, K., Oh, D. 2016. Complete genome sequences of Escherichia coli O157:H7 strains SRCC 1675 and 28RC that vary in acid resistance. Genome Announcements. 4:4. https://doi: 10.1128/genomeA.00743-16.
- Gunther, N.W., Reichenberger, E.R., Bono, J.L. 2016. Complete genome sequence of UV-resistant Campylobacter jejuni RM3194, including an 81.08-kilobase plasmid. Genome Announcements. 4(2):e00305-16.
- Fratamico PM, DebRoy C, Liu Y., Needleman DS, Baranzoni GM, Feng P. 2016. Advances in molecular serotyping and subtyping of *Escherichia coli.* Front Microbiol. 7, 644. https://doi: 10.3389/fmicb.
- Li, X., Liu, Y., Jia, Q., LaMacchia, V., O'Donoghue, K., and Huang, Z. 2016. A systems biology approach to investigate the antimicrobial activity of oleuropein. Journal of Industrial Microbiology and Biotechnology. 43(12):1705-1717.
- Liu Y, Xu A, Fratamico PM, Sommers, CH, Rotundo L, Boccia F, Jiang Y, Ward TJ. 2018. Draft whole-genome sequences of seven *Listeria monocytogenes* strains with variations in virulence and stress responses. Microbiol. Resour Announc 7: e01038-18. https://doi.org/10.1128/MRA.01038-18.
- Liu Y**.,** Yoo B. B, Hwang C-A, Suo Y, Sheen S, Khosravi P and Huang L. 2017. LMOf2365_0442 encoding for a fructose Specific PTS permease IIA may be required for virulence in *L. monocytogenes* Strain F2365. Front. Microbiol. 8:1611. https://doi: 10.3389/fmicb.2017.01611.
- Liu, Y., *McKeever, LC.* and Malik, NS. 2017. Assessment of the antimicrobial activity of olive leaf extract against foodborne bacterial pathogens. Front. Microbiol. 8:113. https://doi: 10.3389/fmicb.2017.00113.
- Liu, Y., Yoo, B., Hwang, C., Suo, Y., Sheen, S., Khosravi, P., Huang, L. 2017. LMOf2365_0442 encoding for a fructose specific PTS permease IIA may be required for virulence in L. monocytogenes Strain F2365. Frontiers in Microbiology. 8:01611.
- Suo, Y., Gao, S., Xie, Y., Liu, Y., Qu, Y., Shen, Y., Zhou, C. 2017. A multi-pathogen selective enrichment broth for simultaneous growth of Salmonella enteria, Escherichia coli O157:H7 and Shigella flexneri. Journal of Food Safety. https://doi:10.1111/jfs.12388.
- Gunther, N.W., Reichenberger, E.R. 2017. Complete genome sequence of Campylobacter jejuni RM1246-ERRC that exhibits resistance to Quaternary Ammonium Compounds. Genome Announcements. https://doi.org/10.1128/genomeA.00978-17.
- Baranzoni, G., Fratamico, P.M., Kim, G., Reichenberger, E.R., Funk, J., Manning, S. 2017. Genome sequences of 34 Shiga toxin-producing E. coli isolated from swine and other sources. Genome Announcements. https://doi.org/10.1128/genomeA.01214-17.
- Uhlich, G.A., Reichenberger, E.R., Cottrell, B.J., Fratamico, P.M., Andreozzi, E. 2017. Whole-genome sequence of Escherichia coli serotype O157:H7 strain B6914-ARS. Genome Announcements. https://doi.org/10.1128/genomeA.01191-17.
- Yan, R., Liu, Y., Gurtler, J., Fan, X. 2017. Sensitivity of pathogenic and attenuated E. coli O157:H7 strains to ultraviolet-C light as assessed by conventional plating methods and ethidium monoazide-PCR. Journal of Food Safety. https://doi: 10.1111/jfs.12346

- Uhlich, G.A., Paoli, G., Zhang, X., Dudley, E.G., Figler, H.M., Cottrell, B.J., Androzzi, E. 2017. Whole-genome sequence of Escherichia coli serotype O157:H7 strain PA20. Genome Announcements. https://doi: 10.1128/genomeA.01460-16.
- Yoo, B.K., Liu, Y., Juneja, V.K., Huang, L., Hwang, C. 2017. Effect of environmental stresses on the survival and cytotoxicity of Shiga toxin-producing Escherichia coli. Food Quality and Safety. 1(2):139-146. https://doi: 10.1093/fqsafe/fyx010.
- Suo, Y.. Gao, S., Baranzoni, G. M., Xie, Y. and Liu, Y. 2018. Comparative transcriptome RNA-Seq analysis of *Listeria monocytogenes* with sodium lactate adaptation. Food Control. 91, 193-201.
- Rotundo, L., Boccia, F, Fratamico, PM, Xu, A., Sommers, CH, Liu, Y, Bono JL, Pepe T. 2018. Draft genome sequences of seven strains of Shiga toxin-producing *Escherichia coli* O111 with variation in their sensitivity to novobiocin. Microbiol. Resour Announc 7: e01030-18. https://doi.org/10.1128/MRA.01030-18.
- Amagliani, G., Rotundo, L., Carloni, E., Omiccioli, E., Magnani, M., Brandi, G., Fratamico, P.M. 2018. Detection of Shiga toxin-producing Escherichia coli (STEC) in food: evaluation of culture enrichment conditions. Food Research International. 103:398-405.

The (Paoli) project objectives were to: (1) Molecular identification and characterization of the genetic factors that influence biofilm formation by Shiga toxin-producing Escherichia coli (STECs) through genomic, transcriptomic, and molecular analyses; (2) Examination of the influence of extrinsic (biotic and abiotic) and intrinsic factors on biofilm formation by STECs; and (3) Qualitative and quantitative characterization of microbial communities associated with beef, and how the various populations influence the presence of STECs through, 16S rDNA-targeted metagenomic studies of microbiomes on ground and intact beef, with beef slaughter facilities (and their correlation with the presence of STEC), and of biofilm forming bacteria associated with beef and beef slaughter facilities.

Examples of Accomplishments

- **Transmission of antibiotic resistance within biofilm.** The complex microbial communities (biofilms) found in foods and food processing environments provide a physical barrier that can protect bacteria from a variety of stresses, including antibiotics, and may provide conditions that promote for the spread of genes encoding antibiotic resistance. To better understand the contribution of gene transfer in biofilms to antibiotic resistance in foodborne pathogens, ARS conducted studies on the mobility of plasmid-associated antibiotic resistance genes between pathogens in biofilms. The researchers found that many of the plasmids examined were capable of transferring antibiotic resistance to other bacteria within biofilms at equal or greater frequency compared to experimental controls on agar plates.

- **Naturally occurring variants of E. coli O157:H7 form strong biofilms.** The formation of biofilms is important for the persistence of pathogens in foods and food processing environment. Early events in biofilm formation involve stable binding of bacteria to surfaces and similar binding events are known to contribute bacterial persistence during the establishment of an infection. Nevertheless, most strains of the major foodborne pathogen E. coli O157:H7 are generally weak biofilm-formers under environmental conditions. ARS had previously described several genetic defects that contribute to reduce biofilm formation by pathogenic E. coli. Here we report additional genetic and environmental factors necessary for biofilm formation by these deadly foodborne pathogens ascertained by careful physiological and genetic characterization of naturally-occurring biofilm-forming variants of E. coli O157:H7. The regulatory mechanisms reveal elements that are shared among cellular processes related to biofilm formation, responses to environmental stress, and pathogen virulence. The mechanisms remain unknown in approximately 25% of the variants and await further analyses by WGS.

- **Genomic studies of pathogenic E. coli strain reveal distinct gene regulation.** Some STEC strains are harmful to humans and can be acquired through the consumption of contaminated foods. The persistence of these STEC in foods is aided by the ability of some strains to form or associate within microbial biofilms. To aid in our understanding of biofilm formation by this pathogen, studies determined the complete closed genome sequence of a well characterized clinical isolate of STEC (strain PA20), and deposited the annotated genome in a publicly available DNA sequence database (GenBank). Using the complete genome as a reference, gene expression studies were conducted to examine the response of biofilm- and virulence-associated genes when the cells were subjected to antimicrobials. Under the applied antimicrobial stress, strain PA20 decreased the expression of biofilm genes while dramatically increasing the expression of numerous virulence genes in a time dependent manner. While the physiological relevance of the observed over-expression of virulence genes by STEC is not fully understood, the decrease in their capacity to form biofilm suggests an additional mechanism by which antimicrobial or sanitation processes might mitigate the presence of this pathogen in foods.

- **Antibiotic stress increases virulence in E. coli.** The ability to attach to surfaces and form biofilms may be a contributing factor in the persistence of pathogenic E. coli O157:H7 in foods and food processing environments. ARS is studying E. coli containing biofilms in order to understand their role in environmental persistence and human pathogenesis. Genome-wide gene expression was examined in E. coli O157:H7 in the presence of antibiotics with the potential to affect the capacity for biofilm formation. The results indicated that, when subjected to DNA damaging antibiotic stress, E. coli responds by increasing expression of virulence genes, but genes involved in biofilm formation are repressed. In this study, key regulators of virulence gene expression were identified.

- **First report of closed DNA sequences of Brochothrix thermospacta genomes, an important food spoilage organism.** The ability of bacteria to associate in biofilms and/or aggregates is an important factor for their persistence in the environment. In food and food processing environments these complex bacterial structures can potentially trap pathogens and provide protection against intervention processes. Studies previously isolated strains of Brochothrix thermosphacta, an important food spoilage organism, some of which prefer to grow in aggregated clusters or webbed films in liquid cultures, in contrast to other strains that grow uniformly dispersed in solution. In order to provide a basis to study this unique growth, the complete genome sequences of two strains of B. thermosphacta were determined, reported and deposited in the GenBank public DNA sequence database. One strain forms complex multicellular structures, while the other grows normally. These DNA sequences are the first two complete and closed genomes for this species and provides an important resource for studies on food spoilage and the potential for this organism to contribute to pathogen persistence in foods.

- **Citrus oil spray disrupts E. coli biofilm formation.** Bacteria live in complex communities in the environment and bacteria in these communities use chemical signals to communicate their presence to one another. These chemical signals regulate various behaviors such as motility, biofilm formation, and virulence characteristics of harmful bacteria (pathogens). Compounds capable of inhibiting bacterial cell-to-cell communication have been explored for their potential to reduce the persistence of pathogens in foods, or as potential alternatives to antibiotics. ARS in collaboration with Purdue University studied a fine emulsion of a chemical found in citrus peels (D-limonene) for its ability to disrupt one pathway of intercellular communication in pathogenic E. coli. Very low concentrations of D-limonene were able to interfere with cell-to-cell signaling and alter cellular physiology including disrupting formation of biofilms. These results indicate that D-limonene emulsion could be applied to food processing surfaces to minimize bacterial biofilm formation, reduce virulence, or as an alternative when antibiotics are not suitable.

- **Field portable DNA sequencing for pathogen detection.** In order to prevent the distribution of contaminated foods and reduce the burden of foodborne illness, food producers and regulatory agencies need rapid, accurate and cost-effective methods for the identification of bacterial foodborne pathogens. Recently, WGS has been broadly adopted by regulatory and public health agencies to characterize bacterial pathogens and track outbreaks of foodborne illness. Nevertheless, due to expense and technical limitations, these genomic technologies have not been adopted for rapid foodborne pathogen detection and identification. Recently, an inexpensive and portable DNA sequencing device, the Oxford Nanopore MinION DNA sequencer, which overcomes several of these limitations, was introduced. The MinION was used to identify the 7 different types of pathogenic E. coli that are currently not allowed in foods in the U.S. within a complex mixture.

Outcomes and Impacts

- Conducted studies on the mobility of plasmid-associated antibiotic resistance genes between pathogens in biofilms. Studies found that many plasmids were capable of transferring antibiotic resistance to other bacteria within biofilms at equal or greater frequency compared to experimental controls.
- Determined additional genetic regulatory mechanisms and environmental factors necessary for biofilm formation by foodborne pathogens.
- Determined the complete closed genome sequence of a well characterized clinical isolate of STEC (strain PA20). Observed that under antimicrobial stress, strain PA20 decreased the expression of biofilm genes while dramatically increasing the expression of numerous virulence genes in a time dependent manner.
- Undertook the complete genome sequences of two strains of B. thermosphacta, one strain forms complex multicellular structures, while the other grows normally. These two DNA sequences provide an important resource for studies on food spoilage
- Determined that a fine emulsion of D-limonene found in citrus peel was able to interfere with cell-to-cell signaling and alter cellular physiology including disrupting formation of biofilms.
- Determined that (in our studies) the MinION sequencing technology has the potential for inexpensive, rapid, specific, and field -portable detection and identification of foodborne bacterial pathogens

Examples of Relevant Publications

- Uhlich, G. A., G.C. Paoli, C.-Y. Chen, B. J. Cottrell, X. Zhang, and X. Yan. 2016. Whole-Genome Sequence of *Escherichia coli* Serotype O157:H7 Strain EDL932 (ATCC 43894). Genome Announcements. 4(4):e00647-16. https://doi:10.1128/genomeA.00647-16.
- Uhlich, G. A., C.-Y. Chen, B. J. Cottrell, C. S. Hofmann, X. Yan, and L. Nguyen. 2016. Stx_1 prophage excision in *Escherichia coli* strain PA20 confers strong curli and biofilm formation by restoring native *mlrA*. FEMS Microbiology Letters. fnw123. https://doi: 10.1093/femsle/fnw123.
- Chen, C.-Y., L.-H. Nguyen, B. J. Cottrell, P. L. Irwin and G. A. Uhlich. 2016. Multiple mechanisms responsible for strong Congo red-binding variants of *Escherichia coli* O157:H7 strains. FEMS Pathogens and Disease 74: ftv123.
- Uhlich, G.A., Reichenberger, E.R., Cottrell, B.J., Fratamico, P.M., Andreozzi, E. 2017. Whole-genome sequence of *Escherichia coli* serotype O157:H7 strain B6914-ARS. Genome Announcements. https://doi.org/10.1128/genomeA.01191-17.
- Uhlich, G.A., Paoli, G.C., Zhang, X., Dudley, E.G., Figler, H.M., Cottrell, B.J., Andreozzi, E. 2017. Whole-genome sequence of *Escherichia coli* serotype O157:H7 strain PA20. Genome Announcements 5(2):e01460-16.DOI: 10.1128/genomeA.01460-16
- Uhlich, G., Chen, C.-Y., Cottrell, B., Andreozzi, E., Irwin, P., Nguyen, L. 2017. Genome amplification and promoter mutation expand the range of *csgD*-dependent biofilm responses in a STEC population. Microbiology 163:611-621.

- Peritz, A., Chen, C., Paoli, G., Gehring, A.G. 2018. Serogroup-level resolution of the "Super-7" Shiga toxin-producing *Escherichia coli* using nanopore single-molecule DNA sequencing. Analytical and Bioanalytical Chemistry. https://doi.org/10.1007/s00216-018-0877-1.
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- Andreozzi, E., Gunther, N.W., Reichenberger, E.R., Cottrell, B.J., Rotundo, L., Nunez, A., Uhlich, G.A. 2018. Pch genes control biofilm and cell adhesion in a clinical serotype O157:H7 isolate. Frontiers in Microbiology. 9(2829). https://doi.org

Systems Biology: Produce

Introduction

The rationale for the research focus described here was that enteric pathogens such as enterohemorrhagic Escherichia coli (EHEC), Salmonella and Listeria are some of the most important foodborne pathogens associated with fresh produce consumption. They are capable of survival and proliferation in various adverse environments outside of animal hosts, and it has been postulated that these bacterial pathogens have developed fitness for association with plant growth environments. The mechanisms for these pathogens to attach and adhere to food and environmental matrices, and to survive and persist in such environments are poorly understood and needs increased research.

Studies at WRRC, Albany, CA were conducted through a large multidisciplinary project (Gorski) whose objective was to: (1) Elucidate biological factors and molecular mechanisms that enhance or reduce fitness characteristics related to survival and growth of enteric pathogens in the produce production continuum.

Examples of Accomplishments

- **Virulence potential of endemic, environmental Listeria monocytogenes strains adjacent to active leafy green production fields**. Internalin A, encoded by the inlA gene, is a protein essential for L. monocytogenes virulence in humans, and about 40 to 50 percent of the strains isolated from foods and food processing plants contain truncated inlA genes such that they are essentially non-virulent; however, little is known about the inlA genes in environmental isolates. The inlA genes from a collection of 112 L. monocytogenes strains isolated from public access watersheds surrounding a leafy-greens production region on the Central California Coast during 2011 – 2014 were sequenced. Ninety percent of the isolates had intact inlA genes, which is significantly different from isolates in processing plants. Moreover, the strains carrying these intact genes persisted in the environment for at least 3-years, possibly cycling through animals.

- **Listeria monocytogenes genomic subtypes similar to outbreak strain subtypes detected in waters near produce production in California.** ARS in a collaboration with scientists at North Carolina State University and the FDA conducted a study of L. monocytogenes serotype 4b strains isolated from various sources in North America. Using Multi-locus genome typing, the clonal complex (CC) or sequence type (ST) of the strains was determined. The 25 California L. monocytogenes strains belonged to the emerging, hypervirulent CC4, CC217, ST639, and to ST382 (which has been responsible for 3 produce-related outbreaks in the last 5 years).

- **Identification of interactions between Human Norovirus and corresponding ligands in lettuce.** Human Norovirus (HuNoV) is the most common cause of diarrheal illness in the United States, and HuNoV-contaminated lettuce has caused several outbreaks and sporadic illness. In humans HuNoV binds to Human Blood Group Antigens (HBGA), but it is unclear how the virus attaches to and accumulates on pre-harvest lettuce leaves since HuNoV is very difficult to grow in the laboratory for direct studies. ARS in collaboration with scientists at Shanghai Jiao Tong University and Shanghai Institute of Technology, engineered a bacterium to carry the HuNoV capsid protein (the P protein). The bacteria-surface-displayed P protein (BSD-P) was used to characterize conditions for the P protein binding to and removal from lettuce. The system was further used to isolate and characterize candidate viral receptors in lettuce.

- **Genomic and phenotypic characterization of STEC.** STEC consists of a group of diverse strains that are continually evolving. A total of 53 environmental STEC O145 strains recovered from a major produce production region in California were compared with STEC O145 outbreak strains to assess the pathogenicity of the environmental strains. Although the core virulence determinants of enterohemorrhagic E. coli (EHEC) are conserved in all environmental strains, there is large variation in expression of the virulence traits. Only a few cattle isolates exhibited key virulence traits comparable with the outbreak strains, implying the emergence of hyper-virulent strains in a clonal population. Such outliners were also observed in a clonal population of STEC O121 strains. Among the strains with the same genotype, variants with increased metabolic capacity for several carbon substrates that naturally occur in plants were detected, implying a potential niche for selection of variants with improved fitness.

- **An effective biocontrol agent and antibiotic alterative to kill non-O157 Shiga toxin-producing Escherichia coli.** The number of cases of STEC-related foodborne infections (which include the predominant serogroups O157, O26, O45, O103, O111, O121, and O145) increases each year, with an estimated 176,000 cases, 2400 hospitalizations, and 20 deaths annually in the U.S. Besides STEC O157, STEC O145 is the most widespread pathogen among the top six non-O157 STEC serogroups associated with foodborne outbreaks in the U.S., and STEC O145 was responsible for a multistate outbreak associated with lettuce consumption in the US in 2010. We isolated, identified, and genomically characterized a novel bacteriophage (Ro145clw) from the environment that specifically kills STEC O145, and evaluated its physiological features. The phage had a big burst size of 192 phages per infected cell, a short replication cycle (latent period of

21 min) and could sustain a wide range of pH (3-10) and temperature (-80°C - 73°C). Furthermore, phage R0145clw could render strong lytic activity against both E. coli O145:NM (environmental strain) and E. coli O145:H28 (outbreak strain), with 6.2 log and 3.5 log reduction after 6 h of incubation at 25°C, respectively.

Outcomes and Impacts

- The data on source and virulence potential of Listeria strains is being used by the FDA in the development of risk assessment for science-based irrigation water policy decisions.
- Determined that Listeria subtypes ST382 and ST639 had a higher propensity among water isolates than food or human clinical strains. CC4, a clonal type that emerged first in Europe, is present in the US in environmental samples near produce production fields.
- Identified an oligosaccharide on the lettuce responsible for HuNoV binding as well as molecules similar to (HBGA) produced by bacteria naturally present on the lettuce surface, indicating that HuNoV has multiple binding sites on pre-harvest lettuce surfaces. These binding compounds provide protection and a means of transmission of HuNoV and are critical in developing interventions to reduce HuNoV contamination.
- Determined that although the core virulence genes of enterohemorrhagic Escherichia coli (EHEC) are conserved in all environmental strains, there is large variation in expression of the virulence traits. Substrates that naturally occur in plants were detected, implying a potential niche variation for selection of variants with improved fitness.
- Isolated a phage variant (R0145clw) with physiological features rendering it resistant to a wide range of pH and temperatures. This phage may be a promising biocontrol agent against E. coli O145.

Examples of Relevant Publications

- Niu M, Yu Q, Tian P, Gao Z, Wang D, Shi X (2015) Engineering bacterial surface displayed human norovirus capsid proteins: a novel system to explore interaction between norovirus and ligands. Front Microbiol 6 (1448)
- Carter MQ, Quiñones B, He X, Zhong W, Louie JW, Lee BG, Yambao JC, Mandrell RE (2016) An environmental Shiga toxin-producing *Escherichia coli* O145 clonal population exhibits high-level phenotypic variation that includes virulence traits. *Appl Environ Microb* 82:1090-1101.
- Gorski L, Parker CT, Liang AS, Walker S, Romanolo KF (2016) The majority of genotypes of the virulence gene *inlA* are intact among natural isolates of *Listeria monocytogenes* from the Central California Coast. *PLoS ONE* 11 (12):e0167566. https://doi:10.1371/journal.pone.0167566
- Wang M, Rong S, Tian P, Zhou Y, Guan S, Li Q, Wang D (2017) Bacterial surface-displayed GII.4 human norovirus capsid proteins bound to HBGA-like molecules in romaine lettuce. *Front Microbiol* 8:251.

- Xu Q, Ni P, Liu D, Yin Y, Li Q, Zhang J, Wu Q, Tian P, Shi X, Wang D (2017) An advanced bacterial surface display system to express cleavable capsid proteins of human norovirus: a novel system to discover candidate receptors. *Front Microbiol* 8:2405
- Lee S, Chen Y, Gorski L, Ward TJ, Osborne J, Kathariou S (2018) *Listeria monocytogenes* source distribution analysis indicates regional heterogeneity and ecological niche preference among serotype 4b clones. *mBio* 9:e00396-00318. https://doi:10.1128/mBio.00396-18.
- Liao Y-T, Salvador A, Harden LA, Liu F, Lavenburg VM, Li RW, Wu VCH (2019) Characterization of a lytic bacteriophage as an antimicrobial agent for biocontrol of Shiga toxin-producing *Escherichia coli* O145 strains. *Antibiotics* 8:74
- Carter MQ, Tan ZF, Pham A, Carychao DK, Cooley MB (2019) A clonal Shiga toxin-producing *Escherichia coli* O121:H19 population exhibits diverse carbon utilization patterns. *Foodborne Path Dis* 16:284-393. https://doi:10.1089/fpd.2018.2567
- Rong S, Zhou Y, Wang M, Guan S, Zhang S, Cai B, Wang D, Tian P, Li Q (2019) Characterization of conditions for bacteria-human norovirus capsid P protein complex (BPC) binding to and removal from romaine lettuce extract. *Intl J Food Microbiol* 298:11-19.

Studies at BARC, Beltsville, MD were conducted through a large multidisciplinary project (Patel) whose objectives were to: (1) Investigate the mechanism(s) of introduction, transference, and survival of enterohemorrhagic Escherichia coli (EHEC), Salmonella, and Listeria to fresh produce at the farm level; (2) Determine the effects of multispecies biofilm formation on the survival, persistence, and dissemination of pathogenic bacteria in fresh produce processing environments and on contamination of fresh produce; and (3) Assess of microbial safety of fresh produce grown under non-conventional farming practices.

Examples of Accomplishments

- **Location, season, and manure type affect survival of pathogens in manure-amended soils.** The Produce Safety Rule of FSMA states that untreated manure must be applied 90 or 120 days prior to the harvest of edible produce crops to minimize contamination from pathogens potentially present in untreated manure. However, this interval was not scientifically validated. ARS studies showed that spatiotemporal factors (site, year, and season) affect survival durations of E. coli in manure-amended soils more than agricultural factors (manure type, organic or conventional management of soils, and depth of application) or weather effects.

- **Organic fertilizers can affect survival durations of bacterial pathogens in soils and on leafy greens.** Bacterial pathogens can be introduced to produce-growing environments through contaminated irrigation water, animal intrusions, or soil/ manure runoff. ARS studies showed that Salmonella Newport can grow to high populations in soil runoff containing heat-treated poultry pellets (HTTP), a commonly used organic fertilizer in vegetable production. Soils amended with HTTP also supported longer

survival durations of Salmonella Newport than unamended soils and promoted more transfer of the pathogen from soils to leaves of spinach plants.

- **Environmental bacteria can enhance biofilm formation by foodborne pathogens.** Ralstonia insidiosa isolated from produce packing facilities promoted the incorporation of the disease-causing E. coli O157:H7 into a dual species biofilm, which is adherence of bacteria to surfaces including those in packing plants. R. insidiosa also enhanced the formation of biofilm by other pathogenic E. coli strains, Salmonella, and Listeria monocytogenes strains in dual species cultures. The bacterium seems to play the role of "bridge bacteria" in multispecies biofilm formation. R. insidiosa induced aggregation, a key step in biofilm formation, by L. monocytogenes in mixed cultures. This information is useful for developing new antimicrobial wash treatments.

- **Microbiological quality of spinach irrigated with reclaimed waste water and roof-harvest water.** A single irrigation with alternative water containing higher populations of total and fecal coliform bacteria did not necessarily result in higher populations of the coliform bacteria on spinach leaves. However, repeated irrigation with reclaimed wastewater resulted in higher numbers of E. coli positive spinach samples. Irrigation waters containing higher populations of total and fecal coliforms did not necessary result in higher populations of these bacteria on the spinach leaves. Roof-harvest water had higher microbial quality than the reclaimed wastewater. Roof-harvest water irrigation did not increase the populations of fecal bacterial indicators on the irrigated spinach plants.

- **Methods to determine the microbial quality of alternative irrigation waters.** A concentrator pipette method was evaluated to determine the microbial quality of wastewater, rainwater, and creek water in comparison to the membrane filtration method. No significant differences were observed concerning bacterial populations and pathogens. Recovery of fecal coliform bacteria in wastewater was lower than that found in filtered water samples.

Outcomes and Impacts

- Provided critical information to growers on potential risk of produce contamination with specific raw animal manure application. The FDA will use these data to develop food safety standards within the Produce Rule for FSMA, for controlling bacterial contamination of fresh produce from soil.
- Determined that soils amended with heat-treated poultry pellets (HTTP), supported longer survival durations of Salmonella pathogens than unamended soils, and promoted more transfer of the pathogen from soils to leaves of spinach plants. This information provides farmers with a specific factor that affects and promotes pathogen survival in pre-harvest produce growing environments.
- Determined that Ralstonia acts as a "bridge bacteria" in biofilm formation. R. insidiosa induces aggregation, a key step in biofilm formation. This information is useful for developing new antimicrobial wash treatments.

- Determined roof-harvest water (RHW) had higher microbial quality than the reclaimed wastewater. Irrigation with RHW did not increase the populations of fecal bacterial indicators on the irrigated spinach plants and showed the potential for its use in produce production. The limitation of RHW was the quantity available for production rather than quality.
- Determined that the concentrator pipette method to evaluate the microbial quality of wastewater, rainwater, and creek water was comparable to the membrane filtration method. No significant differences were observed concerning bacterial populations and pathogens., however, recovery of fecal coliform bacteria in wastewater was lower than that found in filtered water samples.

Examples of Relevant Publications

- Keelara, S., Thakur, S., Patel, J. R. 2016. Biofilm Formation by Environmental Isolates of Salmonella and their Sensitivity to Natural Antimicrobials. Foodborne Pathogens and Diseases 13 (9) 509-516.
- Yin, H., Nou, X., Gu, G., Patel, J. 2018. Microbiological quality of spinach irrigated with reclaimed wastewater and roof-harvest water. Journal of Applied Microbiology. 125: 1: 133-141.
- Yin, H., Patel, J. 2018. Comparison of methods to determine the microbial quality of alternative irrigation waters. Agriculture Water Management. 201:38-45.
- Zhou, B., Luo, Y., Bauchan, G., Feng, H., and Stommel, J. 2018. Visualizing pathogen internalization pathways in fresh tomatoes using MicroCT and confocal laser scanning microscopy. Food Control. 85:276-282.
- Marik, C.M., Anderson, B., Gartley, S., Craighead, S., Bradshaw, R., Kulkarni, P., Sharma, M., Kniel, K.E. 2019. The efficacy of zero valent iron-sand filtration on the reduction of Escherichia coli and Listeria monocytogenes in surface water for use in irrigation. Environmental Research. 173:33-39.
- Shah, M.K., Bradshaw, R., Nyarko, E., Handy, E.T., East, C., Millner, P.D., Bergholz, T.M., and Sharma, M., 2019. Salmonella enterica in soils amended with heat-treated poultry pellets survived longer and more readily transferred to and persisted on spinach. Applied and Environmental Microbiology. 85: e00334-19.
- Shah, M.K., Bradshaw, R., Nyarko, E., Millner, P.D., Neher, D., Weicht, T, Bergholz, T., Sharma, M. 2019. Survival and growth of wild-type and rpoS-deficient Salmonella Newport in soil extracts prepared with heat-treated poultry pellets. Journal of Food Protection. 82: 501-506.
- Neher, D., Cutler, A., Weicht, T., Sharma, M., Millner P.D. 2019. Composts of poultry litter or dairy manure differentially affect survival of enteric bacteria in fields with spinach. Journal of Applied Microbiology. 126:1910-1922.
- Sharma, M., Millner, P.D., Hashem, F., Vinyard, B.T., East, C.L., Handy, E.T., White, K., Stonebraker, R., Cotton, C.P. 2019. Survival of Escherichia coli is affected by spatiotemporal, agricultural, and weather factors in the Mid-Atlantic United States. Applied and Environmental Microbiology. 85: e02392-18.

Problem Statement 3. Microbial Contaminants: Technologies for Detection and Characterization

Goal

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
The challenge is the rapid, and unequivocal detection and characterization of pathogenic microorganisms at the earliest possible stage of the food continuum to provide the necessary data for targeted interventions, and reducing the need for recall of food products. Research would focus on innovative technologies (depending on the matrix) or point of use, and how the technology would be used; that is for baseline studies, traceability and/or attribution forensics. This requires that decisions be made relative to what should be detected, and the required level of detection and characterization. It should be stressed that technologies that have the highest level of detection/characterization capability might not necessarily be the most practical, useful, rapid, economically viable, or easily implemented. High-through-put analysis is important, but it may be impractical under certain circumstances. Further, changes within certain public health regulatory agencies to culture-independent diagnostic tests (CIDTs) requires a reconsideration of some technologies utility and need.

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Traditional microbial culture methods can detect and identify a single specific bacterium, but may require days or weeks, and often do not produce quantitative data. Thus, there was a quest for faster, quantitative "rapid methods", however, the high levels of non-target microorganisms and other materials in food samples often interfered with detection limits required for food safety. Physical and/or microbiological processes are required which separate and concentrate the target pathogen from the sample matrix and/or other microorganisms are essential for effective detection, in order to preserve the unique genetic and phenotypic features. The development of novel biorecognition elements such as generated PCR and/or hybridization probes as well as stable aptamers were also areas of need. This research would assist in consolidating our findings with existing technologies in order to ultimately generate novel typing platforms whether they-be variations on existing typing methods such as MLST, RFLP or greatly expanded DNA, RNA and/or antibody (aptamer) microarrays.

While the "well known" conventional (medium based) and molecular methods/technologies still need development and refining to ensure their continued efficacy, and or use in new situation or circumstances. The Program also realized that there was still a need to consider developing faster, reliable, cost effective methods using more innovative ideas and technologies, realizing the changes in detection needs and the utilization of genomics; and more importantly collaborating with other researchers in diverse fields.

Bacterial foodborne pathogens are highly adaptable with a dynamic/flexible genetic makeup that allows these organisms to persist, colonize and grow in multiple niches. This variability however, also poses challenges for the design of detection and typing methods that are crucial for epidemiology and outbreak trace-back investigations. As befitting organisms with such high clinical importance, significant amounts of "omics" data (including whole genome sequences) are available. However, substantial knowledge gaps remain regarding virulence determinants, gene expression and the genetic networks that are vital for the adaptability, persistence and pathogenicity of these organisms. The Program proposed to: develop novel typing methods that were more focused on identification of clinically-relevant genogroups and virulence genes; analyze the genetic content, variability and gene expression that was the basis for adaptability and virulence; and characterize clinically-relevant genes and proteins, such as virulence or resistance genes, and toxins.

While focusing on "omic" technologies and genomic data was important under the umbrella of Gen-FS "Interagency Collaboration on Genomics for Food and Feed Safety" and regulatory agency needs, new areas of research also needed consideration.

Hyperspectral microscopic imaging (HMI) alone or combining HMI with markers from fluorescence in-situ hybridization (FISH). FISH probes, and especially multiplex FISH (m-FISH) probes with spectral imaging, have the potential to increase both sensitivity and selectivity of HMI. Hyperspectral imaging (HI) systems have the potential to correctly classify foodborne pathogens, however, there are limitations with existing agars, which will need modification. Surface Enhanced Raman Scattering (SERS) can be used in either labeled or label-free platforms, both of which are suitable for rapid screening and characterization at low concentrations. Combining SERS with aptamer-based binding can also help improve classification by reducing non-specific binding from background microflora. In addition to aptamers, labels can further enhance detection limits while still maintaining a rapid method.

Additionally, the Program has had a long-term collaboration with Purdue University's Center for Food Safety Engineering (CFSE) building on each partner's strengths to develop and integrate operational technologies. The importance of collaborations with the CFSE cannot be overstated, due to their expertise in various eclectic fields. The awards and technologies garnered/patented are indicative, for example, two of the five finalists in the first-ever FDA Food Safety Challenge 2014 are previously-funded CFSE technologies, while one CFSE Technology was the winner; and the BARDOT technology (U.S. Patent No. 7,465,560) licensed for commercialization by Hettich, Germany.

Further, the focus of methods development also included viruses and parasites; predominantly subtyping, detection, and pathogenic potential. Standard subtyping methodologies differ among the viruses, for example HuNoV, and zoonotic protists. In order to process the large numbers of these "pathogens" we are isolating from our survey work, faster and more refined methods for detection and subtyping are needed for characterization, to determine what strains are of the most risk, and to test intervention methods to reduce the chance of contamination of pre-harvest produce. These methodologies would be of use to growers, testing labs, and public health agencies.

This wide-ranging research was conducted through projects at several different research locations; WRRC, Albany, California; MARC, Clay Center, Nebraska; U.S. NPRC, Athens, Georgia, BARC, Beltsville, Maryland; ERRC, Wyndmoor, Pennsylvania; and the CFSE, Purdue University, Indiana. Each location has specific expertise and collaborations related to methods development.

Research at WRRC, Albany, CA was conducted by two projects (Parker and Gorski)

The (Parker) project objective was to: (1) Develop improved identification technologies for human bacterial and viral pathogens to replace current testing methodologies.

Examples of Accomplishments

- **Accurate detection of foodborne enteric viruses in a major agricultural region.** Human noroviruses are responsible for 19-21 million illnesses and for $2 billion in direct and indirect costs annually in the U.S. Methods were optimized for the efficient recovery and identification of noroviruses from water samples, isolated from public waterways adjacent to a major production region for leafy greens in California. A novel algorithm was developed for designing a virus typing tool that allowed the simultaneous identification in a single test of multiple virus genotypes associated with foodborne illness with an analytical sensitivity at or below the virus infectious dose. The virus typing tool enabled an analysis of the temporal and spatial prevalence of relevant types of noroviruses in waterways proximal to agricultural fields. Norovirus detection rates were highest in the fall, followed by spring and winter, while it was lowest in the summer.

- **Optimized methods for assessing the relative potencies of bacterial toxins.** Most quantitative assays that monitor the toxin-induced inhibition of protein synthesis and resulting cell death average the results from a population of cells; therefore, it is not possible to differentiate between intoxicated and unintoxicated cells within the population. As an alternative approach, methods were developed for the use of mammalian cell sorting (flow cytometry) with an unstable fluorescent reporter for quantifying the relative potencies of the bacterial toxins, cholera toxin and Shiga toxin subtype 1a, in individual mammalian cells. This research quantified the amounts of bacterial toxins required for complete inhibition of protein synthesis and/or cell death and demonstrated for the first time that mammalian host cells can recover when exposed to sub-lethal toxin concentrations and survive the toxin effects.

- **Shiga toxin (Stx) expressed from environmental Shiga toxin-producing Escherichia coli (STEC).** ARS studies analyzed environmental STEC isolates collected from California agricultural produce regions for Stx expression using antibiotic induction, top-down/middle-down proteomic analysis and gene/genomic sequencing. Thirty-five STEC strains were tested, and only clinical types/subtypes: Stx2a and Stx2c were detected. In one STEC strain, both Stx2a and Stx2c were differentially expressed using different antibiotics.

- **Proteolytic surface-shaving of Salmonella enterica enterica and SPI-1 expression.** Salmonella enterica enterica (SEE) is comprised of over 2500+ different serovars. However, not all SEE serovars are considered robust human pathogens. ARS conducted studies to identify proteins exposed on the surface of live SEE cells using proteolytic surface-shaving (PSS) and high-resolution mass spectrometry (HR-MS). Interestingly, whereas SEE Newport and Thompson showed proteolytic fragments of the effector/invasion proteins (SipA-D) of the Salmonella pathogenicity island (SPI-1), SEE Kentucky did not. The absence of SipA-D detection for SEE Kentucky is consistent with previous assessments of SEE Kentucky indicating that, although it is prevalent in poultry, it is not considered to be a robust human pathogen. These preliminary experiments suggested either that the brief PSS step induced expression of SipA-D or that the injectosome complex (comprised of SipA-D) existed prior to the PSS step

Outcomes and Impacts

- Identified high prevalence of human noroviruses on surface waters at sites adjacent to leafy green fields, indicating that contamination of fresh produce fields could potentially result from runoff after heavy rain events. These findings subsequently contributed to establishing a CRADA for designing aptamer-based biosensors for pathogen capturing to be used by leafy greens processors
- Determined that the use of flow cytometry enabled the quantification of the inhibition of protein synthesis in sub-populations of cells exposed to bacterial toxins. This provided fundamental information on the amounts of bacterial toxins resulting in reversible toxin effects and established that toxin neutralization at a post-exposure stage could be an effective strategy for the treatment of STEC infections and other toxin-mediated diseases.
- Determined by WGS that the stx_{2a} and stx_{2c} genes for this strain are in two different bacteriophage genomes present in the bacterial genome. This technique demonstrated a rapid method for detecting and identifying Shiga toxin expression from suspected STEC strains.
- Demonstrated that proteolytic surface-shaving (PSS) can provide evidence for the existence of surface-exposed higher order protein structures (injectosome, flagella, etc.) that can confirm pathogen virulence and motility. This compares favorably with traditional techniques for assessing Salmonella virulence which involves in vitro mammalian cell assay, or the mouse model are time-consuming, labor-intensive and costly.

Examples of Relevant Publications

- Tian, P., Yang, D., Shan, L., Wang, D., Li, Q., Gorski, L., Lee, B. G., Quiñones, B., Cooley, M. B. 2017. Concurrent detection of human norovirus and bacterial pathogens in water samples from an agricultural region in Central California Coast. Frontiers in Microbiology 8:1560.

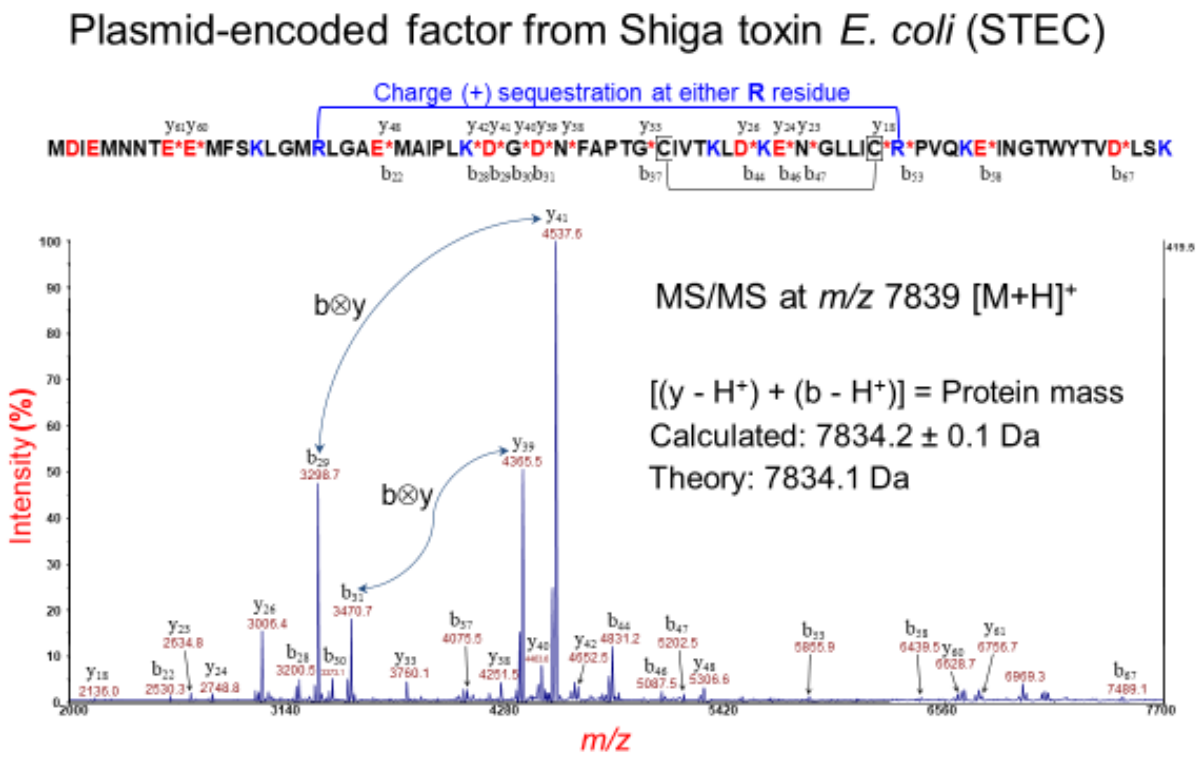
- Quiñones, B., Lee, B. G., Martinsky, T. J., Yambao, J. C., Haje, P. K., Schena M. 2017. Sensitive genotyping of foodborne-associated human noroviruses and hepatitis A virus using an array-based platform. Sensors 17(9):2157.
- Miller, W.G., Yee, E., Lopes, B.S., Chapman, M.H., Huynh, S., Bono, J.L., Parker, C.T., Strachan, N.J.C., Forbes K.J. 2017 Comparative genomic analysis identifies a *Campylobacter* clade deficient in selenium metabolism. Genome Biology and Evolution 9(7): 1843-1858.
- Miller, W.G., Yee, E., Chapman, M.H., Bono, J.L. 2017. Complete genome sequence analysis of all three *Campylobacter sputorum* biovars and a novel cattle-associated *C. sputorum* clade. Genome Biology and Evolution 9(6): 1513-1518.
- Miller, W.G., Chapman, M.H., Yee, E., Revez, J., Bono, J.L., Rossi, M. 2017. Complete genome sequence of the hippuricase-positive *Campylobacter avium* type strain LMG 24591. Genome Announcements 5(43): e01221-17
- Cherubin, P., Quiñones, B., Teter, K. 2018. Cellular recovery from exposure to sub-optimal concentrations of AB toxins that inhibit protein synthesis. Scientific Reports 8:2494.
- Fagerquist, C.K., Zaragoza, W.J., Lee, B.G., Yambao, J.C., Quinones, B. 2018. Clinically-relevant Shiga toxin 2 subtypes from environmental Shiga toxin-producing Escherichia coli identified by top-down/middle-down proteomics and DNA sequencing. *Clinical Mass Spectrometry*. 11:27-36. https://doi.org/10.1016/j.clinms.2018.12.001.
- Fagerquist, C.K., Zaragoza, W.J. 2018. Proteolytic surface-shaving and serotype-dependent expression of SPI-1 invasion proteins in Salmonella enterica subspecies enterica. *Frontiers in Nutrition*. 5:124.
- On, S.L.W., Althus, D., Miller, W.G., Wong, S.G.L., Lizamore, D., Chelikani, V., Carter, G. 2019 *Arcobacter cryaerophilus* isolated from New Zealand mussels harbour a putative virulence plasmid. Frontiers in Microbiology 10:1802.
- Hsu, T., Gemmell, M.R., Franzosa, E., Berry, S., Mukhopadhya, I., Hansen, R., Michaud, M., Nielsen, H., Miller, W.G., Nielsen, H., Bajaj-Elliott, M., Huttenhower, C., Garrett, W.S., Hold, G.L. 2019. Comparative genomics and genome biology of *Campylobacter showae*. Emerging Microbes & Infections, 8(1): 827-840.
- Cherubin P., Quiñones B., Elkahoui S., Yokoyama W., Teter K. 2019. A cell-based fluorescent assay to detect the activity of AB toxins that inhibit protein synthesis. Methods in Molecular Biology 1600:25-36.

The (Gorski) project objectives were to: (1) Develop methods for the detection and subtyping of enteric bacterial and viral pathogens from produce production environments; to aid epidemiological investigations and to distinguish pathogenic from non-pathogenic strains; and (2) Develop of immuno-, bacteriophage-, and mass spectrometry-based methods for rapid detection of foodborne pathogens.

Examples of Accomplishments

- **Developing assays for detection of HuNoV.** ARS in collaboration with Shanghai Jiao Tong University, China, developed two assays for detection of HuNoV in food and environmental samples. One assay, a receptor-mediated capture assay (ISC-RT-qPCR), was developed to estimate the level of HuNoV infectivity in food and environmental water samples. This assay was used in China to detect norovirus in oysters, and detect HuNoV in environmental water sample in California. The second developed is the immune-chromatographic assay (ICA) for rapid detection of HuNoV, which can be performed in 15 minutes, and a shelf-life up to 2 years at room temperature.

- **Rapid detection of Shiga toxins in complex matrices.** Shiga toxins are the most important virulence factor of Shiga toxin producing Escherichia coli (STEC). A rapid (< 6 hours) mass spectrometry-based method was developed to detect and distinguish among the Shiga toxins present in complex samples. ^{15}N-labeled peptides (internal standards) are produced by a synthetic gene, which allows new peptides to be incorporated into the method as novel Shiga toxins are discovered. This method was adapted to detect previously undetectable type 2 Shiga toxins in human serum using mass spectrometry or monoclonal antibodies. It was recently used to quantify the Shiga toxins produced by environmental STEC that were not outbreak strains but were isolated from environments where green leafy vegetables are grown in Central California.



- **Bacteriophage-based Electrochemical Biosensor**. A portable wireless-based bacteriophage-electrochemical biosensor for the detection of viable STEC was successfully developed. The biosensor can detect as low as < 10-100 viable cells of STEC strains (O26, O157, O179) in 1 ml or gram of ground meat, apple juice, and environmental water samples. Taking only 30 minutes to perform and costing only $3.28/test, this new biosensor is 7-88% cheaper than traditional culture-based, PCR-based, and antibody-based tests, and yields results hours to days faster than existing tests.

Outcomes and Impacts

- Developed the ICA technology for norovirus detection which can greatly reduce the detection time and keep the sensitivity comparable to other immunological kits without using special equipment and reagents. A Patent is being filed for the ICA assay.
- Developed the rapid, cost efficient, portable and wireless bacteriophage based STEC detection technology. The technology was selected for the USDA ARS Innovation Fund Award in 2019 to enhance commercialization potential and enable the adoption of the technology by the industry.

Examples of Relevant Publications

- Silva CJ, Erickson-Beltran ML, Skinner CB, Patfield SA, He X (2015) Mass spectrometry-based method of detecting and distinguishing type 1 and type 2 Shiga-like toxins in human serum. *Toxins (Basel)* 7:5236-5235
- Tian P, Wang D, Shan L, Wang D, Li Q, Gorski L, Lee BG, Quiñones B, Cooley M (2017) Concurrent detection of human norovirus and bacterial pathogens in water samples from an agricultural region in Central California Coast. *Front Microbiol* 8:1560. https://doi:10.3389/fmicb.2017.01560
- Zhou Z, Tian Z, Li Q, Tian P, Wu Q, Wang D, Shi X (2017) *In situ* capture RT-qPCR: a new simple and sensitive method to detect human norovirus in oysters. *Front Microbiol* 8:554
- Tian P, Yang D, Lei S, Li Q, Liu D, Wang D (2018) Estimation of human norovirus infectivity from environmental water samples by *in situ* capture RT-qPCR method. *Food Environ Virol* 10:29-38
- He X, Ardissino G, Patfield SA, Cheng LW, Silva CJ, Brigotti M (2018) An improved method for detection of Shiga toxin 2 in human serum. *Toxins (Basel)* 10:pii:E59
- Silva CJ (2018) Food forensics: using mass spectrometry to detect foodborn protein contaminants, as exemplified by Shiga toxin variants and prion strains. *J Food Ag Chem* 66:8435-8450
- Wu VCH, Quintela IA (2018) Bacteriophage-based electrochemical biosensor. USA Patent 12617, Quintela IA, de los Reyes BG, Lin C-S, Wu VCH (2019) Simultaneous colorimetric detection of a variety of Salmonella spp. in food and environmental samples by optical biosensing using oligonucleotide-gold nanoparticles. Front Microbiol 10:1138

- Silva CJ, Lee BG, Yambao JC, Erickson-Beltran ML, Quiñones B (2019) Using nanospray liquid chromatography and mass spectrometry to quantitate Shiga toxin production in environmental *Escherichia coli* recovered from a major produce production region in California. *J Food Ag Chem* 67:1554-1562.
- Quiñones B, Yambao JC, Silva CJ, Lee BG (2019) Draft genome sequences of non-outbreak *Escherichia coli* O157:H7 strains recovered from a major produce-production region in California. *Microbiol Research Announcements*. 8:e00644-00619

Studies at MARC, Clay Center, NE (Bosilevac) addressed the following objective to: (1) Develop improved sampling, detection, and tracking technologies to identify points, including biofilms, where pathogens persist and contaminate in the production of red meat.

Examples of Accomplishments

- **A novel continuous sampling device (CSD) and a manual sampling device (MSD) for beef trim microbiological testing.** Beef trim sampling for pathogen testing is one of the final steps in the food safety system beef processors have implemented to keep meat safe and wholesome for consumers. Traditional methods of sampling for pathogen testing examine less than one pound (325g or 150g) of trimmings from a 2,000-pound combo bin of beef trimmings destined for ground beef. A new trim sampling method using a CSD or an MSD was developed and validated. The CSD and MSD sample a much greater proportion of the trim and are non-destructive. Results from over 1400 samples on numerous days across multiple companies, processing plants, and lean types demonstrated that both the CSD and MSD provide an equal or better level of performance for detecting pathogen contamination in beef trim compared to the existing methods.

- **Improving pathogen detection in beef samples.** The first step of testing beef for E. coli O157:H7 involves growing the meat in a liquid media to enrich the bacteria. This is generally followed by a molecular test targeting E. coli O157:H7. The most commonly used liquid media is modified tryptic soy broth (mTSB) which is non-selective and makes identification of pathogens difficult. A new STEC and Salmonella selective media (SSS broth) was found to control background bacteria growth and allow mostly STEC and Salmonella to grow out for detection. Many molecular tests for E. coli O157:H7 cannot distinguish E. coli O157:H7 from other E. coli of the same O157-serogroup, thus, producing false positive results. A new commercial test kit that can more accurately distinguish E. coli O157:H7 from the other E. coli was developed, validated, and compared to other tests and the gold standard, culture isolation. The new test is based on the identification of a single nucleotide polymorphism (snp) in the rfbO157-gene that is specific to only E. coli O157:H7. The comparative sensitivity, specificity, false-positive rate, and false-negative rate against culture was found to be 92%, 99%, 0.77%, and 8.3%, respectively.

Outcomes and Impacts

- Implementation of the MSD and CDS have been commercialized through a CRADA partner. The new trim sampling methods are resulting in improved beef safety with additional benefits in reduced labor and other costs, and improved worker safety.
- Numerous large and small beef processors have adopted the CSD or MSD method for their sample collection. The MSD and CSD are being validated for microbiological sample collection in pork, poultry, and other food stuffs.
- The CSD and MSD method has been recognized with the 2019 "Excellence in Technology Transfer Award" by the Federal Laboratory Consortium for Technology Transfer.
- When used by beef processors for E. coli O157:H7 testing, the new Real-time PCR linked with melt peak method will not produce the false positive results often seen with other test methods and will implicate less beef products are adulterated requiring disposition.

Examples of Relevant Publications

- Eggers, J., Feirtag, J.M., Olstein, A.D., Bosilevac, J.M. 2018. A novel selective medium for simultaneous enrichment of Shiga toxin-producing *Escherichia coli* and *Salmonella* in ground beef. Journal of Food Protection. 81:1252-1257. https://doi: 10.4315/0362-028X.JFP-17-520.
- Wheeler, T.L., and Arthur, T.M. 2018. Novel continuous and manual sampling methods for beef trim microbiological testing. J. Food Prot. 81:1605-1613. https://doi: 10.4315/0362-028X.JFP-18-197.
- Bosilevac, J.M., Dwivedi, H.P., Chablain, P., Ullery, M., Bailey, J.S., Dutta, V. 2019. Comparative performance evaluation of real-time PCR and dual labeled fluorescence resonance energy transfer probe-based melt peak analysis for the detection of Escherichia coli O157:H7 in beef products. Journal of Food Protection. 82(3):507-512. https://doi.org/10.4315/0362-028X.JFP-18-366.

Research at U.S. NPRC, Athens, GA was conducted by two projects (Park and Buhr)

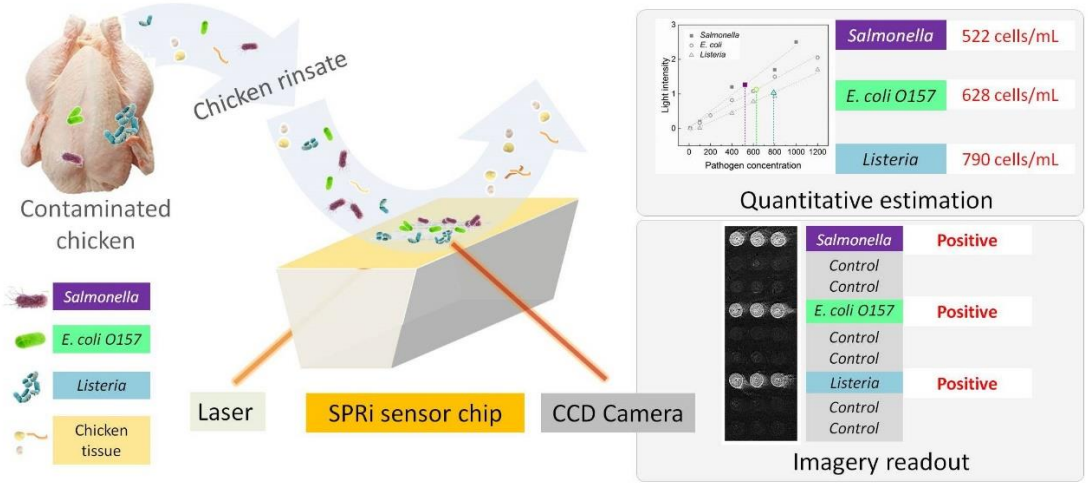
The (Park) project addressed the following objectives to: Develop high-speed imaging methods for rapid detection of pathogens in live poultry flocks, and foodborne hazards, including foreign materials, in processed poultry products; (2) Develop rapid methods and protocols for early detection, identification, and quantification of pathogens in poultry products (foods) using imaging spectroscopy; and (3) Develop methods to detect biofilms in poultry processing facilities with optical technologies.

Examples of Accomplishments

- **Multiplex and label-free screening of foodborne pathogens using surface plasmon resonance imaging (SPRi)**. Surface plasmon resonance imaging (SPRi) is an emerging optical technique, which allows for label-free screening of multiple targets simultaneously with minimum or no sample preparation. Studies evaluated the

feasibility of SPRi for simultaneous detection of four important foodborne pathogens, Salmonella spp., Shiga-toxin producing E. coli, Listeria monocytogenes, and Campylobacter jejuni. The SPRi-biochip was functionalized with corresponding polyclonal antibodies and blocking agents. Performance of the multiplex SPRi method was tested with the bacterial cells while the influence of antibody concentration and immobilization pH was optimized, and the specificity was evaluated. The results suggest that the new SPRi technique has the potential for multiplex pathogen screening, and with further improvements in sensitivity, this platform could provide a flexible and automated means for pathogen detection in food matrices.

Figure: SPRi screening technique



- **Bacterial cell classification using hyperspectral microscopy and machine learning with convolutional neural networks.** Hyperspectral microscope imaging (HMI) has the potential to be an early, rapid, and sensitive presumptive detection tool for classifying pathogenic bacteria on a cellular level. Classification accuracy of HMI was high at the serotype level of the same species of bacteria for Salmonella. For robustness of the HMI technology, ARS investigated the impact of environmental growth stresses such as growth media, incubation temperature and pH on HMI cellular spectra. Testing the same Salmonella strain over a variety of environmental factors found that acidic pH values and high incubation temperatures influence the cellular spectra, most likely due to the outer membrane of the cell generating acid and heat response proteins. The bacteria's growth media suggested that the cell's spectra did change slightly with media, while media with similar color indicators were spectrally similar. These results suggest that given a range of pH and temperature values which are not inducing cellular stresses, pathogens can be classified with high accuracy. Machine learning algorithms, including U-net for segmentation and 1D-CNN for classification, enhanced performance of HMI data analysis for classification of foodborne bacteria to 93.9% accuracy compare to conventional algorithms such as k-nearest neighbor (79.6%) and support vector machine (82.8%).

- **Detection and characterization of Salmonella with immunomagnetic separation (IMS) and surface enhanced Raman spectroscopy (SERS).** ARS developed a method combining the advantages of immunomagnetic separation (IMS) and surface enhanced Raman spectroscopy (SERS) techniques with the goal of detecting and characterizing Salmonella in 24 hours with high sensitivity and minimal sample preparation. After selectively capturing the pathogen from food samples with IMS and overnight culture, SERS spectra were collected and analyzed with multivariate statistical models. The detection and characterization accuracies were confirmed by traditional methods and validated in mixed-culture samples consisting of common foodborne bacteria. The specificity for detecting Salmonella from other species was higher than accuracies between individual Salmonella serotypes. While the initial approach provides an inexpensive alternative to current methods, the expansion of spectral libraries to include additional pathogen serotypes can improve the specificity.

Outcomes and Impacts

- Developed and evaluated a Surface Plasmon Resonance Imaging (SPRi) biochip for simultaneous detection of four important foodborne pathogens. Results suggest that with further improvements in sensitivity, this platform could provide a flexible and automated means for pathogen detection in food matrices.
- Evaluated whether Hyperspectral Microscope Imaging (HMI) has the potential to be an early, rapid, and sensitive presumptive detection tool for classifying pathogenic bacteria on a cellular level. Results suggest that given a range of pH and temperature values which are not inducing cellular stresses, pathogens can be classified with high accuracy.
- Developed machine learning algorithms, including U-net for segmentation and 1D-CNN for classification, enhanced performance of HMI data analysis for classification of foodborne bacteria to ~94% accuracy compare to conventional algorithms such as k-nearest neighbor (~80%) and support vector machine (~83%). A prototype system is being developed with a CRADA partner for commercial use.
- Developed a method combining immunomagnetic separation (IMS) and surface enhanced Raman spectroscopy (SERS) to detect and characterize Salmonella in 24 hours. Specificity for detection from other species was higher than accuracies between individual Salmonella serotypes. The approach provides an inexpensive alternative to current methods, and with the expansion of spectral libraries can improve the specificity.

Examples of Relevant Publications

- Eady, M., Park, B. 2016. Rapid identification of Salmonella serotypes through hyperspectral microscopy with different lighting sources. Journal of Spectral Imaging 5(1):1-10. https://doi.org/ 10.1255/jsi.2016.a4
- Chen, J., Park, B., Eady, M. 2017. Simultaneous detection and serotyping of Salmonellae by immunomagnetic separation and label-free surface enhanced Raman spectroscopy. Food Analytical Methods 10(9): 3181-3193. https://doi: 10.1007/s12161-017-0870-x

- Chen, J., Park, B. 2017. Effect of immunomagnetic bead size on recovery of foodborne pathogenic bacteria. International. Journal of Food Microbiology. 267: 1-8. https://doi.org/10.1016/j.ijfoodmicro.2017.11.022
- Chen, J., Park, B. 2017. Label-free screening of foodborne Salmonella using surface plasmon resonance imaging. Analytical and Bioanalytical Chemistry. 410(22): 5455-5464. https://doi.org/10.1007/s00216-017-0810-z
- Chen, J., Park, B., Huang, Y.W., Zhao, Y, Kwon, Y. 2017. Label-free SERS detection of Salmonella Typhimurium on DNA aptamer modified AgNR substrates. Journal of Food Measurement and Characterization 11(4): 1173-1779. https://doi:10.1007/s11694-017-9558-6
- Chu, X., Wang, W., Yoon, S.C., Ni, X., Heitschmidt, G.W. 2017. Detection of aflatoxin B1 (AFB1) in individual maize kernels using short wave infrared (SWIR) hyperspectral imaging. Biosystems Engineering, 157: 13-23. https://doi.org/10.1016/j.biosystemseng.2017.02.005
- Wang, B., Park, B., Xu, B., Kwon, Y.K. 2017. Label-free biosensing of Salmonella enterica serovars at single-cell level. Journal of Nanobiotechnology 15:40.
- Jiang, H., Yoon, S.C., Zhuang, H., Wang, W., Yang, Y. 2017. Evaluation of factors in development of Vis/NIR spectroscopy models for discriminating PSE, DFD and normal broiler breast meat. British Poultry Science. 58(6):673-680. https://doi.org/10.1080/00071668.2017.1364350
- Jiang, H., Yoon, S.C., Zhuang, H., Wang, W., Yang, Y. 2017. Evaluation of factors in development of Vis/NIR spectroscopy models for discriminating PSE, DFD and normal broiler breast meat. British Poultry Science. 58(6):673-680. https://doi.org/10.1080/00071668.2017.1364350
- Park, B., Seo, Y., Eady, M., Yoon, S.C., Hinton, A., Lawrence, K.C., Gamble, G. 2017. Classification of Salmonella Serotypes with Hyperspectral Microscope Imagery. J. Annals of Clinical Pathology 5(2): 1108-1116.
- Eady, M.B., Park, B. 2018. Unsupervised classification of Salmonella Typhimurium from mixed bacteria cultures with hyperspectral microscope imaging. Journal of Spectral Imaging. https://doi.org/10.1255/jsi.2018.a6
- Seo, Y., Park, B., Yoon, S.C., Lawrence, K.C., Gamble, G.R. 2018. Morphological image analysis for foodborne bacteria classification. Transactions ASABE. 61(1): 5-13.
- Park, B., Eady, M., Oakley, B., Yoon, S.C., Lawrence, K.C., and Gamble, G. 2019. Hyperspectral microscope imaging methods for multiplex detection of *Campylobacter*, Journal of Spectral Imaging 8, a6, https://doi.org/10.1255/jsi.2019.a6
- Eady, M., Setia, G., and Park, B. 2019. Detection of *Salmonella* from chicken rinsate with hyperspectral microscope imaging compared against RT-PCR. Talanta 195: 313-319.
- Eady, M., Park, B. 2019. The effect of environmental growth conditions on Salmonella spectra obtained from hyperspectral microscope images. Food Analytical Methods. https://doi.org/10.1007/s12161-019-01618-0
- Kang, R., Park, B., Chen, J., Chen, K.J. 2020. Identifying non-O157 Shiga toxin-producing Escherichia coli (STEC) using deep learning methods with hyperspectral microscope images. Spectrochimica Acta Part A: Molecular and Biomolecular, 224: 117386, https://doi.org/10.1016/j.saa.2019.117386

The (Buhr) project became involved in methods development and validation during the research cycle as a result of concerns regarding antimicrobial wash carryover and Salmonella verification in poultry.

Examples of Accomplishments (see also Interventions)

- **Development of neutralizing Buffered Peptone Water (nBPW).** ARS developed a neutralization solution (nBPW) for use by commercial poultry processors in Salmonella verification testing. Commercial poultry processors use chemical sanitizers during processing to reduce contamination of carcasses by human foodborne pathogens. However, if traces of these sanitizers are carried-over into testing samples used to determine contamination of poultry carcasses and parts by Salmonella, the results of these tests may be inaccurate.

- **Novel selective medium for growth of Campylobacter.** Campylobacter research is limiting because the pathogen must be grown under artificial atmospheres on media supplemented with blood. ARS developed a novel selective bacterial medium that allows growth of Campylobacter in containers incubated aerobically. The formulation enables the use of aerobic isolation of Campylobacter from samples that also contained other bacteria. Three antibiotic mixtures were tested as supplements that could be added to the medium to allow the growth of Campylobacter while inhibiting the growth of other bacteria. Results indicated that while the other bacteria could grow in media that contained no antibiotics, the growth of most of these bacteria was inhibited in the media supplemented with antibiotics. It was also determined that when Campylobacter was grown in cultures containing other bacteria, Campylobacter could outgrow the other bacteria in the media supplemented with the Bolton antibiotics, but not in the non-supplemented medium.

Outcomes and Impact

- Developed and validated a new Campylobacter medium that does not require the utilization of artificial atmospheres or blood. Furthermore, the addition of selective agents to the medium enables researchers to isolate the bacterium from environmental samples.
- Determined and validated that supplementing the new Campylobacter medium with Bolton's antibiotics produces a selective medium that could be used for aerobic incubation to isolate Campylobacter.

Example of Relevant Publications

- Hinton Jr, A., Cox Jr, N.A. 2018. Selective medium for aerobic incubation of Campylobacter. Journal of Food: Microbiology, Safety, and Hygiene. 3(1):1-6. doi: 10.4172/2476-2059.1000131
- Hinton, Jr. A., Gamble G.R., Berrang, M.E., Buhr, R.J., Johnston, J.J., 2019. Development of neutralizing buffered peptone water for salmonella verification testing in commercial poultry processing facilities. Submitted to Journal of Food: Microbiology, Safety, and Hygiene.

- Hinton, Jr. A., Gamble G.R., Berrang, M.E., Buhr, R.J., Johnston, J.J., 2019. Development of neutralizing buffered peptone water for salmonella verification testing in commercial poultry processing facilities. Journal of Food: Microbiology, Safety, and Hygiene. 10(30):359. doi:10.4172/2155-9597.10.359.

Parasitology methods research was conducted at BARC, Beltsville, MD through the (Santin) project where the objective was to: (1) Develop a unique and highly sensitive assay to detect the zoonotic protists Cryptosporidium, Giardia, Blastocystis, Encephalitozoon and Enterocytozoon in food and environmental samples by targeting intracellular viral symbionts of these parasites and water-borne pathogens.

Examples of Accomplishments

- **Sensitive molecular assay to detect the parasite Cryptosporidium in source and finish water.** Cryptosporidium is responsible for the largest waterborne outbreak in the history of the U.S., and is estimated to cause 30,000 cases of severe diarrhea annually in the U.S. The parasite is resistant to most standard water disinfectants, can cause clinical infection at very low numbers, and no effective drug or vaccine exists. ARS developed a new RNA assay that targets a virus, Cryspovirus, found inside all species of Cryptosporidium in thousands of copies per parasite and tested its efficacy to detect the parasite in water inoculated with various numbers of Cryptosporidium oocysts.

Outcomes and Impacts

- Developed an RNA assay that targets a virus, Cryspovirus, found inside all species of Cryptosporidium. The assay was more sensitive than current gold standard tests and could be used for water screening by regulatory and public health agencies to detect and track Cryptosporidium in untreated and drinking water.

Examples of Relevant Publications

- Jenkins, M., O'Brien, C., Fetterer, R., Santin, M. 2016. RT-PCR specific for Cryspovirus is a highly sensitive method for detecting *Cryptosporidium parvum* oocysts. Food and Waterborne Parasitology 5:14-20.
- de Souza, M.S., O'Brien, C., Santin, M., Jenkins, M. 2019. A highly sensitive method for detecting *Cryptosporidium parvum* oocysts recovered from source and finished water using RT-PCR directed to Cryspovirus RNA. Journal of Microbiology Methods. 156: 77-80.

Research at ERRC, Wyndmoor, PA was conducted by the (Gehring) project addressing the following objectives to: (1) Develop rapid and efficient techniques that separate and concentrate and/or quantify targeted pathogens from food matrices; (2) Develop and validate field testing kits that rapidly screen for the presence and quantification of pathogens and/or indicator microorganisms in foods at the initial processing level; and (3) Develop and validate rapid methods for the identification of pathogens and/or indicator microorganisms in foods for application in either the field or testing laboratories.

Examples of Accomplishments

- **A novel method for rapid enrichment, amplification, and DNA sequence-based typing of foodborne pathogens**. ARS and a biotech company developed a novel enrichment, amplification, and DNA sequence-based typing (EAST) method that required 3 days or less to complete and provided strain resolution enough for source tracking and epidemiological investigation. EAST was applied to the detection of Salmonella-spiked ground turkey and Yersinia enterocolitica-spiked ground pork demonstrating a very high sensitivity and specificity for the target pathogens. Compared to existing typing technology (e.g., pulsed-field gel electrophoresis-based PulseNet, EAST is more sensitive, specific, and simple as well as a relatively rapid, and less costly method. EAST can be used to both detect and type important foodborne pathogens directly from food enrichments containing background bacteria.

- **WGS and analysis of Campylobacter coli YH502 from retail chicken**. ARS applied next-generation DNA sequencing for detection, genotyping, and characterization of Campylobacter strains isolated from retail meat. This analysis, in conjunction with multilocus sequence typing yielded very subtle differences were exploited to delineate seemingly identical Campylobacter strains.

- **Evaluation of antibody-based tests specific for Shiga toxin 1 and 2 in food and water samples**. Antibody-based detection methods referred to as enzyme-linked immunosorbent assays (ELISAs) have been commercially developed to detect, but not distinguish Shiga toxin producing E. coli (STEC) that may produce either one or both toxins: Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Stx2 is 400 times more toxic than Stx1, and the ability to differentiate STEC by their toxin production assists in epidemiological investigations. ARS in collaboration with a biotech company and CRADA partner developed and commercialized two new ELISAs that, unlike other similar rapid test products, could distinguish STEC that produce either Stx1 or Stx2 in food and environmental samples (beef, Romaine lettuce, recreational water, and pasteurized milk were tested). Each ELISA protocol incorporated monoclonal antibodies (demonstrated to bind all known Stx1 or Stx2 variant subtypes) and an innovation of using a bacterial protein extraction reagent that proved to be effective in releasing and thus accurate detection of cell-bound Stx1. Ultimately, detection and differentiation of the Shiga toxin types was achieved with good overall sensitivity, specificity, variance, and reproducibility.

- **Disease-causing potential of Campylobacter isolates from beef and poultry.** ARS used a novel method to isolate 27 Campylobacter strains from meat (chicken and beef), and the strains were identified as either C. jejuni or C. coli by molecular methods, including DNA next generation sequencing. The whole genome sequences and encoded proteins of C. jejuni strain YH001 and C. coli strain YH501 were determined and have been deposited into the NCBI Genbank database under the accession numbers CP010058 and CP015528, respectively. Bioinformatics analysis revealed a significant genetic distance between the C. jejuni and C. coli species, and the isolates from the same food source appeared to be related more closely to each other than those from different sources. Comparative sequence analysis indicated potentially higher disease-causing potential and drug resistance for a strain (C. jejuni YH001) that was found to contain a key virulence gene cluster (cdtABC) and genes for a multidrug efflux pump (cmeABC).

- **Novel test for detection of a bacterial toxin in food.** Production of Shiga toxin (Stx) is both an important virulence factor for the pathogenic bacterium Escherichia coli, and a distinguishing feature routinely screened for in meat samples by the FSIS. Under a collaborative agreement between ARS and Abraxis LLC a novel antibody-based test (amplified luminescent proximity homogeneous assay or AlphaLISA) was developed for the detection of Shiga toxin 2 (Stx2) generated by Stx-producing E. coli (STEC) in foods (Romaine lettuce, ground beef). Efficacy and sensitivity trials showed that not only was AlphaLISA as sensitive as the industry standard ELISA test, but it also demonstrated a superior signal-to-noise ratio with the ability to distinguish high concentrations of the toxin. These features in combination with the reduced hands-on workflow and amenability for automation, make the AlphaLISA method a more economically viable choice for Stx2 detection in foods by both regulatory agencies and testing labs alike.

- **Absolute quantification of Shiga-toxin producing E. coli in beef with ddPCR.** Because harmful bacteria often possess a combination of distinguishing traits/markers that allow them to cause disease, screening systems such as that utilized by FSIS capitalize on the existence of these traits and can delineate potential disease-causing E. coli strains based on the presence of 3 such genetic markers. However, false positives result when a single sample contains more than one bacterium that possess 1 or 2 of the markers (but not all 3 of them) since the screening method does not define the specific organism from which each gene was derived. To overcome this shortfall, a new screening system known as Droplet Digital PCR (ddPCR) with the ability to determine when multiple genes are contained within a single source organism was developed and tested by the Food Science Division at Bio-Rad Laboratories, Inc. (Marnes-la-Coquette, France) in partnership with ARS. Ultimately, this system results in cost-savings by reducing both wasted man-hours and expenses associated with subsequent evaluation of false-positive samples and testing kits are expected to be released for purchase by Bio-Rad Laboratories, Inc. Fall 2019.

- **Rapid flow-through immunoelectrochemical detection of low numbers of bacteria in large volumes.** To address the safety of the food supply chain, the size of raw meat samples collected for testing was increased from ~0.9 ounces to ~11.5 ounces. Although testing involving larger samples sizes can increase the likelihood of detecting pathogens present at low levels, it also creates an unmet need for rapid detection methods that can be used on large volume food samples. In response to this need, an electrochemical sensor was engineered in order to provide a testing method that can accommodate the larger sample size. The newly designed sensor consisted of a porous transducer (sensing element) that allowed for 1 L of sample to be filtered through within one hour. This sensor demonstrated the ability to detect different common foodborne pathogens in food samples that are aligned with protocols currently employed by FSIS.

- **Rapid detection of salmonella via emission from dynamic double emulsion droplets.** A new sensing model for the early-stage detection of foodborne pathogens that is based on the unique chemical-structural-optical coupling in chemical (boronic acid)-functionalized fluorescent double emulsions was developed. This novel sensor demonstrated the ability to detect Salmonella in both enrichment media, and chicken rinse samples and the results of this testing were published in a high impact factor, peer-reviewed, scientific journal.

Outcomes and Impacts

- Developed a novel enrichment, amplification, and DNA sequence-based typing (EAST) method that requires 3 days or less to complete. Compared to existing PFGE-based PulseNet, EAST is more sensitive, specific, simple, relatively rapid, less costly, and can detect directly from food enrichments containing background bacteria.
- Applied next-generation DNA sequencing for detection, genotyping, and characterization of Campylobacter strains isolated from retail meat.
- Developed and commercialized two new ELISAs that, unlike other similar rapid test products, could distinguish STEC that produce either Stx1 or Stx2 in food and environmental samples. The ELISA's have high detection, differentiation, sensitivity, specificity, variance, and reproducibility.
- Conducted WGS of C. jejuni strain YH001 and C. coli strain YH501. Comparative analysis indicated potentially higher disease-causing potential and drug resistance for the strain (C. jejuni YH001).
- Developed in collaboration with Abraxis LLC a novel antibody-based test to detect Shiga toxin 2 (Stx2). The test is highly sensitive, demonstrated a superior signal-to-noise ratio, and can be automated for regulatory use.
- Developed and tested a new screening system which can determine when multiple genes are contained within a single source organism. This system results in time cost-savings, and expenses associated with subsequent evaluation of false-positive samples. Testing kits are expected to be released for purchase by Bio-Rad Laboratories, Inc. Fall 2019.
- Developed an electrochemical sensor to accommodate large samples aligned with protocols currently employed by regulatory agencies. Under provisional Patent application.

- Developed a new sensing model for the early-stage detection of pathogens based on the unique chemical-structural-optical coupling in chemical functionalized fluorescent double emulsions. This novel sensor demonstrated the ability to detect Salmonella in both enrichment media, and chicken rinse samples

Examples of Relevant Publications

- Zhang, D., Coronel-Aguilera, C., Romero, P., Perry, L., Minocha, U., Rosenfield, C., Gehring, A.G., Paoli, G., Bhunia, A.K., Applegate, B., 2016. The use of a novel nanoLuc-based reporter phage for the detection of Escherichia coli O157:H7. Scientific Reports. https://doi: 10.1038/srep33235.
- Gurtler, J., Doyle, M.P., Kornacki, J.L., Fratamico, P.M., Gehring, A.G., Paoli, G., 2017. Advantages of virulotyping foodborne pathogens over traditional identification and characterization methods. Foodborne Pathogens Virulence Factors and Host Susceptibility. New York, NY: Springer Publishing. p. 3-40.
- Capobianco, J., Martorano, C., DeClement, D., 2017. Multilayer devices and methods of manufacturing. Patent Cooperation Treaty (PCT) 62/452,335
- Edlind, T., Brewster, J.D., Paoli, G.C., 2017. Enrichment, amplification, and sequence-based typing of *Salmonella enterica* and other foodborne pathogens. Journal of Food Protection. 80(1):15-24.
- Ghatak, S., He, Y., Reed, S., Strobaugh, T., Irwin, P., 2017. Whole genome sequencing and analysis of *Campylobacter coli* YH502 from retail chicken reveals a plasmid-borne type VI secretion system. Genomics Data. 11:128-131.
- Gehring, A.G., Fratamico, P.M., Lee, J., Ruth, L.E., He, X., He, Y., Paoli, G.C., Stanker, L.H., Rubio, F.M., 2017. Evaluation of ELISA tests specific for Shiga toxin 1 and 2 in food and water samples. Food Control. 77:145-149.
- Xie, Y., He, Y., Ghatak, S., Irwin, P.L., Yan, X., Strobaugh Jr, T.P., Gehring, A.G., 2018. Whole genome sequencing and annotation of Staphylococcus aureus strain SJTUF_J27 isolated from seaweed. Data in Brief. 20:894-898. https://doi.org/10.1016/j.dib.2018.08.084.
- Armstrong, C.M., Ruth, L., Capobianco Jr, J.A., Strobaugh Jr, T.P., Fernando, R., Gehring, A.G., 2018. Detection of Shiga toxin 2 produced by Escherichia coli in foods using AlphaLISA. Toxins. 10(11):422. https://www.mdpi.com/2072-6651/10/11/422
- Song, M., Li, Q., He, Y., Feng, Z., Lan, L., Fan, Y., Liu, H., Chen, D., Yang, M., 2019. A comprehensive multilocus sequence typing scheme for identification and genotyping of Staphylococcus strains. Foodborne Pathogens and Disease. 16(5):331-338. https://doi.org/10.1089/fpd.2018.2565.
- Armstrong, C.M., Capobianco, J.A., Gehring, A.G., 2019. Magnetic Bar Capture Device. US Provisional Patent Application No. 62/737,212; USDA DN: 0068.18.
- Capobianco, J.A., Gehring, A.G., Lee J., Armstrong, C.M., 2019. Flow-Through Electrochemical Sensor. US Provisional Patent Application No. 62/821,624; USDA DN: 0021.18.

- Zeininger, L.; Nagelberg, S.; Harvey, K.S.; Savagatrup, S.; Herbert, M.B.; Yoshinaga, K.; Capobianco, J.A.; Kolle, M.; Swager, T.M. 2019 Rapid Detection of Salmonella enterica via Directional Emission from Carbohydrate-Functionalized Dynamic Double Emulsions. ACS Central Science, 5, 789-795. doi:10.1021/acscentsci.9b00059.

Research at CFSE, Purdue University (coordinated through ERRC, Wyndmoor, PA) was conducted by a multidisciplinary project (Mauer) in collaboration with several NP108 research scientists. The project addressed the following objectives: (1) Development and evaluation of technologies for sample preparation, detection (label-based and label-free), and characterization of microbial, chemical, and biological contaminants of concern in foods that can be implemented for improved food safety and food defense; (2) Application of CFSE developed technologies either alone or in combination with existing methods to evaluate microbial populations and the microbial ecology of foods during production and processing..

Examples of Accomplishments

- **Smartphone-based technology pathogen detection:** Bacteriophage have been used to detect foodborne pathogens though use of a bioluminescent indicator transferred into the bacteriophage so that it becomes detectable once the bacteriophage has infected its target pathogen. A challenge for this detection is that it requires sensitive and costly instruments to detect the extremely dim level of luminescent light. Thus, portability and the ability to link a small optics device to any smartphone could facilitate food safety analyses in a variety of environments. The CFSE at Purdue using ARS funding developed a bioluminescence detection system using silicon photomultiplier (SiPM) sensors. The newly proposed system maintains the portability while providing more sensitive detection of the bacteriophage luminescence from E. coli O157:H7. Testing on spiked ground beef samples demonstrated positive signal detection within 10-12 hours of inoculation of cells. This development is a significant advantage compared to the previously CFSE developed smartphone-based bioluminescence detection system called BAQS (Bioluminescent-based Analyte Quantification by Smartphone). Studies also developed an optical imaging system for lateral flow assays using smartphone imaging. A unique imaging box has been designed for imaging the strip and a signal analysis method was proposed for quantitative analysis of cell numbers based on color density of the positive test lines.

- **Method for detection of zero tolerance Shiga toxin producing E. coli.** The USDA and FDA have adapted rigorous testing protocols and adopted a zero-tolerance policy for STEC on/in a variety of food products. Sample sizes vary for the food to be tested however there cannot be one viable pathogen present. Even using highly sensitive assays for detection of low numbers of organisms, the ability to detect one organism in 25 grams is the equivalent of finding a needle in a haystack. ARS and CFSE adapted their previously developed low cost bacteriophage-based technology that produces an optical signal in the presence of STECs to standard protocols. Specifically, protocols

have been developed to facilitate detection during sample shipping (Figure 1) and quantification of environmental samples significantly reducing analysis time and cost.

Figure 1. Ground meat samples tested for E. coli O157:H7 during shipment using a luminescent based optical phage detection system. A) Samples imaged in light. B) Samples imaged in absence of light.

A B

- **BARDOT-based screening of indicator bacteria from food, environmental and clinical samples.** Detection of bacteria provides a continuous monitoring system which can be used to minimize the chances of outbreaks and financial loss to food industry due to recalls. Using the elastic light scatter "BARDOT" (Bacterial Rapid Detection using Optical Scattering Technology) the CFSE at Purdue using ARS funding developed a light scattering image library for bacterial pathogens representing the family Enterobacteriaceae (EB). The library consists of ~1600 scatter images from 14 genera and 31 species. The technology was used to detect EB in artificially inoculated and naturally contaminated food samples, resulting in positive predictive values (PPV) in some cases to 97% when BARDOT libraries were matched against spiked food samples with mixed cultures; and some PPV values to 100% from individual culture analysis. Additionally, the BARDOT technology could also be used for both food and clinical pathogens. For example, it was possible to identify six different Staphylococcus species with an accuracy of > 87%; coupled with a multi-pathogen selective medium for concurrent detection. BARDOT was also capable of detecting and distinguishing in a single assay the three major pathogens, Salmonella enterica, STEC and Listeria monocytogenes from food based on colony scatter signature patterns. This innovative BARDOT multi-pathogen detection platform can reduce turn-around-time and economic burden on food industries, biotechnology companies, and public health laboratories by offering a label-free, non-invasive on-plate multi-pathogen screening technology for reducing microbial food safety and public health concerns.

- **Shiga-toxin (Stx) enrichment by phage induction methods.** Immunological or molecular approaches, although rapid, cannot provide a correlation with the disease or presence of active Shiga- toxin (Stx). The Vero cell (kidney cell line) cytotoxicity assay is the gold standard for Stx detection. The CFSE at Purdue using ARS funding, modified this Vero cell assay for improved performance using phage induction and improved toxin release from E. coli cells during sample enrichment with the addition of Mitomycin C and chloroform. UV, ciprofloxacin, and polymyxin B were also screened for their ability to induce late phage genes for toxin production. Using the modified method that incorporated Vero cells after antibiotic and chloroform treatment, it was possible to detect active Stx from test samples within 16-18 h.

- **Design of HESPI by hyperspectral laser:** Purdue (CFSE) in collaboration with ARS, developed an enhanced and alternate version of the BARDOT instrument. The new technology called "BEAM" utilizes a newly introduced supercontinuum laser which enables the system to use 50-100 different laser lines. One of the critical issues in dealing with a wide range of wavelengths is that the imaging spot laterally translates due to diffraction phenomena from the filters. Engineers designed an optical relay system that can minimize this spot movement such that hyperspectral imaging from a single colony can be achieved within 40-100 ms. Using BEAM technology studies established optical scatter pattern libraries composed of ~8,500 scatter patterns from 29 bacterial strains. These patterns were validated using ~15,300 blind samples with an average error per class of less than 10%. A major limitation of the current software is the inability to classify colonies as "unknown" (belonging to a genus not present in the training library). When BEAM technology is applied for classification of microbial communities from environmental samples, the development of libraries is under continuous improvement by including less abundant, site-specific or not previously observed genera. This validation process demonstrated the potential and the limitations to be addressed for the BEAM technology to become a rapid tool of characterization of culturable bacterial communities from environmental samples.

- **Development of deep-learning-based tools for visualization of complex phenotypic datasets collected for detection, enumeration, and recognition of emerging foodborne pathogens.** A major issue in any type of human disease prevention (including foodborne illness) is the ability to recognize new and emerging threats from a vast and complex sea of data. The CFSE at Purdue using ARS funding carried out an analysis of biophysical data describing phenotypic characteristics of known and unknown microorganisms in order to extract data patterns associated with emerging pathogens. The major goal was to develop the ability of the AI-enhanced data processing pipeline to enumerate the classes of microorganisms and recognize the presence of new (i.e., previously unknown or emerging) types of microbes which are not represented in the early-warning databases. Studies developed a dimensionality-reduction engine able to ingest the phenotypic datasets containing biophysical measurements of the biological samples and represent them in a fashion enabling further automated classification and recognition of new emerging patterns. The software developed at Purdue integrates the cutting-edge machine learning tools developed by the

industry as well as in academia with a unique optical and spectroscopic phenotypic biosensing devices created at Purdue. The result demonstrated the tremendous potential of machine learning technologies in the areas of bio-surveillance, biothreat detection, and agricultural biosafety. Additionally, the developed software has applicability far exceeding the field of agriculture and food safety, and it could be potentially used for visualization and interaction with clinical samples processed using -omics technologies.

- **Affordable, reliable, sensitive, and repeatable biosensor.** The CFSE at Purdue using ARS funding designed a cost-efficient, reliable, and repeatable approach for the detection of whole-cell foodborne pathogens in real food samples. Whole cell detection of pathogens limits the number of sample preparation steps and significantly shortens the testing time. DNA-based aptamers specific for E. coli O157:H7 were applied to paper using a novel, optimized inkjet process. Controlled bioprinting of the test strips with novel carboxyl-functionalized aptameric inks allowed quantitative detection of E. coli O157:H7 in ground beef with an extremely low level of detection (10^2 CFU/ml). This printed aptamer-based platform achieves the lowest limit of detection of the state of the art for aptamer based optical detection of whole cell E. coli O157:H7, with enough evidence to prove its specificity at genus, species, strain, and serotype level. Developing assay strips that provide accurate, quantitative detection with low variability is a critical, new and inexpensive technology for food safety testing.

- **Development of high-resolution, low-powered, networked, time-temperature monitoring sensor.** Temperature abuse of food products has been established as one of the most important causes for foodborne diseases outbreaks. Regulatory efforts focus within food production but there is a lack of control measures outside the production plant. Therefore, distribution and retail are considered as the weakest links in the food safety management system, creating a pressing need for new management tools. Correlation of microbial growth profiles to temperature history requires a sensor that can collect and transmit measurements without interfering with the conditions of the sample under study. The CFSE at Purdue using ARS funding developed a high-resolution, low-powered, networked sensor for time-temperature monitoring (TTM) that provided temperature measurements with high resolution and the ability to transfer the data wirelessly to a base-station. Additionally, a simple interface was developed that allows a wireless connection to all sensors within range, which provided simultaneous monitoring of multiple sensors with a single handheld device. The sensors are calibrated in a wide range of temperatures (-40 to 40 °C) and exhibited resolution better than 1 °C. The sensors underwent real-world testing to define product temperature changes over time, which allowed accurate profiles of the temperature changes that food products are exposed to over time; and modeling of bacterial growth in those products, which in turn could predict changes in shelf-life or risk of pathogen growth. Multiple TTM sensors were deployed and provided continuous temperature data for over two months in a deli case of a commercial store. The measurements, which were acquired on a weekly base through a smartphone, demonstrated the temperature variations in three different locations of the deli case. These TTM sensors are ready for application in different environments and have the

potential to assist in efforts to manage the environmental conditions to which food products are exposed during distribution and retail, providing crucial information on the quality and handling of the product.

- **Development and testing of a new bench top Laser-induced breakdown spectroscopy (LIBS) instrument.** Rapid and sensitive detection of human pathogens in food samples is a major challenge for food safety researchers. The CFSE at Purdue using ARS funding developed a new Laser-induced breakdown spectroscopy (LIBS) instrument and demonstrated that it can be used to detect antibodies labeled with metal tags singly and in multiplexed assays using different metal labels. The instrument can detect the metal-labeled antibodies in paper-based immunoassays, opening the door to the development of using this technology in future commercial food safety testing.

- **Microchip device for pathogen detection:** The CFSE at Purdue using ARS funding developed a technique that performs on-chip picoliter real-time reverse transcriptase loop mediated isothermal amplification (RT-LAMP) reactions on a histological tissue section without any analyte purification while preserving the native spatial location of the nucleic acid molecules. The entire process, from tissue loading on microchip to results from RT-LAMP can be carried out in less than two hours. It is anticipated that this technique, with its ease of use, fast turnaround, and quantitative molecular outputs, will become an invaluable tissue analysis tool for researchers and clinicians in the biomedical arena and regulators of food safety. This technology is the first demonstration of performing rapid and quantitative nucleic acid detection from unprocessed solid tissue samples and can be translated to process solid food samples, such as meat or poultry, to identify live pathogens.

- **A net fishing enrichment strategy for pathogen detection.** The relatively low numbers and uneven distribution of pathogenic microorganisms in foods create challenges for reliable and rapid pathogen detection. The CFSE at Purdue using ARS funding developed a unique strategy for the enrichment of pathogens based on a concept that captures target bacterial using a net fishing approach. The functionalized 'net' is immersed into a liquid contaminated food sample and captures the target pathogenic bacteria. The net fishing enrichment requires 2 hours for capture and 30 minutes for detection of a foodborne pathogen (such as E. coli O157:H7). Lower numbers of pathogens can be detected sooner using this approach because they are captured by the 'net' and then detected.

Outcomes and Impacts

- Developed a bioluminescence detection system using silicon photomultiplier (SiPM) sensors, that maintains portability while providing more sensitive detection of the bacteriophage luminescence from E. coli O157:H7. This technology is a significant advantage compared to the previously developed smartphone-based bioluminescence detection system.
- Developed a light scattering image library for the BARDOT system for several bacterial pathogens with high species accuracy (positive predicted values). This innovative label-free, non-invasive on-plate multi-pathogen screening technology will find utility for food safety and public health concerns.
- Modified the Vero cell cytotoxicity assay which is the gold standard for Stx detection for improved performance using phage induction and improved toxin release. Using the modified method, it was possible to detect active Stx from test samples within 16-18 h.
- Developed an enhanced and alternate version of the BARDOT called "BEAM" which enables the system to use 50-100 different laser lines, such that hyperspectral imaging from a single colony can be achieved within 40-100 ms with an average error per class of less than 10%.
- Undertook an analysis of biophysical data describing phenotypic characteristics of known and unknown microorganisms in order to extract data patterns associated with emerging pathogens. Demonstrated the tremendous potential of machine learning technologies in the areas of bio-surveillance, biothreat detection, and agricultural biosafety.
- Designed a cost-efficient, reliable, and repeatable approach for the detection of whole-cell foodborne pathogens in real food samples. This reduces the number of sample preparation steps and significantly shortens the testing time. The printed aptamer-based platform achieves the lowest limit of detection of the state of the art for aptamer based optical detection of whole cell with enough evidence to prove its specificity at genus, species, strain, and serotype level.
- Developed a high-resolution, low-powered, networked sensor for time-temperature monitoring (TTM) that provided temperature measurements with high resolution and the ability to transfer the data wirelessly to a base-station. The TTM sensors are ready for application in different environments and have the potential to assist in efforts to manage the environmental conditions to which food products are exposed during distribution and retail, providing crucial information on the quality and handling of the product.
- Developed a new Laser-Induced Breakdown Spectroscopy instrument and demonstrated that it can be used to detect antibodies labeled with metal tags singly and in multiplexed assays using different metal labels. The instrument can detect the metal-labeled antibodies in paper-based immunoassays, opening the door to the development of using this technology in future commercial food safety testing.
- Developed a technique that performs on-chip picoliter real-time reverse transcriptase loop mediated isothermal amplification (RT-LAMP) reactions on a histological tissue section without any analyte purification while preserving the native spatial location of the nucleic acid molecules. The entire process, from tissue loading on microchip to

results from RT-LAMP can be carried out in less than two hours. This technology is the first demonstration of performing rapid and quantitative nucleic acid detection from unprocessed solid tissue samples to identify live pathogens.
- Developed a unique strategy for the enrichment of pathogens based on a concept that captures target bacterial using a net fishing approach. The functionalized net fishing can capture and detect lower numbers of pathogens using this approach.

Examples of Relevant Publications

- Duarte-Guevara, P., Duarte-Guevara, C., Ornob, A., & Bashir, R. (2016). On-chip PMA labeling of foodborne pathogenic bacteria for viable qPCR and qLAMP detection. *Microfluidics and Nanofluidics, 20*(8), 9. https://doi: 10.1007/s10404-016-1778-2
- Kim, H., Doh, I. J., Sturgis, J., Bhunia, A. K., Robinson, J. P., & Bae, E. (2016). Reflected scatterometry for noninvasive interrogation of bacterial colonies. *Journal of Biomedical Optics, 21*(10), 9. https://doi: 10.1117/1.jbo.21.10.107004
- Kim, H., Jung, Y., Doh, I. J., Lozano-Mahecha, R. A., Applegate, B., & Bae, E. (2017). Smartphone-based low light detection for bioluminescence application. *Scientific Reports, 7*, 11. https://doi: 10.1038/srep40203
- Kim, H., Rajwa, B., Bhunia, A. K., Robinson, J. P., & Bae, E. (2017). Development of a multispectral light-scatter sensor for bacterial colonies. *Journal of Biophotonics, 10*(5), 634-644. https://doi: 10.1002/jbio.201500338
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- Diaz-Amaya, S., Lin, L. K., DiNino, R. E., Ostos, C., & Stanciu, L. A. (2019). Inkjet printed electrochemical aptasensor for detection of Hg2+ in organic solvents. *Electrochimica Acta, 316*, 33-42. https://doi:10.1016/j.electacta.2019.05.079
- Diaz-Amaya, S., Zhao, M., Lin, L. K., Ostos, C., Allebach, J. P., Chiu, G. T., . . . Stanciu, L. A. (2019). Inkjet Printed Nanopatterned Aptamer-Based Sensors for Improved Optical Detection of Foodborne Pathogens. *Small, 15*(24), e1805342. https://doi:10.1002/smll.201805342

- Doh, I. J., Gondhalekar, C., Patsekin, V., Rajwa, B., Hernandez, K., Bae, E., & Paul Robinson, J. (2019). A Portable Spark-Induced Breakdown Spectroscopic (SIBS) Instrument and its Analytical Performance. *Appl Spectrosc, 73*(6), 698-708. https://doi:10.1177/0003702819844792
- Gondhalekar, C., Rajwa, B., Bae, E., Patsekin, V., Sturgis, J., Kim, H., . . . Robinson, J. P. (2019). *Multiplexed detection of lanthanides using laser-induced breakdown spectroscopy: a survey of data analysis techniques* (Vol. 11016): SPIE
- Patsekin, V., On, S., Sturgis, J., Bae, E., Rajwa, B., Patsekin, A., & Robinson, J. P. (2019). *Classification of Arcobacter species using variational autoencoders* (Vol. 11016): SPIE
- Ren, W., Ballou, D. R., FitzGerald, R., & Irudayaraj, J. (2019). Plasmonic enhancement in lateral flow sensors for improved sensing of E. coli O157:H7. *Biosens Bioelectron, 126*, 324-331. https://doi:10.1016/j.bios.2018.10.066
- To, C. Z., & Bhunia, A. K. (2019). Three Dimensional Vero Cell-Platform for Rapid and Sensitive Screening of Shiga-Toxin Producing Escherichia coli. *Front Microbiol, 10*, 949. https://doi:10.3389/fmicb.2019.00949
- Zhou, Y. F., Duan, C. Y., Doh, I. J., & Bae, E. (2019). Exploring the Utility of 3-D-printed Laboratory Equipment. *Applied Sciences-Basel, 9*(5). https://doi:ARTN 937 10.3390/app9050937

Problem Statement 4. Chemical and Biological Contaminants: Detection and Characterization Methodology; Toxicology &Toxinology

Goals

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Toxicology examines the relationship between dose and its effects on the exposed organism, whereas ***Toxinology*** deals specifically with animal, plant, and microbial toxins produced by or accumulated in living organisms, their properties and their biological significance for the organisms involved. Both kinds of studies are required to reduce risks arising from contamination of food by chemical and biological contaminants.

The regulation and control of veterinary drugs, chemical residues, heavy metals, persistent organic pollutants, and biological toxins derived from bacteria, fungi and plants are an integral component of any food safety program. To protect public health and the environment, regulations have been passed and implemented that set limits on contaminants in edible agricultural products. Compliance and enforcement of these regulations is a critical role of the ARS National Program's stakeholders that requires the availability of practical detection and characterization methods for veterinary drugs (antibiotics, beta-agonists), chemical residues (dioxins, pesticides), heavy metals (As, Pb, Cd), and organic pollutants (polybrominated diphenyl ethers). In addition to regulatory monitoring, there is a need to understand the biological effects of any inadvertent contamination by humans or animals. In addition to toxicological and toxinological studies, this Problem Statement also includes research directed towards methods for detection and identification of mycotoxins, toxicity evaluation, and mechanism of action.

Accomplishments and promising technologies within this research area will be quickly advanced through technology transfer and where appropriate, will undergo validation through national or international bodies such as the Association of Official Agricultural Chemists (AOAC). These studies require multidisciplinary approaches to meet the challenge, and accomplishments may have far reaching effects regarding food biosecurity, regulations and trade issues.

The regulation and control of veterinary drugs, chemical residues, heavy metals, and persistent organic pollutants was critical to the program's principal stakeholders (FSIS and FDA). To protect public health and the environment, regulations set limits on contaminants in edible agricultural products. Compliance and enforcement of these regulations continues to be a critical role of the Program's stakeholders that requires the availability of practical detection and characterization methods for chemical residues (dioxins, pesticides), veterinary drugs (antibiotics, beta-agonists), heavy metals (As, Cd), and organic pollutants (polybrominated diphenyl ethers). In addition to regulatory monitoring there was a need to understand the biological effects of any inadvertent human or animal contamination. Included in this section was the development of specific sensing technologies. The use of nondestructive spectral sensing technologies, for example, whole-surface [in-line] hyperspectral imaging is one option for reducing the risks of contamination or food adulteration.

Regulatory agencies have placed more of the burden of inspection responsibility on the producers and processors. Plants are also responsible for meeting other consumer protection issues as determined by regulatory agencies. In essence producers/processors assume the responsibility for inspection, and the regulatory agencies perform oversight and verification to ensure standards are met. Under HACCP, HIMP or GMP, consumers demand safe, high quality food, however, consumer demand for more food increases the need for, and pressure on inspectors. Balancing consumer needs with the capabilities of the inspection agencies and the producers/processors is difficult. To reduce these issues studies are focused on developing automated, low cost, accurate, on-line and hand-held, computerized inspection [sensing] systems. These automated systems operate with minimum human intervention and can function despite changes in physical plant structure, and environmental conditions. A specific need was to continue development and validation where appropriate with the goal of commercial implementation. On-line, computerized sensing-systems placed or used strategically will assist and improve the regulatory and in-house inspection system; minimizing the problems of human error and variability; and increasing commercial processing productivity and profitability.

Research was directed towards developing methods for the detection and identification of mycotoxins; and evaluation of mycotoxin toxicity and mechanism of action. Research on the development of biocontrol technologies, and crop/fungal/toxin relationships is part of this area and may also impact Problem Statement 2 (Systems Biology). Research on production practices, expert systems, and breeding resistant crops, was limited. Toxins derived from bacteria and plants are an integral component of any food safety/food biosecurity program. Research was directed towards developing methods for the detection and identification of toxins.

Chemical and Biological Contaminants Chemical Contaminants

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Chemical contaminants in foods are a concern to producers, processors, public health and regulatory agencies and consumers, both nationally and internationally. Indeed, in the U.S. and Europe consumers rated chemical residues as a greater health risk than foodborne pathogens, even though the incidence of FBI from foodborne pathogens far exceeds illness from chemical residues. Chemical residues in foods (contamination/adulteration in the broadest sense) either found naturally, unknowingly though production or processing errors, or though deliberate action impact the ability to market food and drink products worldwide. Global food/drink trade/market is valued at trillions of dollars, and chemical contamination is extensively monitored worldwide to better ensure food safety and quality, compliance with regulations, and to meet governmental (including legal), industrial and consumer requirements. Several national and international food/drink crises have occurred with respect to contamination/adulteration leading to even greater concerns, with subsequent governmental and legal responses. For example, the UK Food Standards Agency implemented the National Food Crime Unit based in large-part on recommendations in the Elliot Report on food adulteration. In the U.S. implementation of the FDA-Food Safety Modernization Act has

also increased the monitoring rate nationally and with foods imported in to the U.S. In response, food and drink testing by inhouse and 3rd party labs have grown significantly over the past 5 years, into a world-wide billion-dollar market. To meet needs, the detection methods used by laboratories must be fast, simple, reliable, inexpensive, rugged, provide high sensitivity, high-through-put, with quantifiable detection limits for a wide scope of chemicals in all types of foods. Development, validation and transfer of improved (and automated) analytical methods to real-world monitoring labs to meet acceptable performance criteria was a major challenge undertaken by the Program. Research was conducted in-part to meet the needs of the USDA-FSIS, the FDA, and other national and international organizations that monitor chemical residues in food, which also includes industry, consumer groups, and academic scientists worldwide. This lab has a history of highly successful methods developments.

The (Lehotay) project at ERRC, Wyndmoor, PA was directed towards several objectives to: (1) Develop, validate, and transfer: multiclass, multiresidue methods for pesticides, environmental, and emerging contaminants in FSIS-regulated foods, and conduct a survey of food samples for the contaminants; (2) Develop qualitative screening and identification criteria with automated data processing that meets regulatory needs to minimize/avoid false positives and negatives; (3) Develop sample processing methods for regulatory analysis that improve the ability to detect combinations of veterinary drug residues in the same sample preparation; (4) Develop automated high-throughput sample processing, preparation, and analysis methods using flow-injection and/or open probe techniques coupled with mass spectrometry to monitor >500 veterinary drugs, pesticides, and environmental contaminants in foods; and (5) Develop novel analytical methods for inorganic and organometallic heavy metals in foods and supplements

Examples of Accomplishments

- **A new sampling method for residue analysis.** Better monitoring of contaminants in foods to protect human health can be achieved by developing and implementing fast and efficient methods associated with high sample throughput, lower costs, and less labor. ARS developed and validated a robotic method of sample preparation for high-throughput fast analysis of foods to monitor for pesticides and environmental contaminants. The method is being implemented by FSIS for use in the USDA National Residue Program. The automated high throughput sample preparation method is performed sequentially in parallel with the 10-minute analysis method to monitor >300 pesticides and environmental contaminants in the sample. Test foods thus far include fish (catfish, tilapia, salmon), meats (beef, chicken, pork, goat, sheep), and a variety of fruits, vegetables, and other foods (e.g. wheat, black olive, dried basil). The accuracy of the robotic-controlled, high-throughput method is exceptional with minimal labor needed.

- **A new method for lipid sample cleanup.** Efficient removal of fats and other matrix components interfering with ultra-trace analysis of contaminants presents a challenge in analysis of complex food samples. A new commercial product, “enhanced matrix removal of lipids” (EMR-Lipid) was evaluated for sample cleanup of fatty and some nonfatty

foods for hundreds of pesticides, environmental contaminants and veterinary drugs by different methods of instrumental analysis. Efficient removal of co-extracted lipids for fatty samples and chlorophyll in green vegetables was achieved. Better removal of these matrix components leads to improved long-term performance of sophisticated analytical instruments and reduced maintenance needs.

- **Eliminating false results.** Qualitative identifications are essential in regulatory monitoring to avoid detrimental impacts of false positive and false negative results. Missed residues that are present (false negatives) pose concerns in terms of human health, risk assessment, environmental impact, and enforcement actions. On the other hand, incorrect findings of chemicals that are not actually present (false positives) lead to inaccurate food safety assessments, damage reputations of the wrong officials and falsely accused producers and may cause economic losses to producers. The validated methods implemented by FSIS have entailed measurement of rates of false positives and negatives in blind analysis of targeted contaminants in different foods. Scientific assessment of identification criteria has been described and standards set using different analytical tools to ensure that the rates of false positives and negatives are as low and technically and humanly possible in real-world operations.

- **Efficient data processing**. As analytical technologies and techniques have improved, sample throughput in chromatography has become limited by data processing of large generated data sets. Human review and manual corrections of integrated chromatographic peaks has been standard practice for decades, but this is no longer possible in high-throughput applications involving many targeted chemicals. A simple automated summation chromatographic peak integration approach has been evaluated and implemented for chemical residue monitoring, which obviates the need for time-consuming human review and manual reintegration. Improved results were achieved using summation integration in a much more efficient and reliable approach, which is applicable to chromatographic analysis in many fields of investigation.

- **Optimization of sample processing techniques to determine minimum test sample size for analysis of contaminants in foods.** The best way to improve the efficiency in chemical residue analysis of foods is to use the minimum amount of test sample portion that still yields accurate results for the bulk sample. ARS conducted a study involving 10 food commodities and two types of modern commercial sample processing devices to minimize test portions for analysis of added and incurred pesticide residues in the bulk samples. The results indicated that 5 g test samples processed at room temperature from 1 kg typically meets needs for regulatory analysis. This is half the portion commonly analyzed currently, which means that half the level of reagents may be used, thereby saving costs in the future.

- **Effective and efficient analytical methods.** Effective and efficient analytical methods using ultrahigh-performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) were developed for monitoring >175 veterinary drug residues at U.S. tolerance levels in food animal tissues. The methods were validated and

transferred to FSIS, which has now implemented the third iteration (known in the FSIS Chemistry Laboratory Guidebook as CLG-MRM3). Each method has demonstrated a continual expansion in the number of drugs and types of foods monitored, increased quality of results, eased the labor, reduced the time, and lowered the costs involved in the USDA National Residue Program.

- **Inorganic arsenic species**. Inorganic arsenic species are highly toxic and need to be monitored in food to protect human health. A novel and useful quantitative method for inorganic arsenic in rice was developed based on cryogenic focusing followed by hydride generation with detection by either atomic fluorescence spectrometry or inductively-coupled plasma mass spectrometry. The interfering organic arsine components in the food are trapped by a sorbent bed inside a thermoelectric cryotrap, while the inorganic arsenic species of concern pass through the trap for sensitive analysis. Separation based upon boiling points is faster and easier than the traditional chromatography methods used. This approach has significant cost, green chemistry, personnel safety, and sample through advantages, and it has been awarded U.S. Patent No. 10,352,311 B2 (Cryogenic Trap System).

- **Mercury analysis**. Mercury (Hg) is a highly toxic metal, but its analysis can be quite difficult, particularly in being able to distinguish different organometallic forms of Hg in complex food matrices. A new "green" method of speciation analysis of Hg was developed using rapid extraction using a block heater followed by exposure of the extract to ultraviolet light to induce chemical reactions that lead to being able to distinguish Hg ions and methylmercury from each other. The difference in responses using atomic fluorescence detection allowed independent calculation of each component in an elegant procedure. Thus, many chemical reagents used in traditional methods become unnecessary, and this new method incurs low cost and is friendly to both bench workers and the environment. The approach was used to survey fish oil supplements for the presence of the different forms of Hg.

- **Monitoring antibiotics.** Microbial resistance to antibiotic drugs is a major health concern worldwide, and more efficient and effective methods of analysis are needed to monitor and study resistance. In this study, analytical techniques were developed for rapid determination of antimicrobial resistance due to aminoglycoside antibiotics in less than 3 hours following overnight enrichment. After exposing the bacteria to aqueous aminoglycoside solution, mass spectrometric analysis revealed biomarkers useful for rapid detection of the resistant strain. In-house devised software was then used for rapid quantification of the biomarkers. A feasibility comparison study suggested that a cost-effective method could be used for large scale proteomic investigations in the future. This type of instrument-based approach using mass spectrometry possesses advantages over traditional microbiological methods, including complementary value.

Outcomes and Impact

- Developed and validated a new method to monitor for pesticides and environmental contaminants now implemented by FSIS for use in the USDA National Residue Program. The automated approach increases sample throughput while reducing cost of analysis.
- Evaluated and validated a new lipid removing material for efficient cleanup of complex food samples for the analysis of pesticides, environmental contaminants, and veterinary drugs improving long-term performance of analytical instruments, while reducing maintenance needs.
- Identification criteria for residue analysis has been described and standards set to ensure that the rates of false positives and negatives are as low and technically possible.
- Evaluated and implemented a simple automated summation chromatographic peak integration approach chemical residue monitoring, which obviates the need for time-consuming human review and manual re-integrations.
- Optimized a sample processing technique to determine minimum test sample size for analysis of contaminants in foods. The results indicated only 50% of the current amount is needed for regulatory analysis thereby saving costs.
- Developed and validated an effective and efficient analytical method using (UHPLC-MS/MS) for monitoring >175 veterinary drug residues at US tolerance levels in food animal tissues. Methods transferred and implemented in the National Residue Program.
- Developed a quantitative method for inorganic arsenic in rice based on cryogenic focusing, hydride generation and detection by either atomic fluorescence spectrometry or inductively-coupled plasma mass spectrometry. The approach has significant cost, green chemistry, personnel safety, and sample through advantages; awarded U.S. Patent No. 10,352,311 B2 (Cryogenic Trap System).
- Developed a new "green" method of speciation analysis of Hg using rapid extraction using a block heater followed by exposure of the extract to ultraviolet light. The method eliminates many traditional chemical reagents; incurs low cost and is user-friendly.
- Developed an analytical technique for rapid determination of antimicrobial resistance due to aminoglycoside. This type of instrument-based approach using mass spectrometry possesses advantages over traditional microbiological methods.

Examples of Relevant Publications:

- Lehotay, S.J., Han, L. Sapozhnikova, Y. 2016. Automated mini-column solid-phase extraction cleanup for high-throughput analysis of chemical contaminants in foods by low-pressure gas chromatography – tandem mass spectrometry. Chromatographia. 79: 1113-1130.
- Han, L., Sapozhnikova, Y., Lehotay, S.J. 2016. Method validation for 243 pesticides and environmental contaminants in meats and poultry by tandem mass spectrometry coupled to low-pressure gas chromatography and ultrahigh-performance liquid chromatography. Food Control. 66:270-282.

- Han, L., Matarrita, J., Sapozhnikova, Y., Lehotay, S.J. 2016. Evaluation of a recent product to remove lipids and other matrix co-extractives in the analysis of pesticide residues and environmental contaminants in foods. Journal of Chromatography A. 1449:17-29.
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Chemical and Biological Contaminants: Food, Environmental and Other Contaminants

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR); https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf The goal was to provide data that can be used to understand the broad impacts that chemicals play in influencing food, environmental and [human] safety. Rapid screening assays are widely employed by a variety of end-users to test for xenobiotics in animals, and in animal tissues. While many rapid screening assays have been developed to "detect" residues, there are very few data available that evaluate the effectiveness of those screening assays for on-farm detection of residues in animals destined for market. Additionally, understanding the factors that alter rates and routes of metabolism and the disposition of residues in food animals is critical to risk assessors for modelling human exposures to those xenobiotics and for determining proper withdrawal periods after animal exposures have occurred.

Three broad classes of residues were targeted for study: veterinary drugs or feed additives administered to food animals under extra-label use conditions; endogenous steroid hormones; and novel developmental chemicals of potential utility for mitigating the risks of human pathogens from animal or vegetable products. Studies also investigated the sources, levels, metabolism, elimination, and depletion kinetics of some of the most persistent and toxic food contaminants present in U.S. meats including dioxins, furans, brominated flame retardants (BFRs), and perfluorinated hydrocarbons (PFOS, PFOA). Regardless of the chemical class being investigated, the goal was the development, validation and technology transfer of sensitive and accurate analytical tools to detect chemical residues in food animals or food products for either laboratory or field use.

Studies were undertaken at Schafer Agricultural Research Center, Fargo, ND, by two-sister-projects (Smith and Hakk [retired] which were combined (Smith). The objectives were to: (1) Develop and (or) validate sensitive and accurate analytical tools to rapidly detect and quantify chemicals in food animals, food animal products, or other foods; (2) Investigate the kinetics of uptake, metabolism, distribution, and (or) the elimination of chemicals in and from food animals and (or) produce with the goal of reducing public exposure to chemical residues in foods; (3) Determine the fate of endogenous reproductive hormones, antibiotics, and or other chemicals, including biologically-active metabolites or degradation products in wastes of food animal or in food processing systems: (4) Develop and/or validate rapid screening assays for the detection of environmental chemicals relevant to U.S. food production; (5) Determine levels and sources of dioxins and related compounds in the domestic food supply. Provide food safety agencies with data to confirm or refute the wholesomeness and competitiveness of beef, pork, chickens, turkeys and/or catfish; and (6) Determine the uptake, metabolism (in vitro or in vivo), distribution, excretion, and fate of environmental contaminants with the goal of developing pharmacokinetic rate and volume constants pertinent to residue depletion, selection of marker compounds, and calculation of withdrawal intervals.

Examples of Accomplishments

- **Preservation and decontamination of cantaloupe and tomatoes**. Cantaloupe and tomatoes are highly susceptible to spoilage prior to retail marketing and may also harbor pathogens that are harmful or even deadly to humans. Although sanitation of produce with low-dose chlorine dioxide gas is highly effective at eliminating rot organisms and/or pathogens, its use was not approved by regulators because the nature and quantity of chemical residues remaining on chlorine dioxide treated produce was unknown. ARS demonstrated that when low dose chlorine dioxide is properly used, chemical residues in edible cantaloupe or tomato cannot be distinguished from untreated produce, even when very sensitive analytical techniques are used.

- **Fate of estrogen hormones in animal production systems.** Estrogens are natural, but very potent hormones produced by humans and by all food animals. It has been suggested that food animal production systems are a major contributor to estrogenic compounds detected in surface and ground waters across the United States. ARS demonstrated that estrogens are rapidly degraded during animal-waste processing and that trace levels of estrogen bind tightly to soil particles when processed waste is applied to fields. Further, studies demonstrated that soils themselves transform estrogen to less potent metabolites that and runoff from such fields contains insufficient levels of estrogen to cause perturbations to indicator fish. Collectively, these data suggest that properly functioning waste-management facilities in animal production systems contribute insignificant quantities of estrogens to surface and/or ground waters.

- **Rapid detection of chemicals.** Food animals are sometimes exposed to chemicals intentionally (animal drugs) but are often exposed to many other chemicals unintentionally (environmental contaminants). Animal exposure to most chemicals can be measured, but the analytical tools used are very expensive and the assays require a high level of technical skill and a large amount of time. ARS developed a series of rapid assays that can be used on the farm (test strips) or in the laboratory that can detect veterinary drugs or environmental contaminants. On-farm tests can typically be conducted in about 10 minutes and laboratory tests may take as little as 30 seconds, depending on the analyte. Test strip assays are available to animal producers and veterinarians for determining whether chemical exposures have occurred or whether a chemical has depleted from an animal after purposeful or unintentional exposure. Laboratory assays are available to regulatory agencies, import/export officials, and/or scientists for the rapid assessment animal exposure to chemical residues.

- **Fate of chemicals during milk processing.** Dairy animals may be exposed to environmental contaminates and veterinary medicines during cycles of milk production. Some of these chemicals may be secreted into milk. As milk is processed these typically chemicals partition in various degrees into water, fat, or protein based-fractions from which dairy products such as skim milk, sour cream, cheese, and other products are made. Human exposures to such chemicals may be enhanced if a chemical has a propensity to concentrate into one fraction over another. Studies characterized the distribution of 27 chemicals, having a diverse set of uses and physio-chemical properties,

into skim milk, milk fat, curd, whey, and whey fractions of milk. Partitioning characteristics of each chemical were described mathematically using chemical descriptors of each compound (log P, Log D) and a predictive model was proposed.

- **Bioavailability of flame retardants in household dust.** Because uncontrolled house fires represent devastating health risks to families, manufacturers of carpets, furniture, electronics, and other household items have historically incorporated chemical flame-retardants into products during their manufacture. As consumer products are used, normal wear and tear releases dust particles that contain substantial amounts of flame retardant. Because dust particles are small and airborne, a surprising amount of dust is ingested by the average consumer daily. Dust ingestion is thought to be a significant source of human exposure to potentially toxic brominated flame retardants, however the extent of flame-retardant absorption, especially when bound to dust, is unknown. Studies demonstrated that absorption of two important classes of dust-borne brominated flame retardants occurs in rats (a model species), but not equally within, or across, flame retardant class. Further, studies showed that after absorption, flame retardants preferentially migrate to fat, but not liver.

- **Chemical residue transfer to eggs.** Brominated diphenyl ether (PBDE) fire retardants have been banned for use in commercial products for many years because of toxicological concerns. Even with the ban, PBDEs are ubiquitous in the environment because of their slow degradation rates, and the environmental levels of some PBDEs are increasing. Food animals are exposed to flame retardant residues through air and the consumption of feed, water, and soil, but the amount of flame retardant absorbed and transferred into edible products has not been characterized. Studies demonstrated that some, but not all, PBDEs are readily absorbed by laying hens. Highly brominated PBDEs are poorly absorbed and are excreted in feces of dosed hens very rapidly. In contrast, PBDEs containing fewer bromine atoms (four or five) were well absorbed and up to 24% -an extraordinary amount- of the dose was eliminated in eggs during a short 7-day study period.

Outcomes and Impacts

- The US Environmental Protection Agency reviewed residue data. The use of low dose chlorine dioxide gas to preserve and cleanse cantaloupes and tomatoes is now permitted in the U.S.
- Demonstrated that estrogens are rapidly degraded and that soils themselves transform estrogen to less potent metabolites. Collectively, this suggests that properly functioning waste-management facilities in animal production systems contribute insignificant quantities of estrogens to surface and/or ground waters.
- Developed a model that can be used to predict the distribution of chemicals in milk and milk products based on the physical properties of chemical. The availability of such a model is critical to enable risk assessors to quickly determine dairy product safety if contamination events occur.

- Demonstrated that absorption of two important classes of dust-borne brominated flame retardants occurs, but not equally within, or across, flame retardant class.
- Demonstrated that after absorption, flame retardants preferentially migrate to fat, but not liver. This is critical data for globally by regulatory agencies charged with assessing health risks to humans.
- Demonstrated that some, but not all, PBDEs are readily absorbed by laying hens. Highly brominated PBDEs are poorly absorbed and are excreted in feces very rapidly. In contrast, PBDEs containing fewer bromine atoms (four or five) were well absorbed, and a large amount was eliminated in eggs produced over the next week of laying.

Examples of Relevant Publications

- Smith, D.J., Ernst, W., Herges, G.R. 2015. Chloroxyanion residues in cantaloupe and tomatoes after chlorine dioxide gas sanitation. Journal of Agricultural and Food Chemistry. 63(43):9640-9649.
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Chemical and Biological Contaminants: Heavy Metal Contaminants

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Excessive levels of heavy metals in certain plant foods poses a severe risk for consumers. Consequently, producers of crops consumed as food or feeds must consider agronomic management factors which affect the levels of these elements in crops and livestock. An example of a crop of specific concern to public health and agricultural regulatory agencies, industry and consumers is rice and rice products. This commodity accumulates more inorganic arsenic (iAs) than any other crop due to its method of production; commonly grown in flooded soils which may generate arsenite under anaerobic conditions. Levels of (iAs) in rice are of specific concern to the FDA. Other heavy metals such as cadmium (Cd), zinc (Zn) and lead (Pb) are also of concern in a variety of crops, for example: sunflower kernels, leafy greens, and root vegetables. The bioavailability of these heavy metals in feed sources also presumes the accumulation in consumable animal tissue.

Research at BARC, Beltsville, MD was conducted by a project (Codling) [previously Chaney]. The objectives of the work were to: (1) Characterize effects of flood and soil management on As (inorganic and total) and Cd accumulation in rice grain; (2) Characterize competition of other cations with Cd accumulation by rice and vegetables; (3) Characterize effects of soil amendments on Pb, Cd and As accumulation by garden crops to improve advice to urban gardeners regarding risk reduction for contaminated urban soils; and (4) Continue evaluation of the effects of crop species and crop Zn on bioavailability of crop Cd to animals.

Examples of Accomplishments

- **Methods to determine inorganic arsenic (*iAs*) in rice grain**: FAO/CODEX has established limits on *iAs* of (200 ppb) in polished rice for international trade, while the FDA limit is 100 ppb in rice-based infant food. Meeting limits on *i*As in rice is important to producers who need information to assess contamination and management practices. Management practices such as alternate wetting and drying (AWD) for growing rice are being practices saving water and reduce rice grain As concentrations. A new simple, rapid and inexpensive method was developed which uses Hydride Generation to measure only the *iAs* in rice grain.

- **Soil amendments on Cd accumulation by spinach from a Cd-mineralized soil:** Cadmium (Cd)-mineralized soils occur in many nations. When these soils are non-calcareous, crops and especially leafy vegetables such as lettuce (Lactuca sativa L.) and spinach (Spinacia oleracea L.) may accumulate levels of Cd in their edible portions that exceed international standards. Vegetable crops grown in some areas of California absorb an excessive amount of Cd into their edible portions. Agronomic or genetic management alternatives were needed to allow the use of these otherwise highly productive soils for spinach. Studies showed that combinations of biosolids compost

(10%), Mn, Zn, and limestone (5%) could reduce Cd phyto-availability. Cadmium accumulation was suppressed below international guidelines limits when combinations of compost + Zn + limestone or compost + Zn + Mn + limestone were applied.

Outcomes and Impacts

- The level of iAs in rice is of major importance both in the U.S. and internationally. Rice is the second leading food crop in the world and was worth $2.75 billion to the US economy in 2018. Results from ARS research provided important information on how to determine iAs in rice grain. The method developed provides the FDA, rice industries, farmers and researchers with means of quickly determine iAs in rice grain. Scientists at the Dale Bumper National Rice Research Laboratory in Arkansas are using this extraction method to quickly identify low arsenic rice cultivars.
- The management alternatives studies identified new sustainable technologies for stakeholders to produce quality leafy vegetable products, respecting the Codex limitations, in those specific areas with Cd mineralization or low Zn:Cd ratio sources of soil Cd enrichment. This outcome provided valuable information to stakeholders such as vegetable farmers and industries producing agronomic (vegetable) crops in high cadmium soils such as those found in certain areas of California, the principal production area for fresh produce in the U.S.

Examples of Relevant Publications

- Paul, A.L.D and Chaney, R. L 2017. Effect of Soil Amendments on Cd Accumulation by Spinach from a Cd-Mineralized Soil. Journal of Environmental Quality doi:10.2134/jeq2016.07.0251
- Codling, E. E., Onyeador, J. Accumulation of lead and arsenic in Malabar spinach (Basella alba L.) and sweet potato (Ipomoea batatas L.) leaves grown on urban and orchard soils Journal of Plant Nutrition. 40: 2898-2909. doi:10.1080/01904167.2017.1382530. 2017.
- Codling, E. E and Chen, G. 2017. Effects of compost amended lead-arsenic contaminated soils on total and inorganic arsenic concentrations in rice. Journal of Plant Nutrition 40: 2146-2145. doi.10.1080/01904167.2017.1346684.
- Chaney, R. L, Green, C. E and Lehotay, S. J. 2018. Inter-laboratory validation of an inexpensive streamlined method to measure inorganic arsenic in rice grain. Analytical and Bioanalytical Chemistry 5703-5710. doi.10.1007/s00216-018-1075

Chemical and Biological Contaminants: Sensing Technology

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR); https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf Nondestructive spectral imaging technologies including fluorescence, visible/near-infrared (NIR) reflectance, and Raman spectroscopy can be used to identify and facilitate removal of contaminants and contaminated foods at all stages of processing from farm to table. Further these technologies can be applied for the detection of contaminants and defects on food products and processing surfaces. The ARS Food Safety Program is internationally recognized for its sensing technology research (and collaborations), and the effective outcome of this applied engineering to commercialization. It should be recognized that the transfer of sensing and instrumentation technologies takes an extensive period from the initiation of research and is a multifaceted endeavor that requires many stages of work, including fundamental research and prototype development, patent development and submission, testing and validation, component upgrades for real-world use, and interactions with industry partners. Development of both in-line (actively on the processing line) and hand-held imaging inspection devices were the focus of attention.

Research was conducted by a project at BARC, Beltsville, MD (Kim) with several (national and international academic and industry) collaborations. The objectives were to: Advance development and validation of on-line automated whole-surface inspection systems for simultaneous safety and quality inspection of fresh produce in high-throughput commercial processing operations; (2) Develop and validate user-friendly analytical sensing methods and technologies for targeted and non-targeted rapid screening of foods for microbial, chemical, and biological contaminants in laboratory, field, and/or industrial environments; (3) Advance development of portable spectral imaging technologies to allow identification and detection of food contaminants and develop sampling and inspection protocols for implementation of the developed technologies in industry and regulatory applications; and (4) Advance development, test and validate an automated system for detecting contaminants in produce fields and investigate cost and sensitivity trade-offs of different potential system components and configurations with regard to production of a cost-effective commercial system.

Examples of Accomplishments

- **Whole-surface produce inspection.** To perform comprehensive online quality and safety inspection of fruits and vegetables in processing environments, whole-surface sample presentation is critical for image-based inspection. Since no such whole-surface imaging inspection technology exists for industry use, ARS developed two prototype inspection technologies and methodologies, with different sample transport and presentation methods to address the morphological differences present, for whole-surface imaging inspection of flat leafy green vegetables and of round-shaped fruits. The leafy-greens inspection system evaluates both sides of relatively flat, two-sided samples by flipping them between two conveyors that are both scanned by a single line-scan imaging device. The round-fruit inspection system rotates a sample while a patented

optical-mirror assembly and line-scan imaging device captures two side-view images of the rotating fruit to reconstruct the entire surface of the spherical shape. One U.S. Patent for round-shaped object sample presentation and imaging method was issued in 2017 and a CRADA partner is pursuing licensing for the ARS multitask imaging technology that simultaneously acquires multispectral fluorescence and reflectance which is critical for effective online safety and quality inspection.

- **Detection of multiple-species fecal contamination on produce.** Animal feces is the primary sources of E. coli and Salmonella bacteria, which may contaminate produce fields directly via animal intrusion or indirectly via contaminated irrigation water or natural rain/groundwater flow through regions of livestock or wildlife activity. Studies used feces samples from dairy cattle, pigs, chickens, and sheep to simulate contamination scenarios for fresh produce. Spots of both undiluted and diluted feces were applied to romaine lettuce leaves for hyperspectral fluorescence imaging. Image-based algorithms were developed to detect each fecal species as well as for "common" use to detect all four fecal species as one group. The results show that fluorescence imaging methods can effectively detect fecal contamination on green produce surfaces and be used to help prevent harvesting of contaminated crops or cross-contamination in processing plants.

- **Development of enhanced Raman spectroscopy and imaging instrumentation systems for ingredient authentication and adulterant detection.** Imaging technologies/methods were developed to overcome a lack of tools for macro-scale Raman measurement of food samples. A 785 nm line-scan Raman imaging system was developed and patented (U.S. Patent No. 9,927,364) that can analyze 30 ml of food powder within 10 minutes—compared to 24 hours by conventional instruments—without any special sample preparation. The system can visualize the contaminant distribution across the sample area and demonstrated effective detection for chemical contaminants at concentrations as low as 100 ppm (e.g. maleic acid in starch). Following an earlier 785-nm point-scan imaging system, a new 1064-nm point-scan imaging system was developed to analyze highly fluorescing food samples; both were used to develop and test a new method of Spatially Offset Raman Spectroscopy (SORS). To capture signal from material up to several millimeters below the sample surface, SORS uses point-source measurements at multiple locations offset laterally from the laser-excitation point, but conventional SORS technique is limited by fixed offset distances and intervals. This new SORS method can easily change the offset distances and intervals to accommodate different sample types and was demonstrated for analysis of gelatin-encapsulated powder samples such as turmeric, urea, ibuprofen, and acetaminophen. These technological developments fill a need for macro-scale Raman tools that can improve sample testing in the food and pharmaceutical industries to help ensure product safety and quality and detect fraud.

- **Development of Raman-based detection methods for adulterant detection for dry food powders, encapsulated ingredients, milk, and meat.** Operating parameters for the 785-nm line-scan Raman imaging system were optimized for contaminant detection in dry milk powder, starch, and flour (for melamine, maleic anhydride, and benzoyl peroxide, respectively) and demonstrated effective for detecting concentrations as low as 100 ppm. The line-scan system was also used to develop an image processing method to detect veterinary drug residues (oflaxacin, chloramphenicol, and sulfadimidine) in raw pork samples. Spatially Offset Raman Spectroscopy (SORS) measurements were performed using 785 nm to analyze gelatin capsules containing urea, ibuprofen, and acetaminophen powders, and using 1064 nm to analyze capsules of turmeric contaminated with metanil-yellow. The 1064-nm point-scanning was also used to effectively detect contaminants (metanil yellow, Sudan-I dye, Sudan Red G dye, and white turmeric) in yellow turmeric powder and in curry powder. The yellow turmeric powder was also analyzed with Fourier Transform infrared (FTIR) measurements, leading to a dual-modality contaminant detection method that is currently in development to incorporate both Raman and FTIR in one system for enhanced chemical detection. Residues of the antibiotic tetracycline were detectable in cow milk at concentrations as low as 0.01 ppm using 785-nm Raman measurements in conjunction with surface-enhanced Raman scattering (SERS) nanoparticles and a transparent SERS substrate fabricated by a research collaborator. Ractopamine in solution was also analyzed using 785-nm Raman measurements and found to be detectable at10-8 M, which is lower than the industry regulatory standard concentration of 10 μg/L.

- **Gradient Temperature Raman Spectroscopy (GTRS) for detecting food ingredient analysis.** GTRS was developed and patented (U.S. Patent No. 9,706,780 B2) to acquire Raman spectra relative to an applied temperature gradient, which can reveal identifying information about chemicals related to their specific structural/vibration properties that may change at different phase transition temperatures. Melamine, cyanuric acid, biuret, and urea, four nitrogen-rich compounds that can dupe conventional protein tests when they are mixed with milk powder, were found via GTRS to show spectral lines that can be effectively used for their detection in milk and to differentiate between them despite their structural similarities. Clearly identifiable differences were also revealed by GTRS for oleic acid and linoleic acid that are structurally similar in having one vs. two double bonds, respectively, and the same number of carbon atoms; omega-3 and omega-6 linolenic acid which each have only three double bonds but at different locations; docosahexaenoic acid (DHA) and the N-3 and N-6 forms of docosapentaenoic acid which are structurally similar but biochemically/biophysically very different C22 lipids; and docosahexaenoic acid and eicosapentaenoic acid which are both commonly labeled as “fish oil” in health supplements. Although the Raman spectra measured at room temperature may show few differences for the samples in these groupings, laboratory GTRS and NMR experiments can confirm the structure and conformation of each, and that the conformation of polyunsaturated lipids change conformation in mixtures. GTRS shows great promise for use as an analysis tool to address questions of food safety or quality with respect to product reformulations or other cases in which composition verification is needed

- **Handheld fluorescence imaging for meat safety inspection in slaughter plants.** In meat processing plants, current meat inspection for food safety and quality attributes, including potential fecal contamination, is conducted through visual examination by human inspectors working under conditions that are poorly suited to conventional fluorescence detection methods that require ambient darkness. A handheld fluorescence-based imaging device can be used under normal operational ambient lighting in food processing plants to highlight contaminated food and equipment surfaces on a wireless display monitor. An investigation of the effectiveness of the current prototype device in enhancing visual detection of fecal contamination on red meat, fat, and bone surfaces of beef under varying ambient luminous intensities (0, 10, 30, 50 and 70 foot-candles) found that, overall, diluted feces on fat, red meat and bone surfaces of beef under ambient light ranging from 0- to 50-foot-candles were detectable using 670-nm single-band fluorescence images. As an assistive tool, this technology will support and improve meat safety inspection programs as implemented by U.S. processors and regulatory inspectors.

Figure: Hand held device

- **Food processing plant contamination and sanitation inspection.** ARS developed portable handheld imaging technologies to assist in contamination and sanitation inspection applications was tested in cooperation with the USDA-FSIS Office of Policy and Program Development, Risk, Innovations, and Management Staff (RIMS). Seeking new science-based measures to modernize inspection and enforcement policies, RIMS consulted EMFSL on the potential use of the handheld imaging device and challenges of in-plant testing and evaluation. On-site prototype testing at a Ready-to-Eat (RTE) deli meat processing plant and a beef packing plant were jointly conducted to gain insight on in-plant inspection needs and situational considerations of processing environments, and to solicit feedback from FSIS inspection staff who are potential end-users of the device for development of user protocol. In addition, under the interagency agreement with the U.S. Army Natick Soldier Research Development and Engineering Center, (Natick) field testing and validation of the imaging devices was conducted for use in US Army food safety audit programs. In-plant field testing of the updated device was

also conducted in cooperation with Natick collaborators at a large-scale commercial bakery that manufactures products for the military as well as for private sector food and restaurant companies, to investigate the use of the handheld imaging device for inspection of surfaces in conjunction with a novel surface-sampling method developed by the collaborators that may be incorporated into routine procedures. A licensing request for commercialization was granted in 2019.

- **In-field preharvest produce inspection: fecal detection system.** ARS developed an imaging platform that uses a hyperspectral imaging system mounted on a semi-automated cart for detecting fecal contamination in produce fields prior to harvest. Since field contamination is the primary source of contaminated produce, current practices use trained observers on foot to search fields for signs of animal intrusion, which, if found, leads to closer examination of the localized area for evidence of fecal contamination on/near the plants and potential temporary prohibition on harvesting in that area. A novel method for generating a line illumination source was developed using a high-energy pulsed laser for fluorescence measurements and used with a gated camera and nanosecond exposure times to capture hyperspectral fluorescence signals from fecal materials. The measured responses from the fecal materials were magnitudes of order greater in intensity compared to the brightest daylight conditions, thereby enabling outdoor fluorescence imaging irrespective of ambient daylight intensity. The results suggest that this ground-based imaging method may be adaptable for large-scale outdoor crop imaging implemented by using unmanned aerial vehicles (drones), which would increase the efficacy of inspections.

- **Fluorescence imaging for assessing fecal contamination of soil and compost maturity.** Pathogenic microbial contamination of fresh produce can occur through many pathways during pre- and post-harvest operations, such as from livestock or wildlife excrement, immature biological soil amendments, contaminated agricultural water, poor worker health and hygiene, and contaminated field tools. Use of mature manure compost and preventative control of fecal contamination from wildlife and can help to minimize the risk of foodborne illness associated with produce. However, neither traces of animal feces nor the degree of maturity of manure compost can be identified in the field by naked eye. In this study, hyperspectral fluorescence imaging techniques were investigated to characterize fecal samples from bovine, swine, poultry, and sheep species, and to determine feasibilities for both detecting animal fecal presence and identifying the fecal species origin in mixtures of soil and feces. Dynamic and unique fluorescence emission features exhibited by fecal samples were found useful for detecting fecal presence and showed that identification of fecal species origin for soil-feces mixtures is feasible. In addition, the imaging techniques were evaluated for assessing the maturity of manure compost, indicating that using simple single-band fluorescence imaging at the fluorescence emission maximum for animal feces was feasible for this purpose without the need for implementing full-spectrum hyperspectral fluorescence imaging.

Outcomes and Impacts

- Developed for online bulk produce processing, two prototype systems to separately address inspection of the whole surfaces of relatively flat leafy greens and of round-shaped fruits, utilizing two independent sample presentation and imaging mechanisms to accommodate the different morphological properties of the produce.
- Developed a line-scan Raman imaging method and apparatus that can directly and rapidly analyze power samples within minutes, compared to 24 hours for convention instruments, for nondestructive detection of chemical contaminants in food materials.
- Developed a line-scan high-throughput Raman imaging technology. Prototype instruments (bench-top and portable) are available. The technology produced one commercial license (pending), one utility Patent, and various material transfer agreements.
- Developed and validated a spectral library for a variety of food powders and chemical additives and detection algorithms
- The ARS Raman imaging techniques provide tools for real-time food safety testing for qualitative and quantitative detection, including two patented technologies (U.S. Patents No. 9,927,364 and 9,963,882 B2) that can effectively detect contamination and adulteration of food products.
- Developed, validated and licensed a handheld fluorescence-based imaging device. The technology has demonstrated great potential and efficacies for use as an assistive tool for human inspectors in assessing contamination and sanitation of food-contact surfaces and foods in processing facilities, as well as in food safety audit for food preparation facilities serving the US military.
- Developed a handheld imaging device for contamination and sanitation inspection. Several versions of the devices were transferred under material transfer agreements and the patented technology was licensed for commercialization August 2019: USDA License No. 1588-002.
- Developed and validated a system for high-resolution hyperspectral reflectance and fluorescence imaging in outdoor fields using ambient lighting or pulsed laser excitation for in-field preharvest produce inspection for animal intrusion and fecal contamination. The technology tested on a ground-based platform and is undergoing adaptation to a drone platform to enable macro-scale field imaging.

Examples of Relevant Publications

- Everard, C., Kim, M.S., Cho, H.J., and O'Donnell, C. 2016. Hyperspectral fluorescence imaging using violet LEDs as excitation sources for fecal matter contaminate identification on spinach leaves. J. Food Measure. & Characterization. 10(1):56-63.
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Chemical and Biological Contaminants: Biological Toxins (Mycotoxins)

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Certain fungi, principally the Penicillium, Aspergillus and Fusarium genera, infest a variety of food and feed crops producing toxins [mycotoxins] that may be hazardous to animal and human health. These mycotoxins, for example aflatoxins, trichothecenes and fumonisins are secondary metabolites and include compounds that are potent carcinogens, hepatotoxins, nephrotoxins, and neurotoxins. Some mycotoxins are also immune-compromising, and thus can reduce resistance to other diseases. Mycotoxins in commodities and associated products (food and feed) cause extensive economic losses to growers, processors, livestock and poultry producers and food and feed processors. The U.N. Food and Agriculture Organization estimates that annually over 25% of the world's food crops are affected by mycotoxins, and this assessment is increasing as a consequence of global climate change. Although mycotoxin contamination in a major concern globally its effect is different, mycotoxin type (genera-wise), nationally/internationally (country-wise), agriculturally (crop-wise), animal-wise, economically, medically, and long-term. Therefore, despite decades of research, and the introduction of good production, processing, storage, and distribution practices mycotoxins continue to be a problem, if not a greater problem than previously known. Due to the impact of mycotoxin contamination, there are significant efforts associated with keeping contaminated agricultural commodities out of the food and feed supply. Thus, many countries or groups (for example the EU) either regulate or strongly control the permissible levels of mycotoxins in foods and feed.

Research efforts include those addressing development of various intervention/control methods, genomics, detection methods for mycotoxins and derived toxins (masked mycotoxins), monitoring, toxicology and endophytes. This part of the accomplishment report is divided into several area due to the breadth of the mycotoxin research the Program addresses.

Introduction

Intervention and Control
As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Research focused on prevention of mycotoxin contamination, with emphasis on prevention of aflatoxins in the U.S.'s highest valued crops. For example, commodities produced in California include [almonds, (>99% of the U.S. market supply, > 85% of the global supply); walnuts (>95% of the U.S. market supply, >50% of the global supply); pistachios (>95% of the U.S. market supply, >50% of the global supply); table grapes; raisin grapes; figs and pomegranates], valued at ~$10 billion annually which is >20% of the California agriculture GDP. Additionally, there are cotton, corn and peanuts produced in other states such as Arizona, Texas and Georgia whose value is in the billions of dollars. All these are considered high value commodities nationally, and internationally for U.S. trade.

The main research effort involves studies on both pre- and post-harvest fungal control, specifically; disruption of mycotoxin biosynthesis and catabolism, and mycotoxin detection in storage and stockpiles. Insect-feeding damage, for example by the Naval-Orange-Worm (NOW) may create wounds and avenues for fungal infection. Thus, studies were included on the use of natural host plant volatiles for insect control. Certain insect and fungal infections can result in contamination by bacterial pathogens such as Salmonella spp.; thus, efforts to apply fungal control combined with processing technologies were investigated. Ecological studies determined the attributes and changes in the microbial communities in order to understand the dissemination of fungal and bacterial pathogens, and toxins in/on commodities and the interactions and relationships within these communities.

Various alternative interventions were studied, however, the most effective method for preventing mycotoxin (aflatoxin, cyclopiazonic acid, ochratoxin, fumonisins) contamination appears to be a type of biological control that utilizes atoxigenic isolates or other inhibitory microorganisms that do not produce toxins to competitively inhibit, exclude/out-compete toxin producers. ARS has investigated this concept and studies continued to advance biological control through increased understanding of the population biology of, for one example, the use of atoxigenic Aspergillus flavus (Af) and continued engagement of stakeholders such as the Almond Board, the Administrative Committee for Pistachios, the National Cotton Council, Arizona Cotton Research and Protection Council, South Texas Cotton and Grain Association, the Texas Corn Producers Board, and for international studies International Institute of Tropical Agriculture [Africa] and several African nations. In order to make possible application of management procedures in internationally partnerships, additional funding was provided by the Bill & Melinda Gates Foundation; Meridian Institute; US-Agency for International Development; USDA-Foreign Agricultural Service; United Kingdom Department for International Development; and the Austrian Development Agency.

Efforts were directed to development of criteria for selection of optimal biocontrol strains based on phenotypic, genetic, and geostatistical analyses, as well as assessment of adaptation to cropping systems. Multi-season influences of biocontrol were characterized and rationale for the design of strain mixtures with greatest value in target rotations, regions, and environments.

This research was conducted by two projects (McGarvey and Callicott) using differing but complementary approaches.

The (McGarvey) project conducted at WRRC, Albany, CA (specifically focused on California) objectives were to: (1) Develop and implement control measures to reduce, eliminate, or detect contamination of toxin producing fungi of tree nuts, for example the use of host plant- or fungal-derived semio-chemicals to attract or control insect pests, or use of sterile insect techniques to decrease insect pest populations; (2) Elucidate principles of microbial ecology and develop biological control measures to inhibit pathogenic and toxigenic microorganisms, particularly fungi, and can include research on the isolation and development of new biocontrol agents and formulations to control or prevent toxigenic microbes, or survey, identify, and determine ecology of microbial populations for control strategies such as

competitive microorganisms; and (3) Discover natural chemical compounds that enhance the efficacy of established microbe intervention strategies, for instance augment the activity of antimicrobial agents/treatments against pathogens via target-based application of natural chemo-sensitizing agents.

Examples of Accomplishments

- **Biological control agents to reduce human pathogen growth on produce.** A produce associated bacterial culture library containing ~ 10,000 isolates was screened for the ability to inhibit the growth of the human pathogens Salmonella enterica, Listeria monocytogenes, and Escherichia coli O157:H7 in a high throughput, in vitro fluorescence growth assay. Multiple isolates were identified that inhibited the growth of these pathogens in vitro and were further evaluated for the ability to grow, persist and inhibit the growth of these pathogens on fresh, ready to eat produce. The product of this research was the identification of biological control agents that reduce and, in some cases completely inhibit the growth of pathogens on produce. Interestingly, some of these biological control agents also enhance plant growth and have been transferred to interested agricultural industry partners through confidential research and development agreements (CRADA) and material transfer research agreements (MTRA).

- **Volatile compounds as lures to attract navel orangeworm (NOW).** Naturally occurring volatile chemicals identified from almond orchards have been used to attract and monitor populations of NOW under field conditions. The origin of the lure's volatile components was discovered to be derived from almonds with a fungal contamination. This finding demonstrated that the NOW is attracted by olfactory cues to almonds with fungal contamination. It is likely that the NOW is attracted to these fruits because the fungus degrades the protective tissues of the fruits, providing a suitable location for egg laying or overwintering. This strategy of analyzing volatiles from agricultural commodities with fungal damage is being used to identify additional attractants for insect pests of different types of tree nut orchards.

- **Natural compounds with enhanced fungicidal activity.** Contamination of crops and agricultural lands by mycotoxigenic fungi, or fungi resistant to conventional fungicides represents a serious hazard to food safety. ARS identified natural compounds (benzaldehydes and cinnamic acid derivatives) that can rapidly remove fungal contaminants from food and/or environmental matrices. The identified benzaldehydes are redox-active compounds that disrupt the cellular components for oxidative stress resistance in fungi, inhibit mycotoxin production, and prevent fungal tolerance to commercial fungicides. The natural cinnamic derivatives effectively prevent fungal growth by disrupting the cell wall integrity of fungi. These natural compounds also lower the effective doses of fungicides, reducing the required inputs of these toxic compounds, and lowering the environmental and health risks associated with fungicide application.

- **X-ray based irradiators as an alternative to radioisotopes.** Irradiation is an important tool with many applications in agriculture, including control of bacterial infection, phytosanitary treatments to kill insect pests on food products, and sterilization of insects for a common pest control strategy called Sterile Insect Technique (SIT). Traditional gamma ray emitting radioactive materials such as cobalt and cesium are increasingly problematic for use as irradiation sources for many reasons, including increased governmental scrutiny and regulation over homeland security concerns, radioisotope suppliers going out of business, the general aging and weakening (radioactive decay) of existing sources, concern over disposal of radioactive waste, and the general lack of consumer acceptance to food irradiation using radioactive materials. ARS developed irradiators using commercially available low energy x-ray tubes like those used in airport baggage scanning equipment and have demonstrated their use as an alternative to gamma sources for insect sterilization. The design configuration allows insect sterilization with greater dose uniformity and precision as compared to gamma sources, while avoiding the many problems associated with radioisotopes.

Outcomes and Impacts

- Identified biological control agents that reduce and, in some cases completely inhibit the growth of pathogens on produce. Determined that some agents also enhance plant growth and have been transferred to interested agricultural industry. Biocontrol agents to reduce bacterial growth on fresh produce. U.S. Patent No. 10,264,808.
- Demonstrated that the NOW is attracted by olfactory cues to almonds with fungal contamination. This strategy is being used to identify additional attractants for insect pests of different types of tree nut orchards.
- Identified natural compounds that can rapidly remove fungal contaminants from food and/or environmental matrices. Determined these natural compounds also lower the effective doses of fungicides, reducing the required inputs of these toxic compounds, and lowering the environmental and health risks associated with fungicide application.
- Developed irradiators for SIT. The design configuration allows insect sterilization with greater dose uniformity and precision as compared to gamma sources, while avoiding the many problems associated with radioisotopes.

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- McGarvey, Jeffery A., Hnasko, R., Stanker, L.H., and Gorski, L., 2019. Use of phyllosphere associated lactic acid bacteria as biocontrol agents to reduce bacterial growth on fresh produce. U.S. Patent No. 10,264,808.

The (Callicott) [previously Cotty] project at Tuscon/Maricopa, AZ objectives were to: (1) Optimize and expand use of biological control of aflatoxins based on atoxigenic strains of A. flavus in order to improve access, affordability, and area-wide management; (2) Develop an understanding of the distribution of A. flavus genetic haplotypes and vegetative compatibility groups worldwide in order to improve selection of biological control agents; (3) Improve understanding of development, evolution, and stability of populations of A. flavus, including phenomena occurring both within and between VCGs, in order to inform optimization of long-term beneficial effects of atoxigenic strain biocontrol.

Examples of Accomplishment

- **Modular manufacturing process.** Traditionally, aflatoxin biocontrol products have been manufactured through one of two methods, either by coating hulled and de-germed barley (as in the Afla-Guard product) with spores of the biocontrol isolate or through steam sterilization of wheat, followed by inoculation and incubation of the biocontrol isolate (as in AF36). The first suffers from a non-sterile product, while the second is energy- and time-consuming.

Figure: Tucson Aflatoxin Reduction lab members coating roasted sorghum during development of new technique.

A new process (figure above) was developed that uses commercially available equipment to roast grain and coat it with spores of the biocontrol isolate or isolates. Roasting both sterilizes the grain and ensures that it does not germinate. Spores, colorant, and a spreader-sticker are added to the cooled, roast grain using a seed coater.

- **New products and new isolates identified.** Over the past 5-years, there has been an extensive increase in registration and use of biocontrol products in sub-Saharan Africa. Prior to 2016, there was one product registered in Nigeria and one product registered in Kenya. Subsequent sampling by the International Institute of Tropical Agriculture (IITA) and characterization by the Tucson Aflatoxin Reduction lab has led to isolate identification and new product registration in Senegal and The Gambia (2016), Burkina Faso (2017), Ghana (2018), Zambia (2018), Tanzania (2018), and Mozambique (2019). Each product consists of four atoxigenic isolates, and two products were registered in Ghana, Tanzania, and Mozambique. Two additional four-isolate products have completed field testing in Malawi. Hundreds of isolates were genetically characterized from each country to identify the most common atoxigenic (non-aflatoxin producing) genetic types, and final selection of isolates was collaboratively decided by ARS and IITA.

Figure: African farmers applying Aflasafe on corn

- **EUP secured for Texas Corn Producers (TCP).** ARS (Tucson Aflatoxin Reduction lab), TCP, and ACRPC worked to secure an Experimental Use Permit (EUP) from EPA to test a new four-isolate biocontrol product (FourSure). While AF36 has been registered for use on corn in Texas since 2011, the growers represented by the TCP wanted an additional product in the market. The field trials under this EUP began in 2017 and finish with the 2019 season.

- **Identity of aflatoxin biosynthesis cluster deletions.** The genes necessary for synthesizing aflatoxins are present in Aspergillus flavus and its close relatives in a stretch of DNA called the aflatoxin biosynthesis cluster. This cluster contains genes encoding both the enzymes necessary for biosynthesis as well as the regulatory proteins. Previous literature had shown the presence of deletions in some isolates of A. flavus, providing an explanation for why those isolates were atoxigenic. While the cluster deletion patterns could be observed in multiple isolates, it was unknown whether having the same pattern indicated the isolates had the same deletion or just similar ones. Genomic analysis showed that isolates with the same amplification pattern did indeed have the same deletion with the same indel boundaries.

- **Clonal reproduction in natural populations.** A large study of over 2,700 A. flavus isolates from Kenyan agricultural soils was conducted to determine the degree to which natural populations are clonal or sexual. No evidence for sexual recombination could be detected, even when removing as many clonal individuals as possible by including only one isolate from each clonal group. This suggests that if sexual recombination is occurring in natural populations, it is at a low enough rate to be unimportant as a concern for biocontrol technology.

- **Worldwide distribution of genetic types.** Studies characterized over 29,000 A. flavus isolates from around the world using 17 simple sequence repeat (SSR) markers. Characterization of isolates by DNA allows for precise description of isolates and will provide all Aspergillus researchers a means to clearly identify similar isolates without having to share cultures. Characterizing these isolates has revealed a remarkable degree of diversity, with over 13,000 unique haplotypes discovered to date. This methodology and the resulting dataset are being shared with other researchers to document the geographic structure of A. flavus and explore the interactions between environment and genotype.

Outcomes and Impacts

- Developed a new manufacturing process for the aflatoxin biocontrol agents which reduces costs. Implemented by the Arizona Cotton Research and Protection Council (ACRPC) for its AF36 plant in Phoenix and by IITA in Aflasafe plants in Kenya and Senegal.
- Developed a new four-isolate product for the U.S. (FourSure) and two four-isolate products in sub-Saharan Africa.

- Determined through specific aflatoxin cluster deletions in strains with divergent genetic backgrounds that these deletions are ancient and stable and that fears of sudden reversion to aflatoxigenicity are unfounded.
- Determined that the concern that sexual recombination between applied atoxigenic isolates and native toxin producers will lead to the sudden appearance of highly competitive toxigenic isolates is without basis.
- A publicly accessible database of genetically characterized isolates has the potential to be transformative in the Aspergillus flavus research community.
- Registration of biocontrol product (AF36 Prevail) using new technology: https://www3.epa.gov/pesticides/chem_search/ppls/071693-00002-20150625.pdf
- Extension of AF36 Prevail usage label to cover almond and fig: https://www3.epa.gov/pesticides/chem_search/ppls/071693-00002-20170322.pdf
- Experimental Use Permit secured for Texas Corn Producers: https://www.govinfo.gov/content/pkg/FR-2016-09-16/html/2016-22357.htm

Examples of Relevant Publications

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Chemical and Biological Contaminants: Genomics, Plant Fungal Interactions, Breeding and Climate Change

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR); https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf The biosynthesis of aflatoxins (AF), fumonisins (FUM) and trichothecenes (TRI) have been extensively studied by ARS, and the genetics have been elucidated in detail in the genomes of A. flavus, the related aflatoxin-producing species A. parasiticus, A. nomius, the non-aflatoxigenic species A. oryzae; and Fusarium species, in particular F. graminearum which is associated with economically devastating plant disease Fusarium Head Blight (FHB) and the associated toxin deoxynivalenol (DON), and the fumonisin-producing Fusarium verticillioides.

Less is known about what causes the fungi to produce mycotoxins under certain environmental conditions and only on certain plants. Also, it is not fully understood how toxin formation is transcriptionally regulated during conidial, sclerotial, and mycelial development although a strong association with these processes has been proven. Information is still lacking concerning how external stress factors, in particular climate change and climate resilience affects mycotoxin formation although much evidence suggests that there is a direct connection. Furthermore, a better understanding of how interactions with susceptible crops affect transcription of fungal genes involved in mycotoxin formation is needed to improve on intervention strategies.

Studies were undertaken to: determine the dynamics of interaction among the key nutritionally and environmentally induced transcription factors necessary for production of mycotoxins in order to develop novel inhibitors to one or more of these factors to prevent mycotoxin formation in crops; examine the effects of known natural (plant-derived compounds) inhibitors of mycotoxin production on key components of the mycotoxin regulatory machinery to ultimately design safe, inexpensive chemicals that inhibit proteins unique to fungal secondary metabolite biosynthesis; and to identify safe and effective procedures for use on crops intended for consumption by humans or animals.

Research would also address the discovery and direct targeting of novel features of Fusarium, centering on ecological fitness and diverse metabolic activities, including detoxification of xenobiotics allied to the elimination of mycotoxins in commodities through postharvest processing.

The most practical solution to the contamination problem would be to prevent the contamination process in crops before harvest. One of the easiest technologies to implement by growers would be to utilize germplasm with enhanced resistance to fungal growth and mycotoxin contamination. ARS studies had identified several genes in corn associated with mycotoxin resistance that have been used to develop molecular markers to transfer resistance to agronomically desirable corn lines. RNA interference (RNAi) techniques are also being used to develop transgenic corn and cotton lines that demonstrate improved resistance to fungal growth and mycotoxin production. Allied to this was the need for development of rapid, non-

destructive detection methodology based upon hyperspectral imaging technology that could be used remotely, by satellite or drone, or in processing facilities to exclude aflatoxin-contaminated corn from the food stream before harvesting or prior to packaging. Finally, sound risk assessment and regulatory policy remains the first line of defense for public health. Studies will obtain data in the knowledge gaps needed to improve the science-based risk assessment of mycotoxins, with emphasis on fumonisins (FBs). The approach involves the use of fumonisin-specific pathology, biochemical effects, and biomarkers of exposure in well-defined rodent bioassays.

This wide-ranging research was conducted through several projects at three different research locations: the NCAUR, Peoria, Illinois; SRRC, New Orleans, Louisiana; and RRRC, Athens, Georgia.

Studies at NCAUR, Peoria, IL (McCormick and Proctor) focused on Fusarium research.

The (McCormick) project objectives were to: (1) Identify and characterize microorganisms and microbial genes that can reduce trichothecene contamination of grain-based food and feed. (2) Determine the effects of climate change on susceptibility of wheat and corn to contamination by trichothecenes and other Fusarium mycotoxins; and (3) Determine the genomic diversity of Fusarium Head Blight (FHB) pathogens and identify species or population-specific differences in host-pathogen interactions, mycotoxin production, or pathogen fitness under different climatic conditions.

Examples of Accomplishments

- **Genetic tools for molecular surveillance**. Several different fungi can cause FHB and contaminate grain with a vomitoxin or other mycotoxins. Accurate tools to detect, identify, and determine the distribution and toxin potential of FHB pathogens are needed to successfully develop and deploy effective FHB and mycotoxin control strategies. However, an understanding of FHB pathogen diversity, distributions, and toxin potential is incomplete and traditional methods of pathogen identification and toxin analyses have been either ineffective or expensive and time consuming.

- **Novel targets to control mycotoxin contamination of wheat and barley.** Fusarium graminearum is the major cause of FHB a significant disease of wheat, barley, and other cereal crops worldwide. In addition, the fungus contaminates grain with mycotoxins that pose a significant threat to food safety and animal health. The recent appearance of novel pathogen populations and types of toxins in North America is of concern because this diversity may include novel adaptations that enable the fungus to rapidly respond to current FHB control measures. ARS analyzed the genomes of 60 F. graminearum isolates from across North America to identify genes that help this important pathogen adapt to agricultural environments, identified 121 genes that distinguish three distinct pathogen populations in North America, and found 14 regions of the genomes that have population-specific adaptations.

- **The distribution and prevalence of the novel NX-2 mycotoxin revealed through global molecular surveillance of Fusarium head blight pathogens.** Fusarium graminearum and related fungi are responsible for FHB and other economically destructive diseases of cereal crops world-wide. In addition, these fungi contaminate grain with mycotoxins that pose a significant threat to food safety and animal health. Strains of F. graminearum with a previously unknown mycotoxin type, termed NX-2, were recently identified from FHB infected wheat. However, the origin, distribution, and global prevalence of this novel toxin type is unknown. ARS utilized a global collection of more than 2,500 F. graminearum isolates to determine the distribution, prevalence, and evolutionary history of NX-2 strains. The results expanded the known geographic distribution of NX-2 strains to include the northeastern U.S., expanded the known host range of NX-2 strains to include oat and barley, and indicated that the NX-2 toxin type may be restricted to southern Canada and the northern U.S. where it occurs at low frequency on cereal crops. In addition, nine genetic changes in a toxin production gene that are specific to NX-2 strains were identified and demonstrated that this novel toxin has a unique evolutionary history indicating it may provide an advantage to FHB pathogens in some environments.

- **Genes identified to improve mycotoxin monitoring and risk assessment.** Trichothecenes are fungal toxins that frequently contaminate food and feed crops and, as a result, pose health risks to humans and domestic animals. Collectively fungi produce over 150 different trichothecenes, each with a similar but distinct chemical structure that can vary markedly in toxicity and in the risks, they pose to human and animal health. ARS combined chemical, molecular, genetic, and genome sequencing technologies to determine the origins and the genetic basis for this toxin diversity. The study identified specific genes that were acquired, lost, or that changed in function to produce different trichothecene structures.

- **Reliable metabolic markers for predicting wheat resistance to Fusarium head blight.** FHB is a devastating fungal disease of wheat which can reduce yield and contaminate grain with harmful mycotoxins. Because FHB outbreaks are strongly associated with weather, wheat varieties are evaluated for resistance to FHB under temperature and moisture conditions that favor disease spread. Although atmospheric carbon dioxide levels are rising, wheat varieties are not currently being screened under higher carbon dioxide levels. A study to determine if wheat resistance ratings are accurate under elevated carbon dioxide levels, measured the amount of infection, mycotoxin contamination, and natural plant defense metabolites in susceptible and moderately resistance wheat grown under current or elevated amounts of carbon dioxide.

- **Corn defense metabolites that can be targeted to enhance both resistance to climate stress and mycotoxigenic fungi.** Fusarium fungi are economically important fungal pathogens of corn that reduces grain yield by causing tissue rot and contaminates grain with mycotoxins rendering it unsafe for consumption. A study to

determine how environmental conditions affect mycotoxin contamination, found that corn plants grown under drought and elevated carbon dioxide conditions are more susceptible to rot and mycotoxin contamination of grain. Under drought conditions, plants divert resources and accumulate higher amounts of defense metabolites in the roots and corn varieties that accumulate higher levels of these defense metabolites in their roots are also more resistant to root and ear rot.

- **Fungal enzyme targets to improve food safety.** Fusarium graminearum is a fungal pathogen that causes FHB of wheat and other cereals and reduces crop yields and quality by producing mycotoxins. To reduce the incidence of FHB and mycotoxin contamination of grain, we need to understand how Fusarium overcomes plant defenses to cause disease. Salicylic acid is an important plant signaling molecule that regulates how the plant responds to fungal pathogens. A study of the F. graminearum genome identified an enzyme that degrades salicylic acid and found that it weakens coordinated plant defenses against FHB. In addition, the study identified an arabininase, enzyme that degrades plant cell walls during infection and reduced the plant immune response to fungal invasion. Both enzymes, salicylic acid hydrolase and arabininase are new molecular targets for disease control and mycotoxin reduction programs.

- **Fungal mock community developed to improve microbiome research.** Understanding of the role of microbiomes in plant health and food safety may lead to new strategies for supporting the production of a safe and abundant food supply. Current research methods for studying microbiomes are typically based on DNA analysis which offers several advantages over older methods. However, there are still limitations to DNA-based methods, and these can lead to erroneous conclusions. ARS developed artificial mixtures of fungal DNA that can be used as an experimental control to identify sources of error in microbiome studies, so that methods can be improved, and limitations can be considered when interpreting results.

- **Microorganisms isolated from soil detoxifies vomitoxin.** Vomitoxin (deoxynivalenol or DON) is a trichothecene mycotoxin produced when the fungus Fusarium infects small grains including wheat and barley. Ingestion of DON contaminated grain can cause diarrhea, hemorrhaging, and feed refusal. DON helps the fungus to spread into the grain. A study found bacteria collected from agricultural and landscape fields that could detoxify DON both in culture and in contaminated grain.

- **An improved plant enzyme to reduce mycotoxin contamination and Fusarium head blight in cereal crops.** FHB is a devastating disease of small grain cereal crops that causes yield reductions and contamination of grain with vomitoxin and other trichothecenes. These toxins are harmful to the health of humans and livestock because of their ability to block protein synthesis. They are also important virulence factors for FHB, therefore plants that can detoxify DON and NIV, by attaching a sugar to the toxins, have improved resistance to the disease. A study demonstrated that a sugar transfer gene from barley improved resistance to trichothecene toxins and FHB in wheat. X-ray crystallography was used to characterize key structural features of sugar transfer

enzymes and guide the modification of a sugar transfer enzyme from rice plants in order to produce an improved enzyme that can disable a wide variety of trichothecene mycotoxins. The new enzyme provides a means to control FHB across a broad spectrum of Fusarium species and is a good candidate for incorporation into crop improvement programs. In addition, the new enzymes provided improved detection and measurement of masked mycotoxins in food and feed.

Genetic control of trichothecene toxins in biocontrol fungus Trichothecenes are a group of mycotoxins that when present in crops pose health risks to people, pets and livestock. The fungus Fusarium graminearum causes head blight of cereal crops and produces the trichothecene vomitoxin, which is toxic to plants (phytotoxic) and contributes to the ability of the fungus to cause head blight.

In contrast, the biocontrol fungus Trichoderma arundinaceum inhibits the growth of many plant disease-causing fungi and produces a trichothecene that is highly toxic to other fungi but harmless to plants. A study of trichothecenes in pathogenic and biocontrol fungi identified a group of genes in T. arundinaceum that control production of trichothecenes that are not phytotoxic. This research provided targets to improve food safety and reduce contamination of grain by counteracting the phytotoxic effects of trichothecenes.

Outcomes and Impacts

- Developed and validated a new, easy and inexpensive genetic tools to identify strains of FHB pathogens. Determined the extent and distribution of different toxins types within the U.S. as well as Brazil, Uruguay, and Mexico.
- Determined the genes from F. graminearum that assist the pathogen adapt to agricultural environments., and that each population uses a unique set of tools to invade and obtain nutrients from their hosts, compete with other microbes, and adapt to different climatic conditions.
- Determined from a global collection of F. graminearum isolates the distribution, prevalence, and evolutionary history of NX-2 strains. The results expanded the known geographic distribution of NX-2 strains.
- Identified and demonstrated that the novel toxin NX-2 may provide an advantage to FHB pathogens in some environments; and further provide tools needed for efficient and

effective monitoring of mycotoxin contamination and for the development of cereals broadly resistant to FHB infection.

- Determined the origins and the genetic basis for different trichothecenes toxin diversity. Identified specific genes that were acquired, lost, or that changed in function to produce different trichothecene structures.
- Determined the increased overall effect of elevated carbon dioxide on mycotoxin contamination, and that elevated carbon dioxide led to changes in plant defenses and disease severity.
- Demonstrated that resistance ratings developed for wheat grown at current carbon dioxide levels may not apply under future conditions.
- Identified a set of metabolic markers that can be reliably used by breeders to select for FHB resistance even under increased atmospheric carbon dioxide.
- Determined that corn plants exposed to both elevated carbon dioxide and drought have a compromised immune system and are less able to deploy defense metabolites. Identified traits that can be targeted to enhance both resistance to abiotic stress and mycotoxin contamination.
- Determined that F. graminearum degrades salicylic acid and this weakens coordinated plant defenses against FHB. Determined that arabininase degrades plant cell walls during infection and reduced the plant immune response to fungal invasion. Both salicylic acid hydrolase and arabininase can be used as molecular targets for disease control and mycotoxin reduction programs.
- Developed artificial mixtures of fungal DNA that can be used as experimental controls to identify sources of errors in microbiome studies. Identified components of the wheat microbiome that are correlated with the amount of Fusarium biomass and the concentration of Fusarium mycotoxins. Potential new approaches to reduce mycotoxin contamination of small grain cereals via manipulation of the wheat microbiome.
- Demonstrated the potential use of microorganisms from the environment to remediate mycotoxin (for example DON) contaminated materials, such as dried distillers' grains derived from fuel ethanol production and used as feed for livestock. The microbes also serve as a source of new detoxification genes that can be expressed in plants to improve resistance to Fusarium disease.
- Demonstrated that a sugar transfer gene from barley improved resistance to trichothecene toxins and FHB in wheat, providing a means to control FHB across a broad spectrum of Fusarium species. Potential candidate for incorporation into crop improvement programs and improved detection and measurement of masked mycotoxins.
- Identified a group of genes in Trichoderma arundinaceum that control production of trichothecenes that are not phytotoxic providing targets to counteracting the phytotoxic effects of trichothecenes.

Examples of Relevant Publications

- Kelly, A., Proctor, R.H., Belzile, F., Chulze, S.N., Clear, R.M., Cowger, C., Elmer, W., Lee, T., Obanor, F., Waalwijk, C., Ward, T.J. 2016. The geographic distribution and complex evolutionary history of the NX-2 trichothecene chemotype from Fusarium graminearum. Fungal Genetics and Biology. 95:39-48.
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- Wetterhorn, K.M., Newmister, S.A., Caniza, R.K., Busman, M., McCormick, S.P., Berthiller, F., Adam, G., Rayment, I. 2016. Crystal structure of Os79 (Os04g0206600) from *Oryza sativa*: a UDP-glucosyltransferase involved in the detoxification of deoxynivalenol. Journal of Biochemistry. 55(44):6175-6186.
- Wilson, N.M., McMaster, N., Gantulga, D., Soyars, C., McCormick, S.P., Knott, K., Senger, R.S., Schmale, D.G. 2017. Modification of the mycotoxin deoxynivalenol using microorganisms isolated from environmental samples. Toxins 9:141; https://doi:10.3390/toxins9040141.
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- Vaughan, M.M., Block, A., Christensen, S., Allen, L.H., Schmelz, E.A. 2017. The effects of climate change associated abiotic stresses on maize phytochemical defenses. Phytochemistry Reviews 17:37-49.
- Garmendia, G., Umpierrez-Failache, M., Ward, T.J., Vero, S. 2017. Development of a PCR-RFLP method based on the transcription elongation factor 1-a gene to differentiate Fusarium graminearum from other species within the Fusarium graminearum species complex. Food Microbiology. 70:28-32.
- Kelly, A.C., Ward, T.J. 2018. Population genomics of Fusarium graminearum reveals signatures of divergent evolution within a major cereal pathogen. PLoS One. 13(3):e0194616.
- Ceron-Bustamante, M., Ward, T.J., Kelly, A.C., Vaughan, M.M., McCormick, S.P., Cowger, C., Leyva-Mir, S.G., Villasenor-Mir, H.E., Ayala-Escobar, V., Nava-Diaz, C. 2018. Regional differences in the composition of Fusarium head blight pathogens and mycotoxins associated with wheat in Mexico. International Journal of Food Microbiology. 273:11-19.

- Proctor, R.H., McCormick, S.P., Kim, H.-S., Cardoza, R.E., Stanley, A.M., Lindo, L., Kelly, A., Brown, D.W., Lee, T., Vaughan, M.M., Alexander, N.J., Busman, M., Gutierrez, S. 2018. Evolution of structural diversity of trichothecenes, a family of toxins produced by plant pathogenic and entomopathogenic fungi. PLoS Pathogens. 14(4): e1006946. https://doi.org/10.1371/journal.ppat.1006946.
- Bakker, M.G. 2018. A fungal mock community control for amplicon sequencing experiments. Molecular Ecology Resources 18(3):541- 556; https://doi.org/10.1111/1755-0998.12760.
- Christensen, S. A., Huffaker, A., Sims, J., Hunter, C. T., Block, A., Vaughan, M. M., Willett, D., Romero, M., Mylroie, J. E., Williams, W. P., and Schmelz, E. A. 2018. Fungal and herbivore elicitation of the novel maize sesquiterpenoid, zealexin A4, is attenuated by elevated CO2. Planta 247:863-873.
- Hossain, M.Z, McCormick, S.P., Maragos, C.M., 2018. An imaging surface plasmon resonance biosensor assay for the detection of T-2 toxin and masked T-2 toxin-3-glucoside in wheat. Toxins. 10(3)119.
- Lindo L., McCormick, S. P., Cardoza, R. E., Brown, D., Kim, H.-S., Alexander, N. J., Proctor, R. H., and Gutiérrez, S. 2018. Effect of deletion of a trichothecene toxin regulatory gene on the secondary metabolism transcriptome of the saprophytic fungus *Trichoderma arundinaceum*. Fungal Genet. Biol. 119:29-46.
- Lindo L., McCormick, S.P., Cardoza, R.E., Kim, H.-S., Brown, D., Alexander, N.J., Proctor, R.H., Gutiérrez, S. 2018. Role of *Trichoderma arundinaceum Tri10* in the regulation of genes involved in terpene biosynthesis and in the control of the metabolic flux through the pathway Fung. Genet. Biol. 122:31-46.
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- Cuperlovic-Culf, M., Vaughan, M.M., Vermillion, K., Surendra, A., Teresi, J., McCormick, S. 2019. Effects of atmospheric CO2 level on the metabolic response of resistant and susceptible wheat to *Fusarium graminearum*. Mol. Plant Microbe Interactions 32(4): 379-391; https://doi: 10.1094/MPMI-06-18-0161-R.
- Hao, G., Naumann, T.A., Vaughan, M.M., McCormick, S.P., Usgaard, T., Kelly, A., Ward, T.J. 2019. Characterization of a *Fusarium graminearum* salicylate hydroxylase. Frontiers Microbiology. doi: 10.3389/fmic.2018.03219.
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- Lippolis, V., Porricelli, A.C.R, Mancini, E., Ciasca, B., Lattanzio, V.M.T., De Girolamo, A., Maragos, C.M., McCormick, S., Li, P., Logrieco, A.F., Pascale, M.2019. Fluorescence polarization immunoassay for the determination of T-2 and HT-2 toxins and their glucosides in wheat. Toxins 11:380; https://doi:10.3390/toxins11070380.

The (Proctor) project objectives were to: (1) Use comparative phylogenomic approaches to enable accurate identification of mycotoxigenic Fusarium and to elucidate components of Fusarium genomes that are responsible for variation in mycotoxin production; (2) Develop and utilize liquid chromatography-mass spectrometry (LC-MS) approaches for metabolomic analysis of Fusarium verticillioides infection of maize; (3) Identify and characterize plant and fungal factors that can impact mycotoxin contamination via their effects on plant disease development; and (4) Identify and characterize components of fungus-fungus interactions that contribute to or inhibit mycotoxin contamination of crops.

Examples of Accomplishments

- **Zeroing in on fungi that cause fumonisin contamination in corn.** Fumonisins are among the mycotoxins of greatest concern to food safety. Although the fungus Fusarium verticillioides has been considered the primary cause of fumonisin contamination in corn for decades, recent findings that some isolates of another corn-associated fungus, Aspergillus niger, can produce fumonisins has raised concerns that it too is responsible for fumonisin contamination. A multiyear field study in Illinois and Iowa demonstrated that infection of corn ears with A. niger did not result in accumulation of significant levels of fumonisins.

- **A method for reducing fumonisin contamination in corn.** Fumonisins are mycotoxins produced by multiple fungi, including the corn ear rot pathogen Fusarium verticillioides, and are among the mycotoxins of greatest concern to food safety because of their toxicity and frequent occurrence in corn. To determine whether the natural cell defense system known as RNA interference (RNAi) can be used as a control strategy to reduce fumonisin contamination in corn, F. verticillioides was engineered to produce RNAi molecules that targeted two genes essential for fumonisin biosynthesis in the fungus. The resulting RNAi-producing strains of F. verticillioides exhibited reductions in fumonisin production up to 3000-fold less than the un-engineered fungus.

- **Defining diversity of Fusarium species that cause mycotoxin contamination in crops. (see also Genomics)** Many species of the fungus Fusarium are food and feed safety concerns because they contaminate crops with mycotoxins that are health hazards to humans, pets and livestock. An accurate understanding of the identity and diversity of species that cause mycotoxin contamination is an essential component of mycotoxin control strategies. However, there are significant knowledge gaps with respect to which species of Fusarium produce which mycotoxins, and there are potentially hundreds of unidentified species of the fungus that could be contributors to mycotoxin contamination around the world. Using genome sequencing and DNA-based phylogenetic analyses, ARS determined the phylogenetic relationships of over 350 isolates that represent the breath of diversity that exists within the genus Fusarium. Representative species from this phylogenetic study were also examined by mass spectrometry-based metabolomic analyses to assess their ability to produce all known Fusarium mycotoxins.

- **Elucidation of genetic mechanisms responsible for variation in mycotoxin production in Fusarium.** Collectively, species of the fungus Fusarium produce multiple mycotoxins that pose health risks to humans, pets and livestock animals. However, there are substantial qualitative differences within and among species with respect to the kinds of mycotoxins they produce. Using a comparative genomics approach, studies determined the distribution of all known mycotoxin biosynthetic genes in over 150 species that represent the breadth of phylogenetic diversity within the genus Fusarium. The results provide a genetic explanation for much of the qualitative variation in mycotoxin production among species. Extensive phylogenetic analyses have also provided evidence for the genetic mechanisms responsible for variation in production. The results indicate that extensive loss of mycotoxin biosynthetic genes has occurred in most lineages of Fusarium. Therefore, understanding plant host and/or environmental factors that select for loss of mycotoxin biosynthetic genes has potential to contribute to control strategies aimed at reducing mycotoxin contamination in crops. In addition, variation in sequences and genomic positions of mycotoxin biosynthetic genes identified in this research are being used as genetic markers to detect, quantify, and distinguish between mycotoxin-producing Fusarium species. Such markers are valuable tools in efforts to develop strategies that reduce mycotoxin contamination in crops.

- **Elucidation of components of corn-fungus interactions that affect mycotoxin contamination.** Mycotoxin contamination is often associated with crop diseases, and both the contamination and disease are likely affected by interactions of molecules produced by mycotoxin-producing fungi and their host plants. Studies identified corn enzymes (chitinases) that inhibit fungal growth by degrading chitin, an essential component of fungal cell walls. This research has also identified multiple enzymes produced by corn ear rot fungi that degrade corn chitinase and determined how one of the proteases degrades a chitinase. Other research has revealed that some fatty acid-derived molecules known as oxylipins, which are produced by corn, can induce expression of fumonisin biosynthetic genes in Fusarium verticillioides, a corn ear rot fungus that produces fumonisin mycotoxins. These findings indicate the potential of manipulating chitinases and oxylipin content in corn as control strategies to reduce mycotoxin contamination by blocking the ability of fungi to cause disease (chitinases) or by directly suppressing mycotoxin production through inhibition of biosynthetic genes (oxylipin content).

Outcomes and Impacts

- Demonstrated that A. niger is not a significant contributor to fumonisin contamination in corn and efforts should focus on F. verticillioides.
- Demonstrated through engineering F. verticillioides the potential of RNA interference (RNAi) as a control strategy to reduce fumonisin contamination in corn. RNAi-producing strains of F. verticillioides exhibited reductions in fumonisin production up to 3000-fold less than the un-engineered fungus.

- Examined the genetic diversity of Fusarium species which updated the phylogenetic relationships of the species; corrected the species identities of multiple strains used as references in mycotoxin research; identified multiple novel species; and provided an accurate assessment of mycotoxin production abilities facilitating risk assessments.
- The DNA sequences characteristic of the novel species were integrated into online databases to determine the identities of Fusarium isolates that cause mycotoxin contamination. ARS is one of the biggest contributors of Fusarium genome sequences to the DNA sequence database at the National Center for Biotechnology Information.
- Determined using a comparative genomics approach, the distribution of all known mycotoxin biosynthetic genes representing the breadth of phylogenetic diversity within the genus Fusarium. Provided a genetic explanation for much of the qualitative variation in mycotoxin production among species. Extensive phylogenetic analyses provided evidence for the genetic mechanisms responsible for variation in production indicating that extensive loss of mycotoxin biosynthetic genes has occurred in most lineages of Fusarium.
- Elucidated plant host and/or environmental factors that select for loss of mycotoxin biosynthetic genes which has the potential to contribute to control strategies aimed at reducing mycotoxin contaminations. Variation in sequences and genomic positions of mycotoxin biosynthetic genes are being used as genetic markers to detect, quantify, and distinguish between mycotoxin-producing Fusarium species. Such markers are valuable tools in efforts to develop strategies that reduce mycotoxin contamination in crops.
- Identified corn enzymes (chitinases) that inhibit fungal growth by degrading chitin, an essential component of fungal cell walls. Further, identified multiple enzymes produced by corn ear rot fungi that degrade corn chitinase and determined how one of the proteases degrades a chitinase.
- Determined that some fatty acid-derived molecules (oxylipins) produced by corn, can induce expression of fumonisin biosynthetic genes in Fusarium verticillioides, a corn ear rot fungus that produces fumonisin mycotoxins. This indicated the potential of manipulating chitinases and oxylipin content in corn as control strategies to reduce mycotoxin contamination by blocking the ability of fungi to cause disease (chitinases) or by directly suppressing mycotoxin production through inhibition of biosynthetic genes (oxylipin content).

Examples of Relevant Publications

- Susca, A., Proctor, R.H., Morelli, M., Haidukowski, M., Gallo, A., Logrieco, A.F., Moretti, A. 2016. Variation in fumonisin and ochratoxin production associated with differences in biosynthetic gene content in *Aspergillus niger* and *A. welwitschiae* isolates from multiple crop and geographic origins. Frontiers in Microbiology. 7: 1412.
- Brown, D.W., Proctor, R.H. 2016. Insights into natural products biosynthesis from analysis of 490 polyketide synthases from *Fusarium*. Fungal Genetics Biology 89: 37-51.

- Niehaus, E.-M., Munsterkotter, M., Proctor, R.H., Brown, D.W., Sharon, A., Idan, Y., Oren-Young, L., Sieber, C.M., Novak, O., Pencik, A., et al. 2016. Comparative "omics" of the *Fusarium fujikuroi* species complex highlights differences in genetic potential and metabolite synthesis. Genome Biology and Evolution. 8(11):3574–3599.
- Villani, A., Moretti, A., De Saeger, S., Han, Z., Di Mavungu, J. D., Soares, C. M. G., Proctor, R. H., Venâncio, A., Lima, N., Stea, G., Paciolla, C., Logrieco, A. F., Susca, A., 2016. A polyphasic approach for characterization of a collection of cereal isolates of the *Fusarium incarnatum-equiseti* species complex. International Journal of Food Microbiology. 234, 24-35.
- Brown, D.W., Baker, S.E. 2017. Mycotoxins: A fungal genomics perspective. In: Moretti, A., Susca, A., editors. Mycotoxigenic Fungi. Vol 1542. New York, NY: Springer. p. 367-379.

Studies at SRRC, New Orleans, LA were conducted through three projects (Cary, Rajasekaran, and Moore) focused on Aspergillus research and related fungi.

The (Cary) objectives were to: (1) Identify key genes, using transcriptome analysis of Aspergillus flavus and Aspergillus flavus-crop interaction that are involved in fungal growth, morphogenesis, toxin production and virulence which can be used as targets for intervention strategies; (2) Identify metabolites produced by predicted secondary metabolic gene clusters in Aspergillus flavus, characterize the molecular regulation of their biosynthesis, and determine if they contribute to the fungus' ability to survive, colonizes host crops and produce aflatoxin; and (3) Examine the role of climatic and environmental pressures on the growth, virulence, toxigenic potential, geographical distribution and aflatoxin production by Aspergillus flavus.

Examples of Accomplishments

- **Targeting regulatory genes from Aspergillus flavus to enhance resistance in corn to aflatoxin contamination.** Using sophisticated molecular techniques, genes encoding a total of 12 regulators from A. flavus necessary for production of reproductive and survival structures (spores and sclerotia) and numerous metabolites including aflatoxin were identified. In some cases, these regulators were found to play a role in the ability of the fungus to invade plant tissues. Specialized plant plasmid vectors targeting the A. flavus veA and nsdC genes for host-induced gene silencing (HIGS) were developed and transformed into corn (CRIS 025). Analysis of transgenic corn seed targeting the genes encoding these two regulators demonstrated significant reduction in the ability of the fungus to contaminate kernels with aflatoxins (85-90% reduction). Further, the effect that biotic and abiotic stresses have on expression of these key regulatory genes can provide valuable insights into the molecular mechanisms controlling how A. flavus strains respond to alterations in their environments.

- **Identification of an Aspergillus flavus secondary metabolite that functions as a virulence factor on corn**. Molecular and biochemical analysis of a previously uncharacterized secondary metabolite gene cluster in A. flavus showed that it was responsible for the production of aspergillic acid (AA). This metabolite is capable of binding iron and has been shown to be toxic to animals. Many of the AA cluster genes were highly expressed at the earliest stages of A. flavus infection of corn suggesting that AA may aid in the ability of the fungus to colonize corn. In situ corn kernel assays demonstrated that fungal growth and aflatoxin (a potent carcinogen) production were reduced by 44% and 72%, respectively in an AA-nonproducing A. flavus mutant compared to a wild-type (non-mutant) strain. Production of aspergillic acid can be used to screen native A. flavus field isolates and, potential non-aflatoxigenic A. flavus biocontrol strains for desired aggressiveness and fitness traits that correlate with their ability to produce aspergillic acid.

- **The impact of atmospheric carbon dioxide, temperature and water levels on Aspergillus flavus aflatoxin production.** The influence of environmental stress related to increased CO2, temperature and water activity is an emerging food safety and security concern and will undoubtedly impact mycotoxin contamination of food and feed crops worldwide. Chemical analyses of infected corn kernels demonstrated that A. flavus aflatoxin production increased at elevated CO_2 levels under differing temperature and moisture conditions. Additionally, several gene networks controlling fungal biological processes such as DNA replication, amino acid biosynthesis and reproduction were also affected by elevated CO_2 levels. These data will be used by computer modelers to predict the ability of A. flavus to grow and produce aflatoxins in crops such as corn under different environmental scenarios. Computer modeling will also provide insight as to how remediation efforts, such as application of atoxigenic biocontrol strains, will be influenced by predicted future global environmental conditions.

Outcomes and Impact

- Demonstrated that expression of two regulatory genes affect the ability of the A. flavus to contaminate.
- Determined that a secondary metabolite gene cluster in A. flavus was responsible for the production of aspergillic acid (AA). Production of AA may aid in fungus colonization.
- Determined that production of AA can be used to screen A. flavus isolates for non-aflatoxigenic A. flavus biocontrol use.
- Demonstrated that A. flavus aflatoxin production increased at elevated CO_2 levels under differing temperature and moisture conditions.
- Developed models to predict A. flavus (toxigenic/and non-toxigenic-biocontrol strain) growth under different environmental scenarios.

Examples of Relevant Publications

- Gilbert, M.K., Mack, B.M., Wei, Q., Bland, J.M., Bhatnagar, D., Cary, J.W. 2016. RNA sequencing of an nsdC mutant reveals global regulation of secondary metabolic gene clusters in Aspergillus flavus. Microbiological Research. 182:150-161. https://doi: 10.1016/j.micres.2015.08.007.
- Lebar, M.D., Cary, J.W., Majumdar, R., Carter-Wientjes, C.H., Mack, B.M., Wei, Q., Uka, V., De Saeger, S., Diana Di Mavungu, J. 2018. Identification and functional analysis of the aspergillic acid gene cluster in Aspergillus flavus. Fungal Genetics and Biology. 116:14-23. https://doi: 10.1016/j.fgb.2018.04.009.
- Gilbert, M.K., Medina, A., Mack, B.M., Lebar, M.D., Rodriguez, A., Bhatnagar, D., Magan, N., Obrian, G., Payne, G. 2018. Carbon dioxide mediates the response to temperature and water activity levels in Aspergillus flavus during infection of maize kernels. Toxins. 10(1):5. https://doi:10.3390/toxins10010005.
- Chang, P.-K., Zhang, Q., Scharfenstein, L.L., Mack, B.M., Yoshimi, A., Miyazawa, K., Abe, K. 2018. Aspergillus flavus GPI-anchored protein-encoding ecm33 has a role in growth, development, aflatoxin biosynthesis, and maize infection. Applied Microbiology and Biotechnology. 102(12):5209-5220. https://doi: 10.1007/s00253-018-9012-7.
- Majumdar, R., Lebar, M.D., Mack, B.M., Minocha, R., Minocha, S., Carter-Wientjes, C.H., Sickler, C.M., Rajasekaran, K., Cary, J.W. 2018. The Aspergillus flavus spermidine synthase (spds) gene, is required for normal development, aflatoxin production, and pathogenesis during infection of maize kernels. Frontiers in Plant Science. 9:317. https://doi.org/10.3389/fpls.2018.00317.
- Lebar, M.D., Mack, B.M., Carter-Wientjes, C.H., Gilbert, M.K. 2019. The aspergillic acid biosynthetic gene cluster predicts neoaspergillic acid production in Aspergillus section Circumdati. World Mycotoxin Journal. 12(3):213-222. doi.org/10.3920/WMJ2018.2397.

The (Rajasekaran) project objectives were to: (1) Develop aflatoxin-resistant corn with enhanced resistance traits against other mycotoxins and drought tolerance. Identify gene regulatory factors, networks and pathways related to resistance-associated proteins (RAPs). These data are then transferred to others to assist in selection by marker-assisted breeding;(2) Identify resistance associated protein (RAPs) genes from corn and cotton using transcriptomic analyses of the Aspergillus flavus-host plant interaction and evaluate for control of fungal growth and aflatoxin contamination; (3) Develop and evaluate transgenic corn and cotton containing over-expressed identified RAP genes (Objectives 1 and 2) or with RNA interference (RNAi)-based silencing of Aspergillus flavus genes critical to growth and aflatoxin production; and (4) Advance and license the rapid, non-destructive hyperspectral imaging technology; develop and evaluate instruments suitable for different user platforms.

Examples of Accomplishments

- **Development of aflatoxin and fumonisin-tolerant corn lines.** In collaboration with the International Institute of Tropical Agriculture (IITA), Nigeria developed six corn varieties (TZAR101-106) with resistance to contamination by the aflatoxin producing fungus, Aspergillus flavus were developed. In field trials these six lines also demonstrated resistance to another fungus, Fusarium, responsible for producing fumonisin. Using these lines several drought tolerant varieties were also developed by IITA that will be used by growers in African countries to reduce the incidence of aflatoxin and fumonisin contamination in corn. Moreover, several new hybrids developed at IITA are under analysis to establish the genetic basis for resistance.

- **Host-pathogen interaction studies identified pathogen-responsive genes in cottonseed and corn kernels.** A genome-wide gene expression profiling study in cotton was completed which identified several key genes turned on during infection of A. flavus in cottonseed. A comparative gene expression analysis was also performed that identified common genes that were significantly differentially expressed in cotton, corn, and peanut in response to A. flavus. Studies identified 26 genes common across all three crops that were considered candidate A. flavus resistance genes, which could be used to improve resistance to aflatoxin resistance in susceptible crops. Selected genes are being overexpressed in cotton for further analysis. A similar study has also been completed with A. flavus-infected resistant (TZAR 102 or MI 82) or susceptible (Va 35) corn kernels and this analysis will shed light specifically on the mechanisms of fungal pathogenesis and corn resistance.

- **Aflatoxin-resistance conferred by the expression of foreign gene(s)**: Transgenic corn plants were regenerated expressing an antifungal protein from hyacinth beans that inhibits alpha-amylase in A. flavus. Kernels transgenic corn plants and their progenies were screened for their ability to withstand fungal infection and toxin production. Significant reduction was obtained in fungal growth and aflatoxin production (63-88%). Similarly, also developed transgenic corn lines expressing a synthetic antifungal peptide AGM 182, modeled after an antimicrobial peptide from horseshoe crab that is not toxic to animals and humans. Transgenic corn kernels expressing AGM 182 demonstrated a significant reduction in fungal growth and aflatoxin contamination (76-98% reduction). Transgenic corn lines with resistance to aflatoxin contamination will serve as a valuable germplasm for breeding new hybrids. Based on this work, a new CRADA was established to develop corn lines with new antifungal peptides and to improve nutritional value for use as swine/poultry feed.

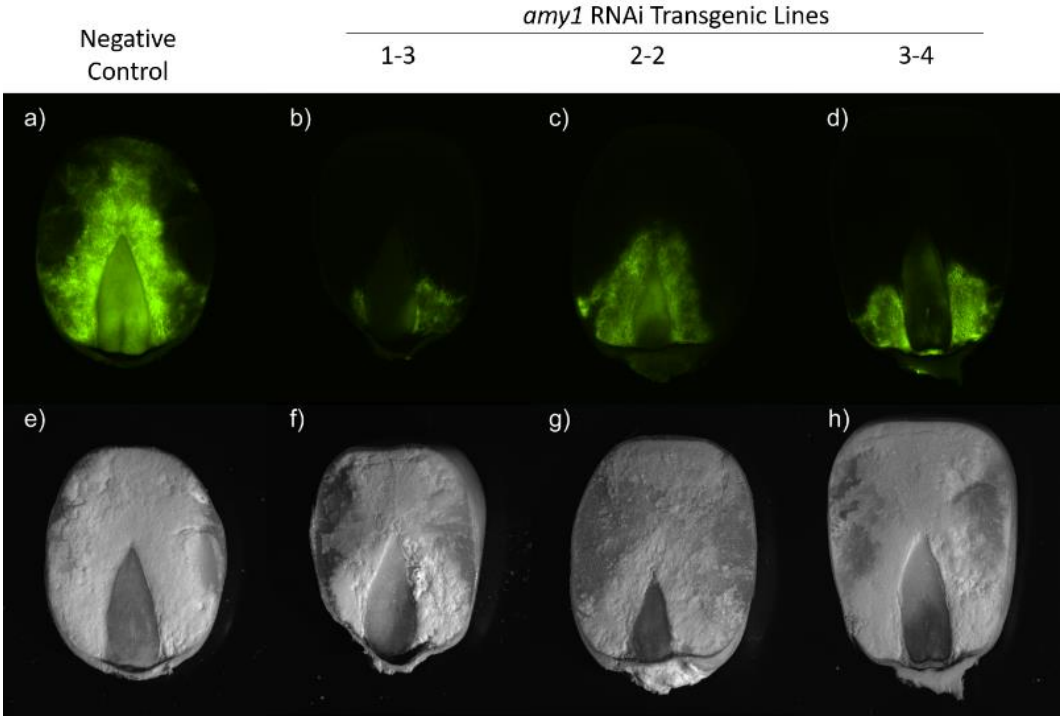

Figure. Transgenic maize plants were generated with RNAi constructs to silence A. flavus α-amylase (amy1). Representative maize kernels from lines expressing the amy1 RNAi construct emit lower fluorescence than a negative control line after infection with an A. flavus strain expressing GFP. Top Row, a-d; fluorescence of the GFP indicates relative growth of the fungus in kernel. The negative control line (a) exhibits higher levels of GFP fluorescence than the amy1 RNA lines (b through d). Bottom Row, (e through h); a light image shows longitudinal sections of the same kernels illustrating GFP fluorescence above.

- **Host-induced silencing of fungal genes to reduce Aspergillus flavus growth and aflatoxin production.** Transgenic corn plants were generated in which gene sequences were introduced to silence the A. flavus α-amylase gene, which is essential for the fungus' ability to colonize and contaminate the crop with aflatoxins. As a result, fungal growth and aflatoxin production were significantly reduced (up to 98%). Additional experiments have generated transgenic corn targeting key fungal genes (identified in the Cary project) required for development and aflatoxin/CPA production for silencing. Corn plants carrying these genes will serve as an excellent parent material to transfer the resistant trait to other commercial varieties.

- **Corn genotypes resistant to aflatoxin contamination have higher levels of polyamines than susceptible lines:** The role of polyamines (PAs) in resistance of corn lines to A. flavus infection and aflatoxin production was determined. Analysis of PA content in both resistant and susceptible corn kernels indicated that resistant varieties showed higher expression of PA biosynthetic genes upon A. flavus infection compared to the susceptible control kernels. The resistant lines accumulated higher amounts of PAs such as spermidine, spermine and specific antimicrobial PA conjugates; showed altered amino acid content and had lower levels of fungal load and aflatoxin contamination.

These results provide a valid approach to controlling A. flavus growth and aflatoxin production in corn through modulation of PA biosynthesis.

- **Detection of aflatoxin contamination using a dual-camera based multispectral imaging system.** Current screening methods for aflatoxin contamination in food and feed products is wrought with detection and sampling problems and the analysis by chemical methods is a time-consuming process. A prototype dual-camera based multispectral imaging system for the purpose of rapidly screening aflatoxin contamination has been designed, developed, and evaluated (U.S. Patent). This technology enables rapid and non-destructive spectral-based detection of aflatoxin contamination and removal in corn to provide toxin-free food supply. Recently, a tablet-based sorting device equipped with UV-LED light source has been developed for use in developing countries to detect aflatoxin-contaminated corn kernels.

- **Peanut lines resistant to aflatoxin contamination:** Peanuts are one of the most important protein-rich edible food around the world. In collaboration with ICRISAT, Donald Danforth Center, and Louisiana State University transgenic peanut lines that are nearly immune to preharvest aflatoxin contamination by A. flavus were produced. Studies identified the target genes critical to fungal growth and toxin production and advised and provided the proper gene constructs to ICRISAT for RNA interference-mediated silencing of fungal genes to reduce fungal growth and toxin production in peanuts.

Outcomes and Impacts

- Developed in collaboration with International Institute of Tropical Agriculture (IITA), Nigeria six corn varieties with resistance to Aspergillus flavus and Fusarium. Several drought tolerant varieties were developed by IITA for growers in Africa.
- Identified key genes turned on during infection of A. flavus in cottonseed. Identified common genes that were significantly differentially expressed in cotton, corn, and peanut in response to A. flavus. Genes common were considered candidate resistance genes, used to improve resistance to aflatoxin in susceptible crops.
- Developed transgenic corn lines expressing a synthetic antifungal peptide. Demonstrated that expressing the peptide in planta significantly reduced fungal growth and aflatoxin contamination.
- Developed transgenic corn lines with resistance to aflatoxin contamination. Implemented a CRADA to develop corn lines with new antifungal peptides and to improve nutritional value for use as swine/poultry feed.
- Generated transgenic corn plants to silence the A. flavus α-amylase gene, essential for the ability to colonize and contaminate the crop with aflatoxins. Plants carrying these genes will serve as parent material to transfer the resistant trait to commercial varieties.
- Determined the role of polyamines (PAs) in resistance of corn lines to A. flavus infection and aflatoxin production. Modulation of PA biosynthesis may provide an approach to controlling growth and aflatoxin production.

- Designed, developed, and evaluated (U.S. Patent) a prototype dual-camera based multispectral imaging system for the purpose of rapidly screening aflatoxin contamination.
- Developed in collaboration with ICRISAT, Donald Danforth Center, and Louisiana State University transgenic peanut lines that are nearly immune to preharvest aflatoxin contamination by A. flavus. Reducing fungal growth and toxin production in peanut, is critical as it is one of the most important protein-rich edible foods.

Examples of Relevant Publications

- Bedre R, Rajasekaran K, Mangu VR, Sanchez Timm LE, Bhatnagar D, Baisakh N (2015) Genome-wide transcriptome analysis of cotton *Gossypium hirsutum* L. identifies candidate gene signatures in response to aflatoxin producing fungus *Aspergillus flavus*. PLoS ONE 10 (9):e0138025. https://doi:10.1371/journal.pone.0138025
- Brown R, Williams W, Windham G, Menkir A, Chen Z-Y (2016) Evaluation of African-bred maize germplasm lines for resistance to aflatoxin accumulation. Agronomy 6 (2):24. https://doi:10.3390/agronomy6020024
- Sharma KK, Pothana A, Prasad K, Shah D, Kaur J, Bhatnagar D, Chen ZY, Raruang Y, Cary JW, Rajasekaran K, Sudini HK, Bhatnagar-Mathur P (2017) Peanuts that keep aflatoxin at bay: a threshold that matters. Plant Biotechnol J.:16: 1024-1033. https://doi:10.1111/pbi.12846.
- Mehanathan M, Bedre R, Mangu V, Rajasekaran K, Bhatnagar D, Baisakh N (2018) Identification of candidate resistance genes of cotton against *Aspergillus flavus* infection using a comparative transcriptomics approach. Physiol Mol Biol Plants 24 (3):513-519. https://doi:10.1007/s12298-018-0522-7
- Majumdar R, Lebar M, Mack B, Minocha R, Minocha S, Carter-Wientjes C, Sickler C, Rajasekaran K, Cary JW (2018) The *Aspergillus flavus* spermidine synthase (*spds*) gene, is required for normal development, aflatoxin production, and pathogenesis during infection of maize kernels. Frontiers in Plant Science 9:317. https://doi:10.3389/fpls.2018.00317.
- Gilbert MK, Majumdar R, Rajasekaran K, Chen ZY, Wei Q, Sickler CM, Lebar MD, Cary JW, Frame BR, Wang K (2018) RNA interference-based silencing of the a-amylase (*amy1*) gene in *Aspergillus flavus* decreases fungal growth and aflatoxin production in maize kernels. Planta 247 (6):1465-1473. https://doi:10.1007/s00425-018-2875-0
- Rajasekaran K, Sayler RJ, Sickler CM, Majumdar R, Jaynes JM, Cary JW (2018) Control of *Aspergillus flavus* growth and aflatoxin production in transgenic maize kernels expressing a tachyplesin-derived synthetic peptide, AGM182. Plant Sci 270:150-156. https://doi:10.1016/j.plantsci.2018.02.006
- Rajasekaran K, Sayler RJ, Majumdar R, Sickler C, Cary JW (2019) Inhibition of *Aspergillus flavus* growth and aflatoxin production in transgenic maize expressing the α-amylase inhibitor from *Lablab purpureus* L. Journal of Visualized Experiments 144:e59169. https://dx.doi.org/10.3791/59169

- Majumdar R, Minocha R, Lebar MD, Rajasekaran K, Long S, Carter-Wientjes C, Minocha S, Cary JW (2019) Contribution of maize polyamine and amino acid metabolism toward resistance against *Aspergillus flavus* infection and aflatoxin production. Front Plant Sci 10:692. https://doi:10.3389/fpls.2019.00692.
- Han, D., Yao, H., Hruska, Z., Kincaid, R., Ramezanpour, C., Rajasekaran, K. and Bhatnagar, D. 2019. Development of high-speed dual-camera system for batch screening of aflatoxin contamination of corn using multispectral fluorescence imaging. Transactions of the ASABE 62(2): 381-391. 2019. https://doi.org/10.13031/trans.13125.
- Tao F, Yao H, Hruska Z, Liu Y, Rajasekaran K, Bhatnagar D (2019) Use of Visible-Near-Infrared (Vis-NIR) Spectroscopy to Detect Aflatoxin B1 on Peanut Kernels. Appl Spectrosc 73 (4):415-423. https://doi:10.1177/0003702819829725.
- Tao, F., Yao, H., Zhu, F., Hruska, Z., Liu, Y., Rajasekaran, K. and Bhatnagar, D. 2019. A rapid and non-destructive method for simultaneous determination of aflatoxigenic fungus and aflatoxin contamination on corn kernels. Journal of Agricultural and Food Chemistry 67: 5230-5239. https://doi:10.1021/acs.jafc.9b01044.

The (Moore) project objectives were to: (1) Determine the mechanism by which atoxigenic strains of Aspergillus flavus reduce pre-harvest aflatoxin contamination by toxigenic strains; and (2) Determine the role of mating-type genes and climatic (environmental) stressors on the ability of Aspergillus flavus biocontrol strains to compete, survive and recombine, thereby impacting the persistence and efficacy of these strains.

Examples of Accomplishments

- **Determination of mechanisms by which Aspergillus flavus biocontrol strains reduce aflatoxin contamination.** The mechanism of biocontrol, and reduction of aflatoxin afforded by atoxigenic A. flavus strains, is not well understood. This study explored reduction of aflatoxin production in response to fungal extrolites (compounds excreted by the atoxigenic fungus). At least one uncharacterized atoxigenic extrolite reduced aflatoxin B1 production (61-78%) by three aflatoxigenic strains on solid YES medium, and reduced cyclopiazonic acid production by 54-68%. LC-MS of spent broth from growth of the atoxigenic strain revealed two unidentified metabolites that may be inhibitory extrolites. The efficacy of biocontrol strains and their ability to produce the inhibitory extrolite(s) can be used to screen for improved efficacy.

Outcomes and Impacts

- Determined that a fungal extrolite reduced aflatoxin B1 production by three aflatoxigenic strains. Biocontrol strain production of extrolite(s) can be used to screen for improved efficacy.

Example of Relevant Publications

- Moore, G.G., Lebar, M.D., Carter-Wientjes, C.H. 2018. The role of extrolites secreted by nonaflatoxigenic *Aspergillus flavus* in biocontrol efficacy. Journal of Applied Microbiology. 126:1257-1264. https://doi.org/10.1111/jam.14175.

Studies at RRRC, Athens, GA (Glenn) focused on Fusarium research: The project's research objectives were to: (1) Determine the evolutionary history and molecular genetics of metabolic and developmental features enhancing the fitness of mycotoxigenic Fusarium species, including such areas as xenobiotic tolerance, denitrification, and nitric oxide detoxification and the contribution to greenhouse gas emission; (2) Evaluate the influence of a common niche on the evolution and adaptation of two co-occurring, seed-borne, metabolically active maize endophytes, Acremonium zeae and Fusarium verticillioides; and (3) Develop and improve control strategies for mycotoxin contamination by targeting fungal-specific enzymatic activities, using molecular technologies such as host-induced gene silencing.

Examples of Accomplishments

- **Suppressing production of fumonisin mycotoxins.** A group of metabolites called pyrrocidines that are produced by the corn kernel inhabiting fungus Sarocladium zeae were found to inhibit the production of fumonisin mycotoxins by Fusarium verticillioides. The potential use of pyrrocidines as inhibitors of fumonisin production is being investigated further and could have immense impact on the safety and value of corn and its processed products by reducing and managing fumonisin contamination.

- **Fusarium species are capable of denitrification.** Genomic and gene transcription studies have shown that Fusarium verticillioides and other Fusarium species have the genetic machinery encoding the physiological pathway for denitrification. This coincides with published studies indicating Fusarium species are among the most frequent denitrifying fungi isolated from soils and thus may be significant contributors to this nitrogen respiration pathway responsible for loss of nitrogen from soil and emission of nitrous oxide to the atmosphere.

- **Utilizing lactamases for resistance to antimicrobials.** The fungal corn pathogen Fusarium verticillioides possesses the gene MBL1 encoding a hydrolytic lactamase that is essential for tolerance to antimicrobial compounds produced by corn plants as a chemical defense strategy. Detoxification of these corn defensive phytochemicals is believed to give F. verticillioides a significant competitive advantage for infecting corn compared to other intolerant fungi. An analysis of fungal genomes found that soil-associated fungi, including a range of Fusarium species, have many lactamase-encoding genes (F. verticillioides has 46 genes; F. solani has 88 genes). The complex soil environment, with its abundance of xenobiotic chemical compounds, is correlated with a dramatic expansion of the lactamase gene family in fungi, presumably so that they can degrade the various antimicrobial compounds encountered.

- **Horizontal gene transfer expands fungal metabolic activity.** Both fungal-to-fungal and bacterial-to-fungal horizontal transfer of genes was documented for Fusarium verticillioides and other fungal corn pathogens. These horizontal transfer events are thought to result from exposure of the fungi to various physiological challenges and demonstrate how fungal genomes are not fixed but expand to facilitate metabolic adaptations to cope with these challenges. One practical example is that protective antimicrobial phytochemicals produced by corn have apparently served as a major evolutionary factor influencing the horizontal transfer of a gene cluster involved in the degradation of the corn compounds. This gene cluster has been found in three different fungal genera that infect corn and may enhance the fitness of the fungi.

- **Mycotoxins as signaling compounds impacting bacterial quorum sensing.** Mycotoxins and other fungal secondary metabolites were found to inhibit bacterial quorum sensing, thus limiting the growth and other biological features of the bacteria. Quorum sensing is a microbial mechanism used to synchronize all physiological activities within a bacterial population in order to enhance their ecological fitness capabilities, and we now have greater understanding of how Fusarium verticillioides may modulate bacterial populations.

Outcomes and Impact

- Discovered a group of chemical compounds that inhibit the production of fumonisins by F. verticillioides. These are the pyrrocidines produced by another corn kernel inhabiting fungus, Sarocladium zeae.
- Determined that Fusarium verticillioides is among a limited number of soil fungi having the full set of denitrification genes that can convert nitrate into nitrous oxide and whose overall fitness may be related to this conversion. The genes responsible were functionally characterized with the goal of identifying inhibitors of denitrification as a strategy to reduce global nitrous oxide emissions and reduce the fitness of F. verticillioides.
- Determined that Fusarium verticillioides possesses 46 genes putatively encoding lactamases that are hypothesized to confer tolerance to antifungal compounds produced by corn or compounds produced by competitor microbes in the cornfield soil environment. Using a gene deletion strategy developed by ARS, mutants were created allowing the testing of various lactam compounds for their antifungal effects and the identification of what genes confer resistance to the lactams.
- Discovered the presence and structural conservation of a gene cluster in Fusarium verticillioides that supports the hypothesis that corn, as the host is a driving force impacting the evolution of the genomes. Acquisition many enhance fitness by conferring the ability to degrade antifungal phytochemicals produced by corn.
- Discovered that some fungal secondary metabolites, some of which are known mycotoxins, may function as quorum sensing inhibitors. Thus, fungi have the potential to affect a biocontrol bacterium's performance via suppression of quorum sensing mechanisms. Understanding this interaction is vital for better biocontrol exploitation.

Examples of Relevant Publications

- Glenn, A.E., Davis, C.B., Gao, M., Gold, S.E., Mitchell, T.R., Proctor, R.H., Stewart, J.E., and Snook, M.E. 2016. Two horizontally transferred xenobiotic resistance gene clusters associated with detoxification of benzoxazolinones by *Fusarium* species. PLoS ONE 11(1): e0147486. https://doi:10.1371/journal.pone.0147486.
- Bacon, C.W., Hinton, D.M., and Mitchell, T.R. 2017. Is quorum signaling by mycotoxins a new risk-mitigating strategy for bacterial biocontrol of *Fusarium verticillioides* and other endophytic fungal species? *Journal of Agricultural & Food Chemistry* 65:7071-7080. https://doi:10.1021/acs.jafc.6b03861.
- Gao, M., Glenn, A.E., Blacutt, A.A., and Gold, S.E. 2017. Fungal lactamases: their occurrence and function. *Frontiers in Microbiology* 8:1775. https://doi:10.3389/fmicb.2017.01775.
- Gao, S., Gold, S.E., and Glenn, A.E. 2018. Characterization of two catalase-peroxidase-encoding genes in *Fusarium verticillioides* reveals differential responses to *in vitro* versus *in planta* oxidative challenges. *Molecular Plant Pathology* 19:1127-1139. https://doi:10.1111/mpp.12591.
- Blacutt, A.A., Gold, S.E., Voss, K.A., Gao, M., and Glenn, A.E. 2018. *Fusarium verticillioides*: Advancements in understanding the toxicity, virulence, and niche adaptations of a model mycotoxigenic pathogen of maize. *Phytopathology* 108:312-326. https://doi:10.1094/PHYTO-06-17-0203-RVW.
- Gao, S., Gold, S.E., Wisecaver, J.H., Zhang, Y., Guo, L., Ma, L-J., Rokas, A., and Glenn, A.E. 2019. Genome-wide analysis of *Fusarium verticillioides* reveals inter-kingdom contribution of horizontal gene transfer to the expansion of metabolism. *Fungal Genetics and Biology* 128:60-73. https://doi.org/10.1016/j.fgb.2019.04.002
- Bacon, C.W., and Hinton, D.M. 2019. Fungal and bacterial maize kernel interactions with the vertically transmitted endophytic state of *Fusarium verticillioides*. Pp. 191-209. In: Verma S., White, Jr J. (eds) Seed Endophytes. Springer, Cham, Switzerland. https://doi.org/10.1007/978-3-030-10504-4_10.

Chemical and Biological Contaminants: Methodology

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR); https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
The economic losses that result from fungal growth in foods and feeds and the subsequent contamination by mycotoxins is substantial. These include losses to the value of affected commodities and foods, losses from livestock diseases, and losses from the costs of monitoring programs that are needed to prevent exposure to mycotoxins. These efforts specifically require development of detection methods for monitoring both mycotoxins and their derived toxins (masked mycotoxins). Studies were aimed at improving or developing methods through the application of new detection technologies, including those based upon biosensors and mass spectrometry. Work focused on the development of multi-toxin assays. Improvements in detection are enabled through the availability of better toxin-binding materials upon which

they are based. To that end, applications in chemistry (analytical, synthetic) and biology (computational, synthetic) improved materials capable of binding mycotoxins. Binding materials also have the potential to be used for remediation of the toxins, for example, developing and applying novel materials for removing toxicants from beverages.

The (Maragos) project at NCAUR, Peoria, IL objectives were to: (1) Improve detection of foodborne toxins through development of novel technologies based upon biosensor platforms and new component materials; (2) Improve detection of foodborne toxins through development of direct detection technologies based upon novel mass-spectrometric platforms; (3) Improve the ability to detect and measure "masked" mycotoxins and biomarkers of mycotoxin exposure in commodities and foods; and (4) Improve toxin detection methods and reduce exposure through the development and application of synthetic materials.

Examples of Accomplishments

- **Rapid methods for detecting the economically important mycotoxins in commodities.** ARS developed a method for detecting three types of toxins simultaneously in wheat using novel biosensor technology known as imaging surface plasmon resonance (iSPR). The technology allows for all three types of toxins to be monitored simultaneously, reducing the time required to test for all three groups of toxins, an important benefit for screening many samples during and after harvest.

- **Rapid methods for detecting masked mycotoxins.** One way that plants mitigate the toxicity of fungal toxins (mycotoxins) is through metabolism. This includes appending sugar residues, which help the plant to sequester the toxin. Animals consume the plants and during digestion certain of the modified forms can be converted back to toxins. ARS developed materials and methods that permitted the detection of these so-called "masked" mycotoxins in corn and wheat. In the case of the fumonisin mycotoxins the researchers developed screening assays (immunoassays) and chromatographic assays (LC-MS) that measured the fumonisins and their masked forms in corn. The LC-MS method was used, in collaboration with researchers at the University of Aberdeen, to demonstrate that the fumonisins were regenerated from their masked forms in an "artificial gut" system. In the case of a second group of toxins (trichothecenes) the same researchers developed a novel sensor based upon imaging surface plasmon resonance (iSPR) to detect low levels of both the initial toxin (T-2 toxin) and its metabolite (T-2-glucoside) in wheat.

- **Rapid detection of emerging mycotoxins.** While certain mycotoxins are known to have significant economic and food safety impacts, there are many more mycotoxins for which the toxicity is known and the occurrence and impact upon safety are not established. These include a neurotoxin produced by certain fungi in corn: cyclopiazonic acid (CPA), which has been shown to co-occur with the more widely known aflatoxins. ARS developed antibodies and a rapid screening immunoassay to detect CPA in maize and to help shed light on CPA possible mechanism of action as a toxin. Studies also

developed a novel way to render CPA fluorescent using lanthanide metals, which greatly facilitated detection of low levels of the mycotoxin.

- **Development of nanomaterials to assist in toxin detection.** A nano-porous material designed to help isolate the mycotoxin ochratoxin A (OTA) in contaminated fruit juice and wine was developed for application in detection methods. A material to selectively isolate OTA from beverages was designed using computational chemistry and machine learning based quantitative structure activity relationship techniques. Analysis of the material through atomic force microscopy indicated nano-sized morphological features that enabled removal of beverage matrix components that interfere with accurate toxin detection. Application of the material in sample clean-up followed by HPLC-fluorescence detection provided a faster, quantitative method that could detect OTA within the ranges of regulated levels.

Outcomes and Impact

- Developed using imaging surface plasmon resonance (iSPR), a novel biosensor for the simultaneous detection of all three types of mycotoxins reducing time and cost
- Developed and validated new materials and methods for detecting masked mycotoxins. Validation was established according to criteria established by the European Union to detect both the primary toxin and its metabolite at levels that ensure the safety of U.S. grain exports.
- Developed antibodies and methods to detect cyclopiazonic acid (CPA); and a novel way to render CPA fluorescent using lanthanide metals, which greatly facilitated CPA detection.
- The ability to visualize CPA as a fluorescent complex assists to determine how the toxin interacts with metals, which are key components of certain enzymes, thereby revealing insights into the potential mechanism whereby CPA exerts its toxic effects, possible mechanism of action, and significance for human health.
- Significant advances were made in the application of machine learning technology to determine more reliable detection properties for foodborne-toxins, including regulated mycotoxins and pesticides. Developed new quantitative structure-activity relationship algorithms through a CRADA with an industrial partner.
- ARS and collaborators from the National Taiwan University applied machine learning technology to identify chemical characteristics for more selective and accurate detection of alternariol and related toxins to address a mandate for more reliable detection of these toxins.

Examples of Relevant Publications

- Maragos, C.M., Sieve, K.K., Bobell, J. 2017. Detection of cyclopiazonic acid (CPA) in maize by immunoassay. Mycotoxin Research. 33(2):157-165.
- Hossain, M., Maragos, C.M. 2017. Gold nanoparticle-enhanced multiplexed imaging surface plasmon resonance (iSPR) detection of Fusarium mycotoxins in wheat. Biosensors and Bioelectronics. 101:246-253. Log No. 343654
- Appell, M., Evans, K.O., Jackson, M.A., Compton, D.L. 2018. Determination of ochratoxin A in grape juice and wine using nanosponge solid phase extraction clean-up and liquid chromatography with fluorescence detection. Journal of Liquid Chromatography & Related Technologies. 41(15-16): 949-954.
- Maragos, C.M., Sieve, K., and Busman, M. 2018. Development of antibodies for N-(1-deoxy-D-fructos-1-yl) fumonisin B_1 and cross-reaction with modified fumonisins. World Mycotoxin Journal 11(4):493-502.
- Hossain, M.Z, McCormick, S.P., Maragos, C.M., 2018. An imaging surface plasmon resonance biosensor assay for the detection of T-2 toxin and masked T-2 toxin-3-glucoside in wheat. Toxins. 10(3)119.
- Maragos, C.M. 2018. Complexation of the mycotoxin cyclopiazonic acid with lanthanides yields luminescent products. Toxins. 10(7):285. https://doi.org/10.3390/toxins10070285.
- Hossain, Md. Z., and Maragos, C.M. 2019. Coordination of mycotoxins with lanthanides in luminescent complexes. Mycotoxin Research 35(3):279-292.
- Hossain, Md. Z., Busman, M., and Maragos, C.M. 2019. Immunoassay utilizing imaging surface plasmon resonance for the detection of cyclopiazonic acid (CPA) in maize and cheese. Analytical and Bioanalytical Chemistry 411(16):3543-3552.

Chemical and Biological Contaminants: Bacterial and Plant Toxins

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR); https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf *Foodborne bacterial disease may be caused by toxins produced by bacteria or in some cases by plants. Most toxins are proteins (exotoxins or endotoxins) that act enzymatically or through direct action upon the host, stimulating a variety of immediate/semi-immediate responses, for example: severe abdominal pain; vomiting; diarrhea and bloody diarrhea; various neurological disorders; massive immune responses due to superantigenic activity; brachycardia/hypertension; paralysis; respiratory arrest; numbness; burning of lips, tongue, throat and fingers; ataxia; staggers; dry-throat and skin; muscular aches; difficulty in speaking; asphyxiation due to respiratory paralysis, often resulting in death; and death. Then there are long-term sequelae (often life-time in length) which may include: rheumatoid disease; autoimmune thyroid disease; inflammatory bowel disease; autoimmunity; renal disease; neural/neuromuscular disorders; organ impairment; heart/vascular disease; nutritional/ gastrointestinal issues; and various personality changes.*

Just as evolution has provided bacteria and plants with survival mechanisms to evade host defenses, their toxins have gained enhanced stability, permitting their activities to persist after even extreme food processing interventions (heat, acid) that are often adequate for killing the bacteria/plant. Many of these toxins exhibit oral bioavailability enough for significant absorption and can cause disease following ingestion. Some toxins are listed as Select Agents (42CFR73) because of their importance not only to food safety but also to biosecurity. Although toxin-producing bacteria are almost ubiquitous and for the most part, are readily detected to certain levels, sensitive toxin detection methods/assays are critically needed since often the amount of toxin required to elicit a response may be at the nanogram level or less. Further, in developing these methods, biomolecules (antibodies) were often developed that bound to the toxins exhibiting inhibitory/neutralization activity. These molecules could potentially serve as medical interventions for incapacitated persons.

This research was conducted at WRRC, Albany, CA (Cheng) where the objectives were to: (1) Advance the development of structure- and activity-based detection methods for protein toxins; (2) Advancing the development of detection methods for non-bacterial toxins; (3) Assessing foodborne risks through examination of toxin stability and bioavailability in relation to intrinsic and extrinsic stresses; and (4) Advance the development of instrumental, portable, and field-deployable testing methods.

Examples of Accomplishments

- **Development of a sensitive detection assay that can identify all known subtypes of Shiga toxins.** Shiga toxin producing E. coli (STEC) are known to produce two Shiga toxins types (Stx 1 and 2), which are further classified into many subtypes. However, there is little information on some of these subtypes and few, if any reagents are available for use in their detection which could result in missed diagnosis. ARS developed a novel universal sandwich ELISA capable of detecting all known subtypes of Stx1 and Stx2 (including Stx1a, 1c, 1d, and Stx2a through 2g). The ELISA assays were highly sensitive, with limits of detection of 10 to 50 pg/mL in phosphate buffered saline, was able to correctly indicate STEC contamination in culture fluids, or even from single bacterial colonies on agar plates without lengthy enrichment in liquid medium and can identify ground beef contamination by a single bacterium. The reagents have been patented and licensed to commercial entities that will manufacture new detection kits. This work lead to a Federal Laboratory Consortium (Far West Region) research award.

Fast and sensitive immunoassays using antibodies against shiga toxins capable of detecting all known subtypes

TechLab Shiga Toxin Quik Chek Assay

- **Development of new sensitive detection assay kits for Shiga toxins produced by non-Escherichia coli.** Shiga toxins (Stx) are the main disease factors for foodborne illnesses caused by pathogenic Escherichia coli. Many new Stx subtypes, including an atypical one made by Enterobacter cloacae have recently been identified. Currently available detection methods, however, do not recognize these newly emerging subtypes of Shiga toxins especially 1e from non-E. coli strains. ARS developed new monoclonal antibodies against Stx1e from Enterobacter cloacae. The technology was transferred to a commercial partner and a new sensitive detection kit capable of detecting all subtypes of Stx1 is now available.

- **Development of sensitive detection assays for abrin toxin.** Abrin is a Select Agent toxin and a potential bioterror weapon. ARS developed new monoclonal antibodies against abrin and assembled a sandwich ELISA capable of detecting a mixture of abrin isoforms. The ELISA can detect as little as 1 nanogram/milliliter of the abrin in phosphate-buffered saline, nonfat milk, and whole milk, significantly below concentrations that would pose a health concern for consumers. Some antibodies can also neutralize abrin toxicity in cell-based assays, and are under consideration for humanization as an intoxication medical intervention.

- **Development of novel antibodies to botulinum neurotoxin serotype E.** Botulinum neurotoxin serotype E (BoNT/E) outbreaks are most often observed in northern coastal regions and are associated with eating contaminated marine animals and other fishery products. ARS developed new mouse monoclonal antibodies for the sensitive detection of BoNT/E. Sandwich enzyme-linked immunosorbent assays (ELISAs) using these reagents detected a little as 0.2 ng/ml in standard buffer matrix and 10 ng/mL in fish product matrices. Development of sensitive and selective mAbs to BoNT/E would help in the initial screening of potential food contamination, speeding diagnosis and reducing the need to use animal bioassays.

- **In vitro methods to detect and quantify active staphylococcal enterotoxins (SE) types D and E toxins as an alternative to animal bioassays.** Food poisoning caused by staphylococcal enterotoxins is among the leading causes of food-borne outbreaks. Current methods only detect the presence of toxins but do not distinguish between inactive and active toxins, the form that causes disease. The current methods for the of detection of active SEs is an emetic in vivo animal bioassay; however, this expensive procedure has low sensitivity and poor reproducibility, requires many animals and is impractical to test on many samples. ARS developed a robust and rapid cell-based assay using genetically engineered T cell-lines expressing the luciferase reporter gene in combination with B-cell lines for presentation of the toxin. Presence of active staphylococcal enterotoxin types D and E will lead to a bioluminescence response that is toxin specific and concentration dependent. As little as 100 ng/mL of active SED and 1 fg/ml of SEE can be detected, providing a significant improvement in sensitivity compared to animal bioassays.

- **Identification of probiotic bacteria that inhibit botulinum neurotoxin (BoNT) intestinal cell binding.** Probiotic microorganisms have been extensively studied for their beneficial effects in protection from allergens, pathogens, and toxins. Studies evaluated the effect of probiotic bacteria on the intestinal binding and absorption of BoNT serotype A. BoNTs are some of the most poisonous natural toxins known to man and are threats to public health and safety. Several probiotics that were tested (Saccharomyces boulardii, Lactobacillus acidophilus, Lactobacillus rhamnosus LGG, and Lactobacillus reuteri) blocked BoNT/A intestinal uptake in a dose-dependent manner whereas a non-probiotic strain of Escherichia coli did not. These results show, for the first time, that probiotic treatment can inhibit BoNT/A binding and internalization in intestinal cells and may lead to the development of new therapies.

- **Effects of food processing on abrin bioavailability.** Abrin is a highly potent plant toxin and a potential bioterror weapon. Studies evaluated the effectiveness of common food processing and pasteurization conditions against abrin in the presence or absence of foods. Selected food processing and pasteurization parameters used by processors for whole milk, non-fat milk, and liquid egg were insufficient to fully inactivate abrin activity. These studies suggest that different food matrices can have significant effects on the biological activity of abrin, information which could be used for food adulteration risk assessments.

- **Death-Cap Mushrooms**. Amatoxins are toxins found in death cap mushrooms and are lethal in very small doses. There is no cure for any intoxication, and the toxins are not destroyed by heat. ARS developed antibodies (mAbs) that sensitively and selectively bind amatoxins. These reagents have been successfully incorporated into ELISA and dipstick lateral flow immunoassays (LFA). Both the ELISA and LFA have a detection limit of 1 ng/mL, and the LFA has been used to detect amatoxins in various sample types including urine and mushroom extracts. To date, 14 different wild mushroom species have been tested using these immunoassays with 100 percent detection accuracy. These

methods and toxin extraction represent a marked improvement over currently used methods that require laboratory expertise and use of expensive instruments.

Outcomes and Impacts

- Developed a novel universal sandwich Enzyme-linked immunosorbent assay (ELISA) capable of detecting all known subtypes of Stx1 and Stx2. The reagents are patented and licensed to commercial entities for manufacture of detection kits. This work received a Federal Laboratory Consortium Award.
- Developed new monoclonal antibodies against Stx1e from Enterobacter cloacae. The technology was transferred to a commercial partner and a detection kit capable of detecting all subtypes of Stx1 is now available.
- Developed new mouse monoclonal antibodies for the sensitive detection of BoNT/E.
- Developed a robust and rapid cell-based assay for active staphylococcal enterotoxin types D and E. This technology eliminates the use of animal-based bioassays.
- Determined that probiotic treatment can inhibit BoNT/A binding and internalization in intestinal cells and may lead to the development of new therapies.
- Determined that different food matrices can have significant effects on the biological activity of abrin, information which could be used for food adulteration risk assessments.
- Developed mAbs for the detection of amatoxins. These reagents have been successfully incorporated into ELISA and dipstick lateral flow immunoassays (LFA) having a detection limit of 1 ng/mL with 100% accuracy.

Examples of Relevant Publications

- Rasooly, R., Do, P.M., Hernlem, B.J. 2016. Low cost quantitative digital imaging as an alternative to qualitative in vivo bioassays for analysis of active aflatoxin B1. Journal of Agricultural and Food Chemistry. 80:405-410.
- Rasooly, R., Do, P.M., Hernlem, B.J. 2016. Sensitive, rapid, quantitative and in vitro method for the detection of biologically active staphylococcal enterotoxin type E. Toxins. 8(5):150.
- Babrak, L.M., Lin, A.V., Stanker, L.H., McGarvey, J.A., Hnasko, R.M. 2016. Rapid microfluidic assay for the detection of botulinum neurotoxin in animal sera. Toxins. 8(1):13.
- Skinner, C.B., Patfield, S.A., Khalil, R., Kong, Q., He, X. 2016. New monoclonal antibodies against a novel subtype of Shiga toxin 1 produced by Enterobacter cloacae and their use in analysis of human serum. mSphere. 1(1):e00099-15.
- Khalil, R., Skinner, C.B., Patfield, S.A., He, X. 2016. Phage-mediated Shiga toxin (Stx) horizontal gene transfer and expression in non-Shiga toxigenic Enterobacter and Escherichia coli strains. Pathogens and Disease. 74(5):1-11.
- Lam, T.L., Tam, C.C., Stanker, L.H., Cheng, L.W. 2016. Probiotic microorganisms inhibit epithelial cell internalization of botulinum neurotoxin serotype A. Toxins. 8(12):377.

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- He, X., Kong, Q., Patfield, S.A., Skinner, C.B., Rasooly, R. 2016. A new immunoassay for detecting all subtypes of Shiga toxins produced by Shiga toxin-producing E. coli in ground beef. PLoS One. 11(1):e0148092.
- Leonardi, W., Zilbermintz, L., Cheng, L.W., et al. 2016. Bithionol blocks pathogenicity of bacterial toxins, ricin, and Zika virus. Scientific Reports. 6:34475. doi: 10.1038/srep34475.
- Rasooly, R., Do, P.M., Hernlem, B.J. 2017. Rapid cell-based assay for detection and quantification of active staphylococcal enterotoxin type D. Journal of Food Science. doi: 10.1111/1750-3841.13634.
- Gehring, A.G., Fratamico, P.M., Lee, J., Ruth, L., He, X., He, Y., Paoli, G., Stanker, L.H., Rubio, F.M. 2017. Evaluation of ELISA tests specific for Shiga toxin 1 and 2 in food and water samples. Food Control. 77:145-149.
- He, X., Patfield, S.A., Rasooly, R., Mavrici, D. 2017. Novel monoclonal antibodies against Stx1d and 1e and their use for improvement of immunoassays. Journal of Immunological Methods. 44:52-56.
- Mavrici, D., Yambao, J.C., Lee, B.G., Quinones, B., He, X. 2017. Screening for the presence of mcr-1/mcr-2 genes in Shiga toxin-producing Escherichia coli recovered from a major produce-production region in California. PLoS One. 12(11):e0187827.
- Tam, C.C., Cheng, L.W., He, X., Merrill, P.A., Hodge, D., Stanker, L.H. 2017. A monoclonal-monoclonal antibody-based capture ELISA for abrin. Toxins. 9(10):328.
- Tam, C.C., Henderson, T.D., Stanker, L.H., He, X., Cheng, L.W. 2017. Abrin toxicity and bioavailability after temperature and pH treatment. Toxins. 9(10):320.
- He, X., Patfield, S.A., Cheng, L.W., Stanker, L.H., Rasooly, R., McKeon, T.A., Zhang, Y., Brandon, D.L. 2017. Detection of abrin holotoxin using novel monoclonal antibodies. Toxins. 9(12):386.
- Rasooly, R., Do, P.M., He, X., Hernlem, B.J. 2017. TCR-Vß8 as alternative to animal testing for quantifying active SEE. Journal Environmental Analytical Toxicology. 7(6):527.
- Rasooly, R., Do, P.M., Hernlem, B.J. 2017. Interleukin 2 secretion by T cells for detection of biologically active Staphylococcal enterotoxin type E. Journal of Food Protection. 80(11):1857-1862.
- Rasooly, R., Do, P.M., Hernlem, B.J. 2017. Low cost bioluminescence imaging as an alternative to in vivo bioassays for quantifying biologically active staphylococcal enterotoxin type E. Sensors and Actuators B: Chemical. 259:357-393.
- He, X., Ardissino, G., Patfield, S.A., Cheng, L.W., Silva, C.J., Brigotti, M. 2018. An improved method for detection of Shiga toxin 2 in human serum. Toxins. 10(2):59.

- Bai, X., Fu, S., Zhang, J., Fan, R., Xu, Y., Sun, H., He, X., Xu, J., Xiong, Y. 2018. Identification and pathogenomic analysis of an Escherichia coli strain producing a novel Shiga toxin 2 subtype. Scientific Reports. 8:6756.
- Tam, C.C., Henderson II, T.D., Stanker, L.H., Cheng, L.W. 2018. Influence of food matrices on the stability and bioavailability of abrin. Toxins. 10(12):52.
- Tam, C.C., Flannery, A.R., Cheng, L.W. 2018. A rapid, sensitive, and portable biosensor assay for the detection of botulinum neurotoxin serotype A in complex food matrices. Toxins. 10(11):476.
- Rasooly, R., Do, P.M., He, X., Hernlem, B.J. 2018. Alternative to animal use for detecting biologically active Staphylococcal enterotoxin type A. Toxins. 10(12):540.
- Bever, C.R., Scotcher, M., Cheng, L.W., Hnasko, R.M., Stanker, L. 2019. Development and characterization of monoclonal antibodies to botulinum neurotoxin type E. Toxins. 11:407.
- Rasooly, R., Do, P.M., He, X., Hernlem, B.J. 2019. T cell receptor Vß9 in method to rapidly detect active staphylococcal enterotoxin type A without live animals. Toxins. 11:399.

Problem Statement 5. Intervention and Control Strategies

Goal

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Mitigation, that is intervention and control strategies can assist in reducing or eliminating pathogens (bacteria, virues and parasites) in food animals and their derived products, seafood, and plant crops (fresh produce) during production and processing.

Mitigation is an issue both pre- and post-harvest and is specific for different food groups. For example, pre-harvest-wise, extensive monies are spent each year to control foodborne diseases in various food animal production systems. The problem is however, that many bacteria which are zoonotic pathogens to humans have developed an intimate (microbial symbiont) relationship with the host. Management strategies are developed to maximize production and improve animal health, while mitigating pathogen levels is a hopeful consequence of these strategies. While some approaches have found utility, for example, the effects different housing systems, on pathogen incidence, unfortunately, others such as feed changes, vaccination protocols, and probiotics have seen limited efficacy, and often cannot control issues such as reducing pathogen shedding. Further, some approaches have led to increased pathogen resistance which long-term exacerbates the problem; since microbial residents found within and surrounding the host continually adapt to survive the extrinsic and intrinsic stresses placed upon them. Additionally, consideration must be made regarding animal production location, animal waste management practices, animal waste and its use as soil amendments, the role of insects, water and wind in the dispersal of foodborne pathogens into the environment. These are critically important issues relative to seafood and produce production systems.

Thus, the challenge is that the pathogen load on a product must be significantly reduced by any mitigation strategy to avoid the consequences in food processing (slaughter/harvesting) resulting from “dirty in, dirty out”. There is also the concern that during processing the initial microbial load can be reduced but recontamination occurs with different pathogens (strains or serotypes) present or resident within the processing environment. Such concerns are valid because there have been numerous observations that both pathogens [and spoilage organisms] present on a product (animal including eggs, plant, seafood) prior to processing are different from those found after processing. This variation in pathogen type has significant public health relevance since those pathogens initially found on the product may not be responsible for any foodborne outbreak and/or clinical outcome.

In postharvest food processing/packaging/storage [mitigation] technologies can inactivate microorganisms to varying degrees. However, the intensities of the technology required can result in adverse functional and/or sensory properties, combined with a significant reduction in quality. Consequently, there remains a continued need to develop and subsequently combine new and/or innovative processing technology systems. Additive and/or synergistic intervention systems are preferred, leading to improved control over microorganism growth without potential changes in food quality or reductions in nutrition value.

Research should also address, where possible, the integrated lethality for an intervention process. The purpose of the process lethality determination is to provide processors with science-based validation of the effectiveness of a specific process to destroy any microorganism of concern. For example, a thermal process needs to account for many variables including the initial pathogen load, multiple pathogens, pathogen strain variability, food structure, and the heating and cooling profile of the product. In-plant validation should be conducted to verify the intervention(s). The entire lethality process is incorporated into a systems approach to developing pathogen intervention and/or control strategies. This Problem Statement addresses a wide range of food products including animals, shellfish-seafood, and plant material (fresh produce). Biocontrol technologies for food crops contaminated by mycotoxins, such a tree nuts, corn and grains are addressed in Problem Statement 4. Data from these studies are utilized in the development and validation of predictive models and incorporated within international databases such as Combase (see Problem Statement 6).

It is critically important within these mitigation studies that for development and validation of any production or processing intervention a common or representative core set of pathogens or surrogates be used. This is critically important in order to make the intervention research results comparable both within and external to the Program. Core sets of strains for different pathogens will be made available through the ARS Bacterial Culture Collection at NCAUR Peoria, IL, and through individual scientists with the Program. If a specific strain was not available in the collection, ONP facilitated obtaining the appropriate isolate. This latter issue included isolates from overseas that were found in comparable systems: for example: STEC isolates from animal systems in the EU.

Intervention and Control Strategies: Beef Cattle and Swine

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
The overall goal was to increase the microbiological safety of beef and swine by reducing or eliminating primary foodborne pathogens. Both animals can serve as reservoirs of foodborne pathogens that can spread through the environment or to meat during harvest. Research continued to be principally focused towards E. coli O157:H7, other STEC's, non-O157 and Salmonella spp., since these pathogens continue to be a high priority for the beef industry, and for the regulatory agency FSIS.

Research was directed towards providing information and technologies to assist the industry in controlling these pathogens throughout the farm to fork continuum, defined by three integrated parts; preharvest, harvest, and postharvest. The complexity of these systems makes it very difficult to control every source of microbial contamination; thus, multiple, and overlapping mitigation measures have been implemented throughout production and processing to ensure the safest final product.

Preharvest research continued to address needs in developing interventions that prevent or mitigate colonization of the gut, particularly the lower GI tract before slaughter or that reduce pathogenic or AMR bacteria in the production environment. The rationale for continuing this work is that although many intervention technologies have been implemented, none are unequivocally effective. Therefore, studies were conducted to understand the biological and ecological factors that affect the ability of foodborne pathogens to colonize, survive and persist in a particular habitat and how this can be interrupted. Other research issues included reduction of pathogen shedding and "super-shedding", antimicrobial intervention, pathogen detection, and host-pathogen interaction. Studies were conducted to develop and evaluate novel interventions for pathogen mitigation for the live animal in production and lairage environments. In conjunction with intervention development, improvements to sampling and detection methodology and pathogen source identification were investigated. Research on host-pathogen interactions, such as immune modulation as an anti-infective therapy would potentially provide the basis for new, improved and specifically targeted developments in pathogen intervention. One problem that persists for example, is that unlike humans, cattle remain asymptomatic following STEC colonization and hence, it is not possible to identify animals harboring STEC based on clinical symptoms.

Interventions at harvest (including slaughter) that are effective in reducing the concentration and prevalence of pathogens are needed since the primary source of contamination is on the hides or skin following transport to the abattoir, and during slaughter and processing from the skin or hide to the carcass surface. Thus, controlling contamination preharvest and during transportation should be a priority in helping reduce contamination at the plant during slaughter and processing.

Postharvest research addressed various needs, including development and evaluation of novel interventions for carcasses and products at multiple stages of processing, and for the finished product. In conjunction with intervention development, improvements to sampling, detection methodology and pathogen source identification were investigated for processing, as well as the finished product.

This wide-ranging research was conducted through several projects at three different research locations; College Station, Texas; MARC, Clay Center, Nebraska; and NADC, Ames, Iowa.

Studies at College Station, TX were addressed by a project (Anderson) with the following objective to: (1) Characterize the biological factors affecting infection and maintenance of Salmonella in lymphatics of food producing animals and elucidate management practices to mitigate infection.

Examples of Accomplishments

- **Establishment of updated best use applications for disinfectants used against foodborne pathogens.** Studies evaluated the susceptibility of historical isolates of major foodborne pathogens from various production environments to contemporarily-used disinfectants, organic acids and antibiotics finding that changes in susceptibilities had occurred to some of these commercial agents.

- **Incidence of Salmonella enterica (SE) in swine**. SE is an important cause of foodborne illness globally every year. ARS in collaboration with researchers from Texas Tech University and Texas A&M University, hypothesized that SE in cattle lymph nodes could pose a risk for human infection if these lymph nodes were included in edible meat products reaching the consumer. Studies examined data from 1200 cheek meat and head trim tissues from pork carcasses collected bi-monthly over a 12-month period from a pork processing plant. The analysis showed high carriage rates of diverse SE serotypes from these pork samples. Most isolations occurred during colder months compared to warmer months and approximately 60% of isolates were multi-drug resistant (resistant to 3 or more antimicrobials).

Outcomes and Impacts

- These findings provide important updated information to help manufacturers and users implement updated best use and abundant care practices in their preparation and application of disinfectants, organic acids and antibiotics to ensure these products provide efficacious control for the continued production of safe, high quality and wholesome food at an affordable cost for the American consumer.
- Determined the potential risk of human exposure to SE if by-product tissues are incorporated into edible meats, and they suggest that further intervention methods, both pre- and post-harvest, should be explored.

Examples of Relevant Publications

- Beier, R.C., Franz, E., Bono, J.L., Mandrell, R.E., Fratamico, P.M., Callaway, T.R., Andrews, K., Poole, T.L., Crippen, T.L., Sheffield, C.L., Anderson, R.C., Nisbet, D.J. 2016. Disinfectant and antimicrobial susceptibility profiles of the big six non-O157 Shiga toxin-producing *Escherichia coli* strains from food animals and humans. Journal of Food Protection. 79(8):1355-1370.
- Beier, R.C., Callaway, T.R., Andrews, K., Poole, T.L., Crippen, T.L., Anderson, R.C., Nisbet, D.J. 2017. Interactions of organic acids with *Salmonella* strains from feedlot water-sprinkled cattle. Journal of Food Chemistry & Nanotechnology. 3(2):60-66. https://doi.org/10.17756/jfcn.2017-038.
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- Beier, R.C., Harvey, R.B., Hernandez Jr, C.A., Hume, M.E., Andrews, K., Droleskey, R.E., Davidson, M.K., Bodeis-Jones, S., Young, S., Duke, S.E., Anderson, R.C., Crippen, T.L., Poole, T.L., Nisbet, D.J. 2018. Interactions of organic acids with *Campylobacter coli* from swine. PLoS One. 13(8):e0202100. https://doi.org/10.1371/journal.pone.0202100.

- Beier, R.C., Harvey, R.B., Hernandez Jr, C.A., Andrews, K., Droleskey, R.E., Hume, M.E., Davidson, M.K., Bodeis-Jones, S., Young, S., Anderson, R.C., Nisbet, D.J. 2019. Disinfectant and antimicrobial susceptibility profiles of *Campylobacter coli* isolated in 1998 to 1999 and 2015 from swine and commercial pork chops. Journal of Food Science. 84(6):1501-1512. https://doi.org/10.1111/1750-3841.14622.
- Beier, R.C., Harvey, R.B., Poole, T.L., Hume, M.E., Crippen, T.L., Highfield, L.D., Alali, W.Q., Andrews, K., Anderson, R.C., Nisbet, D.J. 2019. Interactions of organic acids with vancomycin-resistant *Enterococcus faecium* isolated from community wastewater in Texas. Journal of Applied Microbiology. 126(2):480-488. https://doi.org/10.1111/jam.14145.

Studies at MARC, Clay Center were addressed by a project (Bosilevac) with the following objective to: (1) Develop and validate novel pre- and post-harvest intervention strategies to reduce or eliminate foodborne pathogen colonization and persistence in the animal and on carcasses and meat products.

Examples of Accomplishments

- **Bacteriophage treatment of cattle hides before processing does not improve beef safety.** Several studies have shown that cattle hides are the primary source of beef carcass contamination during processing and that reductions in the E. coli O157:H7 load on the hides of cattle entering processing facilities will lead to reductions in carcass contamination. Bacteriophage, viruses capable of killing bacteria, have been proposed as a novel technology to reduce the levels of E. coli O157:H7 on cattle hides, however the treatment of cattle hides with bacteriophage prior to processing did not produce a significant reduction of E. coli O157:H7 on hides or beef carcasses during processing.

- **Surface pH of fresh beef can be used as parameter to validate effectiveness of lactic acid treatment against E. coli O157:H7 and Salmonella.** The food safety system implemented by beef processors includes use of antimicrobials such as lactic acid sprayed on beef carcasses to mitigate bacterial contamination. Antimicrobial interventions used by beef processors are required to be validated under the actual conditions used. However, antimicrobial intervention sprays are applied under many different parameters (concentration, spray volume and pressure, etc.) making validation studies under commercial conditions cumbersome and expensive. A study demonstrated that surface pH of a beef carcass after applying lactic acid can be used as an effective and inexpensive measurement of antimicrobial efficacy and reduction of pathogenic bacteria if present.

Outcomes and Impacts

- Demonstrated that commercialized bacteriophage applications sprayed onto cattle hides prior to entering beef processing plants do not work. Therefore, treatments using bacteriophage before processing will not improve beef safety.

- Determined that carcass surface pH was effective in validating reductions of both E. coli O157:H7 and Salmonella on beef carcasses. Therefore, surface pH can be used by the beef processing industry to efficiently validate the effectiveness of lactic acid intervention for pathogen reduction in beef. Cargill Meat Solutions is one commercial beef processor now using this method to validate lactic acid applications in their food safety systems.

Examples of Relevant Publications

- Kalchayanand, N., Arthur, T.M., Bosilevac, J.M., Schmidt, J.W., Shackelford, S.D., Brown, T., Wheeler, T.L. 2018. Surface pH of fresh beef as a parameter to validate effectiveness of lactic acid treatment against Escherichia O157:H7 and Salmonella. Journal of Food Protection. 81(7):1126-1133. https://doi.org/10.4315/0362-028X.JFP-17-469.
- Arthur, T.M., Kalchayanand, N., Agga, G.E., Wheeler, T.L., Koohmaraie, M. 2017. Evaluation of bacteriophage application to cattle in lairage at beef processing plants to reduce Escherichia coli O157:H7 prevalence on hides and carcasses. Foodborne Pathogens and Disease. 14(1):17-22. https://doi:10.1089/fpd.2016.2189.

Additional:

Within the (Bono) project at MARC, Clay Center, NE, a small study was conducted whose objective was: (1) An evaluation of a sanitizer against biofilms,

Example of Accomplishment

- **Effectiveness of novel sanitizer product against biofilms.** The effectiveness of a commercial sanitizer product Decon-7™ related to inactivating and removing E. coli O157:H7 and Salmonella biofilm was evaluated. Results indicated that this product is effective, even with short exposure to highly diluted concentrations, in destroying biofilm structure, reducing viable biofilm cells, removing surface colonized biofilm matrix and preventing pathogen post-treatment prevalence. The meat industry needs effective sanitizer products and an easy-to-implement protocol that can be applied to the processing environment and the various equipment. Decon-7™can be deployed as foam or liquid solution to cover the different areas as needed in the plants, thus, this product provides a cost-effective option that the meat industry may consider for their biofilm prevention and control program.

Outcomes and Impacts

- The sanitizer Decon-7™ is effective, while deployed as foam or liquid solution at highly diluted concentrations, against E. coli O157:H7 and Salmonella biofilms, providing a cost-effective option that the meat industry may consider for their biofilm prevention and control program.

Studies at NADC, IA, were addressed by a project (Kudva) with the following objectives to: (1) Understand the impact of the bovine intestinal environment, especially at the recto-anal junction, and the molecular mechanisms that promote or inhibit colonization, adherence, and persistence of STEC in cattle and develop intervention strategies to control STEC colonization; (2) Formulate and assess the efficacy of vaccines for controlling STEC colonization of cattle based on whole-cell and subunit vaccines and identify proteins and epitopes conserved in STEC; and (3) Define potential biomarkers using systems-based approaches that will allow the development of rapid diagnostic tests to identify STEC-colonized cattle.

Examples of Accomplishments

- **Determined the importance of two bacterial cell surface proteins for their distinct role in O157 adherence**. One of these proteins (EspA) promoted O157 adherence to epithelial cells and the other (Curli) contributed to its adherence to non-living materials, such as plastic and glass. This is a significant finding from ARS that delineates the function of two cell-surface proteins of O157, one used for adherence (EspA) to epithelial cells, such as during colonization of intestinal tract of cattle, and the other, presumably for survival in the environment outside the animal in biofilms (Curli). Additionally, studies discovered that Curli limits O157 attachment to bovine recto-anal junction (RAJ) cells where O157 tends to persist. Removing, disrupting or interfering with O157 Curli resulted in better O157 attachment to the RAJ cells. These data will need to be considered, when designing anti-attachment therapies or vaccine.

- **Improved a killed whole-cell E. coli O157 vaccine formulation to enhance immune responses in cattle**. Vaccination of cattle is considered an important option for reducing O157 carriage and thereby reducing human illnesses. ARS performed a study in which cattle were vaccinated with two doses of a vaccine formulation prepared by combining a chemically-inactivated mutant strain of O157 and a commercially available adjuvant. The vaccine-adjuvant combination not only enhanced immunity against O157 but also efficacy. Specifically, fecal shedding of O157 was significantly reduced after experimental challenge in animals that received the adjuvanted formulation. O157 attachment to intestinal cells which leads to intestinal colonization and fecal shedding of O157 in cattle was blocked by one of the immune response factors (antibody IgA) detected only in the feces of vaccinated cattle. Vaccination also induced specific immune cells and factors produced by these cells (interferon gamma) that might also interfere in O157 attachment to cattle intestinal cells. These findings show that vaccine formulations for STEC must be tailored for optimal immune activation and memory response to reduce O157 colonization and fecal shedding in cattle after exposure. Successful vaccination protocols can provide a mechanism for limiting food and environmental contamination, which are critical for reducing transmission of O157 bacteria to humans.

- **Stress alters gastrointestinal microbiome in light-weight dairy calves.** Stress can lead to changes in normal behavior, growth, immunity, and impact gastrointestinal tract (GIT) microbiome which in turn can negatively affect cattle health. Administration

of analgesics may reduce pain, but it's unclear if the biological impact of stress is also mitigated with analgesics. ARS and colleagues at Iowa State University and Kansas State University discovered that dehorning and castration, two common stressors, cause changes in the GIT microbiome regardless of analgesic administration, and these changes are most pronounced in lightweight calves. The work highlights that methods to reduce pain may not alter the biological impact of a stressful procedure, and different approaches may be warranted in animals of different sizes. Reducing stress is important for overall animal health which in turn may influence efficacious immune responses to vaccines and other therapies.

- **Identified E. coli O157 protein involved in initial attachment to human intestinal cells.** E. coli O157 uses a well-characterized mechanism for intimately attaching to human intestinal cells and causing disease. However, prior to intimate attachment there is a process of initial attachment, which is not well understood. ARS along with collaborators at Pennsylvania State University identified an O157 protein involved in the initial attachment known as the carbon starvation-inducible lipoprotein or Slp. Slp interacts with a protein on the human intestinal cells called the polymeric immunoglobulin receptor or pIgR. O157 protein such as Slp involved in the initial attachment maybe targeted for therapeutic interventions, such as vaccines that could interfere with O157 attachment to human intestinal cells and hence prevent disease.

Outcomes and Impacts

- Determined the function of two cell-surface proteins of O157, one used for adherence (EspA) to epithelial cells (colonization), the other outside the animal in biofilms (Curli).
- Discovered that Curli limits O157 attachment to bovine recto-anal junction (RAJ) cells where O157 tends to persist. These data will need to be considered, when designing anti-attachment therapies or vaccine.
- Determined that two doses of a vaccine formulation enhanced immunity against O157 but also efficacy. Indicated that vaccine formulations for STEC must be tailored for optimal immune activation and memory response to reduce O157 colonization and fecal shedding.
- Discovered that dehorning and castration, two common stressors, cause changes in the GIT microbiome regardless of analgesic administration. Highlights that methods to reduce pain may not alter the biological impact of a stressful procedure, and different approaches may be warranted in animals of different sizes.
- Confirmed that reducing stress is important for overall animal health which in turn may influence efficacious immune responses to vaccines and other therapies.
- Identified an O157 protein: carbon starvation-inducible lipoprotein or Slp involved in initial attachment. Slp may be targeted for therapeutic interventions, such as vaccines to interfere with O157 attachment to human intestinal cells and hence prevent disease.

Examples of Relevant Publications

- Sharma, V.K., Schaut, R.G., Alt, D.P., Loving, C.L. 2018. Vaccination with killed whole-cells of *Escherichia coli* O157:H7 hha mutant emulsified with an adjuvant induced vaccine strain-specific serum antibodies and reduced E. coli O157:H7 fecal shedding in cattle. Veterinary Microbiology. 219:190-199. https://doi: 10.1016/j.vetmic.2018.04.003.
- Sharma, V.K., Kudva, I.T., Bearson, B.L., Stasko, J.A. 2016. Contributions of EspA filaments and curli fimbriae in cellular adherence and biofilm formation of enterohemorrhagic Escherichia coli O157:H7. PLoS One. https://doi: 10.1371/journal.pone.0149745.
- Kudva, I.T., Carter, M.Q., Sharma, V.K., Stasko, J.A., Giron, J.A. 2016. Curli temper adherence of Escherichia coli O157:H7 to squamous epithelial cells from the bovine recto-anal junction in a strain-dependent manner. Applied and Environmental Microbiology. 83(1):e02594-16. https://doi: 10.1128/AEM.02594-16.
- Schaut, R.G., Boggiatto, P.M., Loving, C.L., Sharma, V.K. 2019. Cellular and mucosal immune responses following vaccination with inactivated mutant of Escherichia coli O157:H7. Scientific Reports. 9:6401-6411. https://doi.org/10.1038/s41598-019-42861-z.
- Mir, R.A., Kleinhenz, M.D., Coetzee, J.F., Allen, H.K., Kudva, I.T. 2019. Fecal microbiota changes associated with dehorning and castration stress primarily affects light-weight dairy calves. PLoS ONE 14(1): e0210203. https://doi.org/10.1371/ journal.pone.0210203
- Fedorchuk, C., Kudva, I.T., Kariyawasam, S. 2019. The *Escherichia coli* O157:H7 carbon starvation-inducible lipoprotein Slp contributes to initial adherence in vitro via the human polymeric immunoglobulin receptor. PLoS One. 14(6):e0216791. https://doi.org/10.1371/journal.pone.0216791.

Studies were addressed by a project at BARC, Beltsville, MD (Rosenthal) [previously Hill] whose objectives were to: (1) Determine and validate methods for improved inactivation and surveillance of meat-borne exposure to Toxoplasma gondii and Trichinella spiralis; and (2) Identify mitigation strategies that reduce Toxoplasma oocysts contamination on fruits and leafy greens.

Examples of Accomplishments

- **Rapid inactivation of Toxoplasma gondii and Trichinella spiralis during formulation of dry cured ready-to-eat pork sausage.** Food producers require generally-applicable rules for ensuring their meat products are safe for consumption. Studies identified the critical point during preparation of dry cured sausage that inactivates Toxoplasma gondii and Trichinella spiralis, important foodborne parasites. Survival of T. gondii at each stage of preparation was assessed, and it was determined that the parasite cannot survive exposure salt concentrations much lower than those typically used for preparation of dry cured sausage. Salt concentrations above 1.3%, in combination with fermentation to pH 5.2 or below, kills greater than 96% of Trichinella larvae in stuffed sausages within 24-28 hours and inactivates them all within 10-days.

- **Protecting blueberries from parasitic infection.** The increasing consumption of fresh produce has resulted in more outbreaks of foodborne illnesses linked to parasites. Current washing steps in produce processing may not effectively eliminate T. gondii from at-risk produce. Studies evaluated low-dose irradiation to inactivate T. gondii oocysts on blueberries.

Outcomes and Impacts

- The use of dry curing components, specifically NaCl, are effective in controlling parasites in "ready to eat" meats, rendering these meats safe from risk to consumers.
- Low-dose irradiation dramatically reduced viable parasite contamination to well-below detection limits without compromising the quality of the berries, as measured by compression firmness, anthocyanins, or color.

Examples of Relevant Publications

- Hill, D.E., Luchansky, J.B., Porto Fett, A.C., Gamble, H., Juneja, V.K., Fournet, V.M., Hawkins Cooper, D.S., Holley, R., Gajadhar, A., Dubey, J.P. 2017. Curing conditions to inactivate Trichinella spiralis muscle larvae in ready-to-eat pork sausage. Food and Waterborne Parasitology. 6:1-8.
- Hill, D.E., Luchansky, J.B., Porto Fett, A.C., Gamble, H., Urban Jr, J.F., Fournet, V.M., Hawkins Cooper, D.S., Gajadhar, A., Holley, R., Juneja, V.K., Dubey, J.P. 2018. Rapid inactivation of Toxoplasma gondii bradyzoites in dry cured sausage. Food and Waterborne Parasitology. https://doi.org/10.1016/j.fawpar.2018.e00029.
- Lacombe, A.C., Breard, A., Hwang, C., Hill, D.E., Fan, X., Huang, L., Yoo, B.K., Niemira, B.A., Gurtler, J., Wu, V.C. 2016. Inactivation of Toxoplasma gondii on blueberries using low dose irradiation without affecting quality. Journal of Food Protection. 73:981-985. https://doi.org/10.1016/j.foodcont.2016.10.011
- Harito, J.B., Campbell, A.T., Prestrud, K.W., Dubey, J.P., Robertson, L.J. 2016. Surface binding properties of aged and fresh (recently excreted) Toxoplasma gondii oocysts. Experimental Parasitology. 165:88-94.

Intervention and Control Strategies: Poultry

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Contaminated poultry and poultry products continue to be cited as major source/cause of foodborne illness worldwide principally because live birds and eggs can serve as reservoirs for bacteria such as Salmonella and Campylobacter. Contamination through commensalism is not limited and may be found in both broiler and breeder flocks, birds for egg production, and the

eggs themselves. These pathogens can be conveyed onto the processing facility, onto raw poultry product, and onto the final product supplied to consumers.

Despite continued efforts to control preharvest contamination of poultry only minor reductions have occurred suggesting that new approaches needed to be developed to help reduce the spread of any pathogens before entering the processing plant. Reductions in pathogen levels prior to processing is a critical issue, however, it does not preclude efforts to reduce contamination during the animal growth phase. Innate immunity of the animal itself is the first line of defense against pathogens, so activating the immune system through vaccination, or even breeding poultry resistant to specific pathogens are viable research initiatives. Identifying on-farm management and rearing practices, for example, changes to housing systems, stocking densities, better litter management, improvements in dietary regimes combined with implementation of novel alternatives or interventions should reduce pathogen presence.

Specific preharvest interventions are especially important as the trend toward antibiotic free production continues. Optimized preharvest interventions will help reduce the need for antibiotics during commercial poultry production. There were various approaches examined which included for example: novel biocontrol intervention strategies; bacteriophage therapy; probiotics to reduce pathogen GI tract colonization; natural alternatives to antibiotic use; or products for example (peptides and lytic enzymes) that could readily be incorporated into chicken feed. This research is discussed under Problem Statement 7: Antimicrobial Resistance

The processing plant is a postharvest site in which numerous manipulations are made to poultry carcasses and meat, many of which impact the microbial safety of the end products. Research studies were designed to examine the distribution and dispersion of bacterial pathogens in and around poultry processing plants and poultry products. Studies provided a molecular systems approach to foodborne pathogens and their movement in the poultry processing environment. Intervention techniques and processing modifications to lessen microbiological contamination of poultry meat were designed and tested. Research was undertaken into the development of alternative sanitizers that could be used in commercial processing operations. Applied research is also needed to design, develop and test effective means to interrupt the transfer and related postharvest contamination to both raw and cooked products resulting from the transfer of resident pathogens such as Listeria monocytogenes in the processing plant itself (for example, from floor drains).

This research was conducted through several projects at two different research locations; College Station, Texas; U.S. NPRC, Athens, Georgia.

Studies were conducted at College Station, TX (Kogut) where the objective was to: (1) Define the differential host-pathogen interactions between Salmonella and chicken and poultry mucosal immune systems using genomic technologies; and determine the relationship between foodborne pathogens and the mucosal innate immune response, focusing on epigenetic reprogramming of host immune genes in persistent infections.

Examples of Accomplishments

- Host kinome profile of infected cecal tissue (protein phosphorylation patterns) in broilers with a high burden of S. Enteritidis is distinct from that of broilers with lower levels of colonization. As might be expected, the birds with lower loads of S. Enteritidis, meaning the host's immune response restricted colonization, show increased activity in key immune signaling pathways associated with chemokine, Jak-Stat, MAPK, and T cell receptor signaling.

Outcomes and Impact

- Provide groundwork into specific biomarkers to select individual birds that are more resistant Salmonella colonization.
- Demonstrated it is possible to identify key mechanisms and pathways that are associated with increased resistance against Salmonella cecal colonization in chickens.
- Collectively, laid a foundation to determine practical approaches to reduce the incidence of foodborne illnesses associated with poultry-acquired Salmonella.
- Showed that hosts have evolved countermeasures to pathogen invasion, establishment, and replication through two types of defenses: resistance and tolerance. Resistance functions are mediated by the immune system, while tolerance is mediated by different mechanisms that protect host tissues from the virulence factors of a pathogen and those that limit or reduce the damage caused by the host immune and inflammatory responses to the pathogen.
- Determined that pathogens, such as Salmonella, have evolved the capacity to survive the initial robust immune response. Persistence involves a complex balance of protective immunity and immunopathology which allows Salmonella to stay in the avian ceca for months without triggering clinical signs.
- Chronic colonization of the intestinal tract is an important aspect of persistent Salmonella infection because it results in a silent propagation of bacteria in poultry stocks due to the impossibility to isolate contaminated animals.
- Promote the hypothesis that Salmonella have evolved a unique survival strategy in poultry that minimizes host defenses (disease resistance) during the initial infection and then exploits and/or induces a dramatic immunometabolic reprogramming in the cecum that alters the host defense to disease tolerance. Unfortunately, this disease tolerance results in the ongoing human food safety dilemma.

Examples of Relevant Publications

- Kogut. M.H., Arsenault, R.J. 2017. Immunometabolic Phenotype Alterations Associated with the Induction of Disease Tolerance and Persistent Asymptomatic Infection of Salmonella in the Chicken Intestine. Frontiers in Immunology 8: 372 https://doi: 10.3389/fimmu.2017.00372.
- Swaggerty, C.L., Kogut, M.H., He, L.H., Genovese, K.J., Johnson, C., Arsenault, R.J. 2017. Differential levels of cecal colonization by Salmonella Enteritidis in chickens triggers distinct immune kinome profiles. Frontiers in Veterinary Science. 4(214):1-14. https://doi.org/10.3389/fvets.2017.00214.

Studies at U.S. NPRC, Athens, GA were addressed by three projects (Buhr, Berrang and Jones).

The Buhr project addressed the following objectives: (1) Develop reliable and reproducible challenge models with Salmonella and Campylobacter for use in accurately developing, evaluating, and validating processes for reducing pathogen load using various chemical sanitizers; (2) Develop, evaluate, and validate current and novel chemicals, operational protocols, and sampling methodologies used during poultry production and processing of broilers for the reduction and/or control of foodborne pathogens; (3) Identify and evaluate risk factors in the production, management, transportation, or processing that impact bird/egg contamination with foodborne pathogens and develop intervention strategies to control pathogens in the absence of antibiotics; and (4) Determine the extrinsic factors that impact the survival and attachment of microorganisms including evaluating media and growth factors, and develop and validate new improved technologies to isolate and propagate foodborne pathogens.

Examples of Accomplishments

- **Antimicrobial intervention potential carryover.** Determined the amount of water picked up and retained by a carcass during post-chill application, to include the impact of scalding protocol (hard vs. soft scalding), application methods (spraying or dipping), and carcasses post application orientation (hung by the wing or leg). After the five-minute drip time, soft scalded carcasses retained more water than hard scalded carcasses, carcasses that were dipped retained more water than carcasses that were sprayed, and carcasses hung by the leg retained more water that carcasses hung by the wing.

- **Development of neutralizing Buffered Peptone Water (nBPW).** Developed a neutralization solution (nBPW) for use by commercial poultry processors in Salmonella verification testing. Commercial poultry processors use chemical sanitizers during processing to reduce contamination of carcasses by human foodborne pathogens. However, if traces of these sanitizers are carried-over into testing samples used to determine contamination of poultry carcasses and parts by Salmonella, the results of these tests may be inaccurate. FSIS Notice 41-16, July 2016

- **Isolation and identification of amylase-producing bacteria for use as a probiotic.** The isolation of an amylase-producing, endospore-forming bacteria from the intestinal tract of commercially processed broiler carcasses provided a new probiotic bacterium to deliver to live poultry to reduce the colonization by foodborne pathogens, such as Salmonella. Including bacteria that produce the enzyme, amylase, in probiotic cultures might improve the efficacy of these products because amylase can breakdown starch in poultry feed to produce simple sugars that can be used by other beneficial bacteria.

- **Broiler internal Salmonella contamination during catching.** Commercial broilers sampled the week prior or the day prior to catching for transport to the processing plant, revealed that these initial cecal sampling results often fail to accurately predict Salmonella prevalence following feed withdrawal and arrival at the processing plant. To determine if respiratory tract contaminated by dust generated during the catching process was responsible for this discrepancy broilers were sampled prior to, during, and after catching. Dust generated during catching did not result in respiratory tract contaminated with Salmonella, aerobic, or Enterobacter bacteria. These results further complicate the practical implication of logistic slaughter by attempting to process negative and false negative flocks first each processing day.

- **Option for electrical stunning to death of broilers at processing.** The components of electrical stunning systems for broilers commonly used in the U.S. (Direct Current, low voltage, and high frequency) and in the E.U. (Alternating Current, high voltage, and low frequency) resulted in a combined stun-to-death protocol. Research documented that at low voltage DC stun duration of 120-seconds, or a short 5-second AC stun prevented recovery of consciousness and facilitated stun-to-death. By electrically stunning broilers using a combination of DC (10-seconds) followed by the application of AC (5-seconds) confirmed 100% efficacy in induction of a non-recoverable stun-to-death, without inducing carcass damage or hemorrhages. Commercial processing plants can utilize this combined DC+AC stun for any customers that may require an irreversible electrical stun.

- **Litter sampling time does not impact the predicted Salmonella or Campylobacter status of the flock.** Demonstrated that the time of day that litter sampling occurred from feed restricted pullet breeder pens did not alter the Salmonella or Campylobacter recovery from the litter. Litter sampled at various times of the day were all 100% positive for Salmonella in 9, 11, and 17-week-old birds and when sampled for Campylobacter at 17 and 18-week-old birds were also 100% positive. At these ages, the feces and litter contain Salmonella and Campylobacter at levels enough to be recovered from the pen litter prior to the chickens being fed, and at 3- or 6-hours after feeding on the same day.

Outcomes and Impact

- Determined for FSIS the data necessary to calculate the potential amount of antimicrobial that would remain on a carcass and would require rinsing, inactivation, or no action prior to sampling for whole broiler carcass for Salmonella determination.
- Developed and validated a universal neutralizing enrichment broth that is in use for commercial poultry processing plants for daily Salmonella samples sent to FSIS. The utilization of the nBPW improves the accuracy of Salmonella verification testing by inactivating trace-amounts of the sanitizers in the test samples. On July 1, 2016, the FSIS implemented the use of nBPW for regulatory testing

- Determined that once Salmonella or Campylobacter colonize the intestinal tract, both pathogens are shed from the intestinal tract at levels recoverable from the litter at various sampling times.

Examples of Relevant Publications

- Gamble, G.R., Berrang, M.E., Buhr, R.J., Hinton Jr, A., Bourassa, D.V., Johnston, J.J., Ingram, K.D., Adams, E.S., Feldner, P.W. 2016. Effect of simulated sanitizer carryover on recovery of salmonella from broiler carcass rinsates. Journal of Food Protection. 79(5):710-714. https://doi:10.4315/0362-028X.JFP-15-461.
- Bourassa, D.V., Wilson, K.M., Bartenfeld, L.N., Harris, C.E., Howard, A.K., Ingram, K.D., Hinton Jr, A., Adams, E.S., Berrang, M.E., Feldner, P.W., Gamble, G.R., Frye, J.G., Jackson, C.R., Johnston, J.J., Buhr, R.J. 2017. Surface water accumulation and subsequent drip loss for processed broiler carcasses subjected to a post-chill water dip or spray. Poultry Science. 96(1):241-245. https://doi.org/10.3382/ps/pew275.
- Bourassa, D.V., Bowker, B.C., Zhuang, H., Wilson, K.M., Harris, C.E., Buhr, R.J. 2017. Impact of alternative electrical stunning parameters on the ability of broilers to recover consciousness and meat quality. Poultry Science. 96(9):3495-3501. https://doi.org/10.3382/ps/pex120
- Gamble, G.R., Berrang, M.E., Buhr, R.J., Hinton Jr, A., Bourassa, D.V., Johnston, J.J., Ingram, K.D., Adams, E.S., Feldner, P.W. 2017. Neutralization of bactericidal activity related to antimicrobial carry-over in broiler carcass rinse samples. Journal of Food Protection. 80:(4)685-591. https://doi:10.4315/0362-028X.JFP-16-412.
- Bourassa, D.V., Wilson, K.M., Czarick, M., Buhr, R.J. 2018. Microbiological status of broiler respiratory tracts before and during catching for transport to the processing plant. Journal of Applied Poultry Research. https://doi.org/10.3382/japr/pfy029.
- Wilson, K.M., Bourassa, D.V., Mclendon, B.L., Wilson, J.L., Buhr, R.J. 2018. Impact of skip-a-day and every-day feeding programs on the recovery of salmonella and campylobacter following in broiler breeder pullets. Poultry Science. https://doi.org/10.3382/ps/pey150.

The (Berrang) project objective was to: (1) Develop, evaluate and optimize processing treatments to reduce, control and potentially eliminate foodborne pathogens in poultry processing.

Examples of Accomplishments

- **Neutralizing buffer for Campylobacter.** Broiler carcasses are generally sampled by whole carcass rinse and the potential exists for residual levels of antimicrobial processing aid to be carried over into the rinsate. It has been shown that, if un-mitigated, such carryover can interfere with the detection of Salmonella. As demonstrated in this study, carryover of antimicrobial treatment can also interfere with the detection and recovery of Campylobacter in broiler carcass rinse samples. Traditional buffered peptone water was tested and found deficient in neutralizing capability to counteract residual antimicrobial activity of some post-chill processing aids (peroxyacetic acid,

cetylpyridinium chloride, acidified sodium chloride, or a blend of acids) to allow full recovery of Campylobacter. A recently reported formulation for a neutralizing buffered peptone water (currently being used by FSIS) outperformed the traditional carcass rinse medium and allowed significantly improved recovery of Campylobacter even in the presence of 3 of the 4 tested antimicrobial processing aids. Performance of the new carcass rinse medium with the fourth antimicrobial processing aid (acidified sodium chloride) was not different from the traditional formulation. Neutralizing buffered peptone water represents a significant improvement in the broiler carcass rinse method for detection of Campylobacter.

- **Pre-chill application of antimicrobials to broiler carcasses.** Broiler carcasses are treated by cool water pre-chill to begin the chilling process in a commercial slaughter plant. Carcasses take up water during this procedure improving yield. However, pre-chill water does not generally include an antimicrobial chemical; therefore, it may allow viable bacteria to be taken up by carcasses which could be released during sampling of carcasses or cut up parts. A pilot scale model system was developed and used to test application of antimicrobial chemicals during immersion pre-chill. Peracetic acid, chlorine, and a combination of both, with and without a chlorine stabilizing compound (T-128), were tested as pre-chill antimicrobial treatments. Carcasses were subjected to treated pre-chill, subsequent full chill and then cut up for sampling as parts. Addition of chlorine or peracetic acid to the pre-chill can be effective to lessen bacterial contamination of carcasses and related cut up parts.

- **Reduction of Campylobacter on poultry thighs using sequential treatments of antimicrobials.** Campylobacter is a major concern for poultry processors, as USDA performance standards have become stricter. Studies evaluated the use of a low-pH processing aid and peracetic acid (PAA) applied in either individual- or consecutive-dip treatments to reduce Campylobacter in chicken thighs. Thighs were inoculated with a marker strain of C. coli and then dipped into bags containing either the low pH processing aid or the PAA. Combinations of dual low-pH dips, dual PAA dips, low pH then PAA, and PAA then low-pH were all evaluated against a control of dual buffer dips. Peracetic acid followed by low pH dips showed significant reductions compared to all other treatments (99.9% from untreated). This data suggests that treatment with an oxidizing agent (PAA) following by an acidic treatment (low pH) maximizes Campylobacter reduction. Treating with this sequence may allow processors to meet the strict performance standards on Campylobacter in broiler parts.

- **Treatment of chicken parts before incorporation into ground meat products.** Ground chicken meat can be heavily contaminated with bacterial pathogens. Much of this contamination comes from the skin and meat surface which has been handled and subject to potential cross contamination. A pilot scale method was developed to treat inoculated skin and meat with antimicrobial chemicals prior to grinding. This system was tested to determine the efficacy of chlorine and peracetic acid to lower numbers of skin-borne Campylobacter, Salmonella, and Listeria monocytogenes in ground chicken meat product. A 1200 ppm peracetic acid dip was effective to lessen

the numbers of human pathogens on chicken meat and skin and the ground chicken meat product made from it.

- **Treatment of floor drains to prevent contamination by Listeria monocytogenes.** Floor drains tend to collect bacteria in poultry processing plants and can become colonized with L. monocytogenes. This human pathogen can linger in a drain for years and has potential to spread around a processing facility and even contaminate fully cooked ready-to-eat meat. A self-contained chlorine dioxide generating pod for decontamination of floor drains was tested. The pods worked well to greatly lower natural bacterial numbers in floor drains. The technology was further tested against L. monocytogenes inoculated in a model floor drain system. The easy to use and safe to deploy pods were extremely effective for lowering the numbers of L. monocytogenes both in the drain water and as a biofilm attached to the inner wall of the drain pipe by more than 99.9999%.

Outcomes and Impacts

- Determined via validation by FSIS that the formulated neutralizing buffered peptone water outperformed the traditional carcass rinse medium and allowed significantly improved recovery of Campylobacter even in the presence of 3 of the 4 tested antimicrobial processing aids.
- Determined that performance of the new carcass rinse medium with the fourth antimicrobial processing aid (acidified sodium chloride) was not different from the traditional formulation. Determined neutralizing buffered peptone water represents a significant improvement in the broiler carcass rinse method for detection of Campylobacter.
- Determined that treatment with an oxidizing agent (PAA) following by an acidic treatment (low pH) maximizes Campylobacter reduction, which may allow processors to meet the strict performance standards on Campylobacter in broiler parts.
- Developed a pilot scale model system to test application of antimicrobial chemicals during immersion pre-chill. Peracetic acid, chlorine, and a combination of both, with and without a chlorine stabilizing compound (T-128). Determined that addition of chlorine or peracetic acid to the pre-chill can be effective to lessen bacterial contamination of carcasses and related cut up parts.
- Developed a pilot scale method to treat inoculated skin and meat with antimicrobial chemicals prior to grinding. With the system, determined the efficacy of chlorine and peracetic acid to lower numbers in ground chicken meat product. Determined that 1200 ppm peracetic acid dip was effective to reduce human pathogens.
- Developed and validated a self-contained chlorine dioxide generating pod for decontamination of floor drains, especially for Listeria species. The easy to use and safe to deploy pods were effective for reducing L. monocytogenes both in the drain water and as a biofilm attached to the inner wall of the drain pipe by more than 99.9999%.

Examples of Relevant Publications

- Berrang, M.E., Gamble, G.R., Hinton Jr, A., Johnson, J. 2018. Neutralization of residual antimicrobial processing chemicals in broiler carcass rinse for improved detection of Campylobacter. Journal of Applied Poultry Research. https://doi:10.3382/japr/pfx071.
- Steininger, C., Harrison, M., Berrang, M.E. 2018. Application of antimicrobial treatment to whole carcasses during pre-chill can improve microbial quality of broiler parts. Food Microbiology. https://doi.org/10.1111/jfs.12434.
- Park, S., Harrison, M., Berrang, M.E. 2017. Post-chill antimicrobial treatments to control Salmonella, Listeria and Campylobacter contamination on chicken skin used in ground chicken. Journal of Food Protection. 80:857-862.
- Berrang, M.E., Harrison, M., Meinersmann, R.J., Gamble, G.R. 2017. Self-contained chlorine dioxide generation and delivery pods for decontamination of floor drains. Journal of Applied Poultry Research. 26(3):410-415. https://doi: 10.3382/japr/pfx009.
- Landrum, M., Cox Jr, N.A., Cosby, D.E., Berrang, M.E., Gamble, G.R., Da Costa, M.J., Pesti, G.M. 2018. Low pH processing aid to lower the presence of naturally occurring *Campylobacter* on whole broiler carcasses. Advanced Food and Nutritional Sciences. 3:7-13. https://doi.org/10.21065
- Landrum, M.A., Cox, N.A., Wilson, J.L, Berrang, M.E., Gamble, G.R., Harrison, M.A., Fairchild, B.D., Kim, W.K., Hinton, A. Jr. 2019. Reduction of *Campylobacter* on poultry thighs using sequential treatments of antimicrobials. Advanced Food and Nutritional Sciences. 4:1-7. http://dx.doi.org/10.21065

The (Jones) project objectives were to: (1) Assess the impact of dietary regimens, housing systems, and different chicken genetic lines on Salmonella infections of hens, Salmonella contamination of the production environment and eggs, and physical and functional egg quality; (2) Assess the effects of key management practices using experimental and field models of different housing systems on hen health, microbial ecology of foodborne bacteria, and antimicrobial resistance associated with egg contamination.

Examples of Accomplishments

- **Survival and growth of Salmonella Enteritidis strains in egg albumen and yolk.** Salmonella Enteritidis is considered the primary pathogen for shell eggs. FDA regulations allow for shell eggs to remain unrefrigerated up to 36 h post-lay. Ten S. Enteritidis which had been characterized for genetic and cellular characteristics were introduced in low numbers into fresh egg albumen and yolk. The results demonstrated that a few defined genetic characteristics of *S.* Enteritidis strains can affect their survival and growth properties in eggs, but the extent of variation between strains is likely insufficient to influence the effectiveness of egg refrigeration.

- **Salmonella Enteritidis egg contamination in hen housing systems.** Contaminated eggs produced by infected hens can transmit Salmonella Enteritidis to consumers, but the influences of different poultry housing systems and genetic lines of

hens are not fully understood. Two of each brown egg and white egg laying hen genetic lines were housed in both conventional and enriched colony cage housing systems and challenged with S. Enteritidis. S. Enteritidis was found more often inside eggs from the two white egg lines than from the brown egg lines in either type of housing. One brown egg line laid fewer contaminated eggs than any other line and the egg contamination frequencies of the two white lines differed significantly.

- **Shedding of Salmonella spp. by infected laying hens.** Laying hens housed in both conventional and enriched colony cages at low and high stocking densities had varying levels of shedding Salmonella spp. Hens challenged with S. Enteritidis and housed at a high stocking density in enriched colony cage shed more SE, but not has high as hens in conventional cages, regardless of stocking density. S. Heidelberg was shed longer and at a higher frequency than S. Typhimurium. Housing system did not influence ST shedding, but the high stocking density in both housing systems resulted in greater ST shedding.

Outcomes and Impacts

- Demonstrated that a few defined genetic characteristics of S. Enteritidis strains can affect survival and growth in eggs, strain variation does not influence the effectiveness of egg refrigeration.
- Demonstrated that S. Enteritidis deposition inside eggs can vary between genetic lines of egg-laying hens, but different housing systems do not appear to influence these trends.
- Demonstrated that Salmonella spp. are shed at different rates in laying hens depending on stocking density and housing system.

Examples of Relevant Publications

- Gast, R. K., Guraya, R., Jones, D. R., Guard, J., Anderson, K. E., and Karcher, D. M. 2017. Frequency and duration of fecal shedding of *Salmonella* serovars Heidelberg and Typhimurium by experimentally infected laying hens housed in enriched colony cages at different stocking densities. Avian Dis. 61:366-371.
- Gast, R. K., Guraya, R., Jones, D. R., Anderson, K. E., and Karcher, D. M. 2017. Frequency and duration of fecal shedding of *Salmonella* Enteritidis by experimentally infected laying hens housed in enriched colony cages at different stocking densities. Frontiers Vet. Sci. 4:47. https://doi: 10.3389/fvets.2017.00047.
- Gast, R. K., Guard, J., Guraya, R., and Locatelli, A. 2018. Multiplication in egg yolk and survival in egg albumen of genetically and phenotypically characterized *Salmonella* Enteritidis strains. J. Food Protect. 81:876-880. 2018.
- Gast, R. K., Regmi, *P. R.*, Guraya, R., Jones, D. R., Anderson, K. E., and Karcher, D. M. 2019. Contamination of eggs by *Salmonella* Enteritidis in experimentally infected laying hens of four commercial genetic lines in conventional cages and enriched colony housing. Poultry Science. http://dx.doi.org/10.3382/ps/pez222.

Additional

Studies conducted by (Jones) complementary to the main project. The accomplishments can also be considered under Problem Statement 5: Intervention and Control Strategies.

Examples of Accomplishments

- **Equivalency of peracetic acid as an egg shell surface sanitizer.** Eggs processed under USDA Agricultural Marketing Service (AMS) voluntary grade standards must be sprayed with a sanitizing rinse of 100-200 ppm chlorine or equivalence. AMS requested ARS to determine what concentrations of peracetic acid are equivalent to 100-200 ppm chlorine. Using isolates previously cultured from egg production and processing, Salmonella Enteritidis, Typhimurium, and Braenderup, as well as Enterobacter cloacae, were utilized as the challenge organisms. Based on the results, 50-100 ppm peracetic acid was found to reduce the target organisms on the shells of washed shell eggs at a level equivalent to 100-200 ppm. AMS have issued communications to all 220 official shell egg plants utilizing AMS grading services stating the allowable levels of peracetic acid which may be used for rinsing shell eggs.

- **Egg handling and storage conditions impact on egg quality.** The international trade of shell eggs has become more important in recent years in order to feed a growing worldwide population, meet food manufacturing demands, and address supply issues during disease outbreaks or product recalls. The primary barriers for the export and import of shell eggs are; whether to wash eggs and egg storage temperature. A study found the percent egg weight loss was greatest for unwashed eggs at 22°C (15.72 %) and lowest for washed and oiled 4°C (0.33 %). Less than 24 h at 22°C had a greater impact on yolk shape measurement decline than after 15-weeks at 4°C. After 15-weeks, average Haugh unit scores (objective egg grade assessment) for all refrigerated treatments were still Grade A. Unwashed eggs at 22°C dropped from Grade AA to almost Grade B in 1-week. Maintaining eggs at refrigerated temperature has the greatest impact on maintaining egg quality, whether eggs were washed before entering refrigeration. The results are being utilized by AMS and egg producers for international trade development.

Examples of Relevant Publications

- Jones, D. R., Ward, G. E., Regmi, P., and Karcher, D. M. Impact of egg handling and conditions during extended storage on egg quality. 2018. Poultry Science. 97:716-723.

Intervention and Control Strategies: Seafood

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
When adjusted for per capita consumption, seafood is responsible for more cases of foodborne illness than any other food category. Molluscan shellfish (oysters, clams and mussels) are common causes of seafood-associated illnesses and occasional deaths in the U.S. Illnesses are commonly caused by viruses, like norovirus, and from bacteria of the Genus Vibrio. Norovirus is associated with fecal pollution while Vibrio are naturally-occurring in the marine environment. Two Vibrio species (V. parahaemolyticus and V. vulnificus) are particularly problematic because they cause the highest numbers of bacterial-induced illnesses and deaths among shellfish consumers, respectively. Another Vibrio, V. tubiashii, causes high mortalities in larval shellfish in U.S. shellfish hatcheries and has reduced the availability of seed oysters and clams which are needed by the commercial shellfish industry. Unlike other foods, molluscan shellfish bioconcentrate viruses and vibrios as part of their natural feeding processes and retain these pathogens in their edible tissues. Raw shellfish consumption can lead to severe illness, and death, thus innovative-non-thermal intervention strategies using Vibrio predatory bacteria (Bdellovibrio) will be evaluated for reducing human pathogenic vibrios in market-sized oysters. Research is necessary to develop a treatment to reduce vibrios in shellfish hatcheries in order to enhance productivity and ensure the availability of seed oysters and clams needed by commercial shellfish farmers. State-of-the-art techniques using bacteriophage therapy are proposed to eradicate vibrio's in larval shellfish. Human norovirus is the number one cause of foodborne illness in the U.S., causing approximately 58% of foodborne outbreaks. A major issue is that the levels of infectious viruses that are detected in shellfish cannot currently be determined with accuracy, since currently, there are no suitable animal models for assaying HuNoV and in vitro propagation methods have eluded researchers since the virus was first identified in the early 1970s. Non-thermal interventions have been elusive. High pressure processing (HPP) has potential and is currently practiced commercially, its acceptance industry-wide has been limited. Electron beam can inactivate hepatitis A virus a norovirus surrogate on a limited basis, however, its current utility as and intervention is questionable, and activity against HuNoV has not been assessed.

Research was conducted by a project (Richards) based at ERRC, Wyndmoor, PA, but located at Delaware State University. The objectives were to: (1) Develop and evaluate intervention and control strategies for Vibrio species, with specific emphasis on the identification, characterization and application of bacteriophage to remediate shellfish mortalities in hatchery settings, and for use in commercial shellfish processing; (2) Evaluate a modified depuration process with marine Bdellovibrio and related bacteria to eliminate Vibrio in market oysters; (3) Develop and validate technologies to improve current virus detection and testing methods, including distinguishing infectious versus non-infectious virus; technologies for virus replication, for example, development of a cell culture propagation method for human norovirus; virus surrogates; and long-term virus persistence; and (4) Develop and validate emerging technologies for inactivation of enteric virus-contaminated shellfish and

other foods using novel applications of high pressure, E-beam, and laser-induced resonance energy.

Examples of Accomplishments

- **Beneficial predatory bacteria kill pathogenic Vibrio parahaemolyticus.** Vibrio parahaemolyticus is a naturally occurring marine bacterium that is transmitted to humans through the consumption of raw or undercooked oysters and other seafoods. A competing predator of V. parahaemolyticus is Pseudoalteromonas piscicida, whose predatory activity against vibrios was discovered by ARS. P. piscicida was shown to directly transfer enzyme-containing surface vesicles to V. parahaemolyticus where trypsin-like enzymes digest holes in the vibrios. Enzyme testing and genomic sequencing of three P. piscicida isolates revealed a host of enzymes that may control vibrios in various food processing scenarios.

- **Genomic sequencing.** Complete genome sequences of the three strains of Pseudoalteromonas piscicida, a beneficial predatory bacterium active against pathogenic marine bacteria like V. parahaemolyticus, were entered and fully annotated in GenBank. These were the first sequences for this species published in GenBank. Also entered was the whole genome for the larval oyster pathogen Vibrio coralliilyticus strain RE22 and draft sequences for three other V. coralliilyticus strains. Draft genome sequences were also determined for five bacteriophages against V. coralliilyticus revealing a wealth of information on novel enzyme systems, pathogenicity genes and metabolic pathways.

- **Elevated seawater temperature affects larval oyster mortalities.** Larval oysters are susceptible to a host of bacterial pathogens which lead to larval mortalities in hatchery and natural settings. One significant pathogen is Vibrio coralliilyticus, a known coral pathogen, which also infects and kills larval oysters. ARS in collaboration with scientists at Oregon State University evaluated the virulence of four strains of V. coralliilyticus toward larval Pacific oysters (Crassostrea gigas). Mortalities were significantly higher at elevated seawater temperatures where mortalities at 23°C ranged from 23-55%, but at 27°C ranged from 39-94% Consequently, a 4°C rise in seawater temperature increased mortalities by 15-43%, depending on the V. coralliilyticus strain.

- **Noroviruses persist in sewage treatment.** In collaboration with the FDA, ARS developed an assay that was used to evaluate the potential of chlorine-based sewage treatment to inactivate human norovirus.

- **Bacteriophages against pathogenic vibrios.** Pathogenic strains of Vibrio parahaemolyticus are increasingly moving northward as seawater temperatures rise. Oysters were tested for the presence of bacteriophages (phages) against three serotypes of V. parahaemolyticus that are known human pathogens (O1:K1 and pandemic strains O3:K6 and O1:KUT [untypable]). Phages against all three vibrio serotypes were commonly isolated from all three harvesting areas.

- **Male-specific bacteriophages as indicators of human norovirus in oysters.** The presence and levels of RNA and DNA male-specific bacteriophages were evaluated in live oysters and compared with persistence of human norovirus at different water temperatures. Results indicated that these common sewage phages are very persistent within oysters at different environmental temperatures after exposure of the bivalves to contaminated water.

Outcomes and Impact

- The novel P. piscicida cultures were transferred under three Material Transfer Agreements (MTAs) to:
  - Collaborators in Zurich, Switzerland who are determining the ability of Pseudoalteromonas enzymes to catalyze the formation of bioactive products of commercial value
  - Collaborators in California to test the ability of Pseudoalteromonas to control microbial communities
  - Collaborators in Texas who found P. piscicida enzymes useful to digest chitinous waste products from shellfish processing operations to yield commercially valuable chitosan, which has many food processing, biomedical, and pharmaceutical applications.
- Determined that water temperature is a critical issue in shellfish hatchery operations. Maintaining hatchery water below 27°C reduces larval mortalities, minimize production losses and increases the availability of seed oysters needed for commercial shellfish aquaculture.
- Highlighted concerns over the effects of vibrios at elevated seawater temperatures on the survivability of larval oysters, corals and other species in the marine environment.
- Determined that traditional chlorine-based sewage treatment does not substantially inactivate human norovirus.
- Determined that human norovirus and male-specific bacteriophages persist in the same manner in seawater. Thus, monitoring for phage-specific serotypes of V. parahaemolyticus may serve as good indicators for the possible presence of pathogenic vibrio serotypes in oysters.

Examples of Relevant Publications

- Choi, C., Kingsley, D.H. 2016. Temperature-dependent persistence of human norovirus within oysters (*Crassostrea virginica*). Food and Environmental Virology 8(2):141-147.
- Kingsley, D.H., Fay, J., Calci, K., Pouillot, R., Woods, J., Chen, H., Niemira B., Van Doren, J. 2017. Evaluation of chlorine treatment levels on inactivation of human norovirus and MS2 during sewage treatment. Applied and Environmental Microbiology 83(23) doi: 10.1128/AEM.01270-17.

- Richards, G.P., Needleman, D.S., Watson, M.A. 2017. Complete genome sequence of Pesudoalteromonas piscicida strain DE2-B, a bacterium with broad inhibitory activity toward human and fish pathogens. Genome Announcements. 5(33) doi: 10.1128/genomeA.00752-17.
- Kehlet-Delgado, H., Richards, G.P., Hase, C., Mueller, R.S. 2017. Three draft genome sequences of Vibrio coralliilyticus strains isolated from bivalve hatcheries. Genome Announcements. 5(41) doi: 10.1128/genomeA.01162-17.
- Richards, G.P., Watson, M.A., Needleman, D.S., Uknalis, J., Boyd, E.F. Fay, J.P. 2017. Mechanisms for Pseudoalteromonas piscicida-induced killing of vibrios and other bacterial pathogens. Applied and Environmental Microbiology. 83(11) doi: 10.1128/AEM.00175-17.
- Richards, G.P., Needleman, D.S., Watson, M.A. 2017. Complete genome sequence of Pesudoalteromonas piscicida strain DE2-B, a bacterium with broad inhibitory activity toward human and fish pathogens. Genome Announcements. 5(33) doi: 10.1128/genomeA.00752-17.
- Ushijima, B., Richards, G.P., Watson, M.A., Schubiger, C.B., Hase, C.C. 2018. Factors affecting infection of corals and larval oysters by Vibrio coralliilyticus. PLOS One. 13(6). https://doi: 10.1371/journal.pone.0199475
- Richards, G.P., Chintapenta, L.K., Watson, M.A., Abbott, A.G., Ozbay, G., Uknalis, J.A., Oyelade, A.A., Parveen, S. 2019. Bacteriophages against pathogenic vibrios in Delaware Bay oysters (Crassostrea virginica) during a period of high levels of pathogenic Vibrio parahaemolyticus. Food and Environmental Virology. 11:101-112.
- Richards, G.P., Kingham, B.F., Shevchenko, O., Watson, M.A., Needleman, D.S. 2018. Complete genome sequence of Vibrio coralliilyticus RE22, a marine bacterium pathogenic toward larval shellfish. Microbiology Resource Announcements. 7(17) doi: 10.1128/MRA.01332-18.
- Kingsley, D.H., Chen, H., Meade, G.K. 2018. Persistence of MS-2 bacteriophage within Eastern oysters *(Crassostrea virginica*). Food and Environmental Virology 10(1):83-88.
- Richards, G.P., Needleman, D.S., Watson, M.A., Polson, S.W. 2019. Whole-genome sequences of two Pseudoalteromonas piscicida strains, DE1-A and DE2-A, with strong antibacterial activity against Vibrio vulnificus. Microbiology Resource Announcements. 8(1) https://doi: 10.1128/MRA.01451-18.
- Richards, G.P., Needleman, D.S., Watson, M.A., Polson, S.W. 2019. Whole-genome sequences of two Pseudoalteromonas piscicida strains, DE1-A and DE2-A, with strong antibacterial activity against Vibrio vulnificus. Microbiology Resource Announcements. 8(1) https://doi: 10.1128/MRA.01451-18.
- Kingsley, D.H., Chen, H.C., Annous, B.A., Meade, G.K. 2019. Evaluation of a male-specific DNA coliphage persistence within eastern oysters (*Crassostrea virginica*). Food and Environmental Virology 11:120-125.

Intervention and Control Strategies: Plant Crops/Produce

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
A March 2013 report from the CDC showed that almost half [46%] of all foodborne illnesses that led to hospitalization and or deaths between1998-2008 were attributable to fresh produce. Two major E. coli O157:H7 outbreaks (2006) with spinach and cut-lettuce were a major impetus for change by regulatory agencies and industry. The report also caught the attention of consumers that consumption of contaminated fresh fruit and vegetables could make you sick, or even kill you. Subsequent research on contamination pathways (some by this Program) has shown the complexity of the situation, and the need for more specific data on sources, pathways of contamination, and persistence of enteric pathogens on fresh produce. Preharvest research conducted at WRRC, Albany, California relative to produce is found Under Problems Statement 1, Population Systems.

Postharvest studies were conducted to develop and validate new and effective chemical and physical decontamination interventions for produce and/or improve the performance of current interventions to reduce pathogens on fresh produce implemented at fresh-cut packing facilities; understand ecological factors that influence treatment decontamination efficacy; and develop and evaluate process models, including economic analysis models, in order to facilitate technology transfer and commercial adoption of interventions and intervention combinations.

This research was conducted through a project at BARC, Beltsville, MD (Patel) and several projects at ERRC, Wyndmoor, PA (Niemira, Fan, Geveke and Sommers).

Studies at BARC addressed the following objectives through a large multidisciplinary project (Patel) to: (1) Investigate intervention strategies to minimize contamination of EHEC, Salmonella and Listeria on fresh produce at the farm level; and (2) Develop effective intervention technologies to reduce pathogen survival and growth during processing and retail operations.

Examples of Accomplishments

- **Sanitizer concentration is critical during produce wash to reduce food-safety risk.** Improper washing can spread bacteria and increase health-risk associated with consumption of fresh produce. Fresh produce processors traditionally have used a specific free-chlorine level [1 ppm (part per million)] as the "Control Limit" and a re-wash as the "Corrective Action" in Hazard Analysis and Critical Control Points (HACCP) programs. ARS research clearly documented significant risk factors associated with "generally-considered-safe" operating practices. Follow up studies further demonstrated that a minimum of 10 ppm free-chlorine was required to effectively prevent pathogen cross-contamination during washing.

- **Rapid method to determine free chlorine levels in wash water during commercial-scale washing of fresh-cut produce.** Substances released into the water during the cut produce during washing interact with the chlorine, sanitizer and reduce its efficacy. Maintenance of the level of free chlorine, a form that can interact with contaminants, is critical to avoid cross-contamination from bacteria during commercial-scale washing of fresh-cut produce. Controlling the sanitizer levels needed in fresh-cut produce wash water could improve if the chlorine demand is known in real-time. ARS developed a rapid method using ultraviolet light absorbance to estimate chlorine demand for produce wash conditions. Ultraviolet light absorbance of the wash water was measured at two wavelengths. Based on these measurements, a predictive model for chlorine demand was developed and tested. The method shows promise for real-time application during commercial-scale washing of fresh-cut produce.

- **Standard operating procedures are required to control contamination on tomatoes during wash process.** In collaboration with Florida tomato growers and packers, studies identified and quantified key operational parameters to prevent Salmonella survival and cross-contamination during tomato dump tank wash process.

- **Zero-valent iron filtration improves microbial and chemical irrigation water quality.** ARS designed and optimized a zero-valent iron (ZVI) filtration system which can be used by small farmers in their irrigation water systems. Results showed that different ZVI systems consistently reduced levels of antibiotics and bacterial pathogens (E. coli, Listeria monocytogenes) in surface and reclaimed wastewater compared to sand filtration.

- **Natural antimicrobials control enteric pathogens on fresh herbs.** Plant-based essential oils were evaluated on fresh herbs (basil, cilantro, dill, parsley, and tarragon) for their antimicrobial activities against Salmonella and E. coli O157:H7. Treatments with specific concentrations of carvacrol or cinnamaldehyde killed E. coli O157:H7 and Salmonella on fresh herb leaves. There was no visual difference in herbs treated at lower concentrations of cinnamaldehyde or carvacrol. Similarly, cinnamaldehyde and Sporan significantly reduced Salmonella in biofilms formed on equipment surfaces.

Outcomes and Impact

- The research findings were used by the FDA as a scientific basis for the newly released “Draft Guidance for Industry: Guide to Minimize Food Safety Hazards of Fresh-cut Produce” to implement science- and risk-based food safety policies in support of the Produce Rule in FSMA.
- The research findings were used by industry and a multi-agency taskforce to develop "Guidelines to Validate Control of Cross-Contamination during Washing of Fresh-Cut Leafy Vegetables”

- The research findings for tomato's were used by industry to develop "Commodity Specific Food Safety Guidelines for the Fresh Tomato Supply Chain," a critical food safety standard.
- The developed and validated predictive model for chlorine demand is currently being tested for real-time application during commercial-scale washing of fresh-cut produce.
- Designed and determined that Zero-valent iron filtration (ZVI) is a promising mitigation treatment for small farmers to reduce chemical and microbial contaminants in agricultural irrigation waters.
- Determined that organic fresh produce processors can use natural sanitizers to remove biofilm from equipment surfaces and minimize foodborne illnesses associated with consumption of fresh produce. Further, these natural antimicrobials can be used to kill E. coli O157:H7 without affecting the color attributes of fresh herbs.

Examples of Relevant Publications

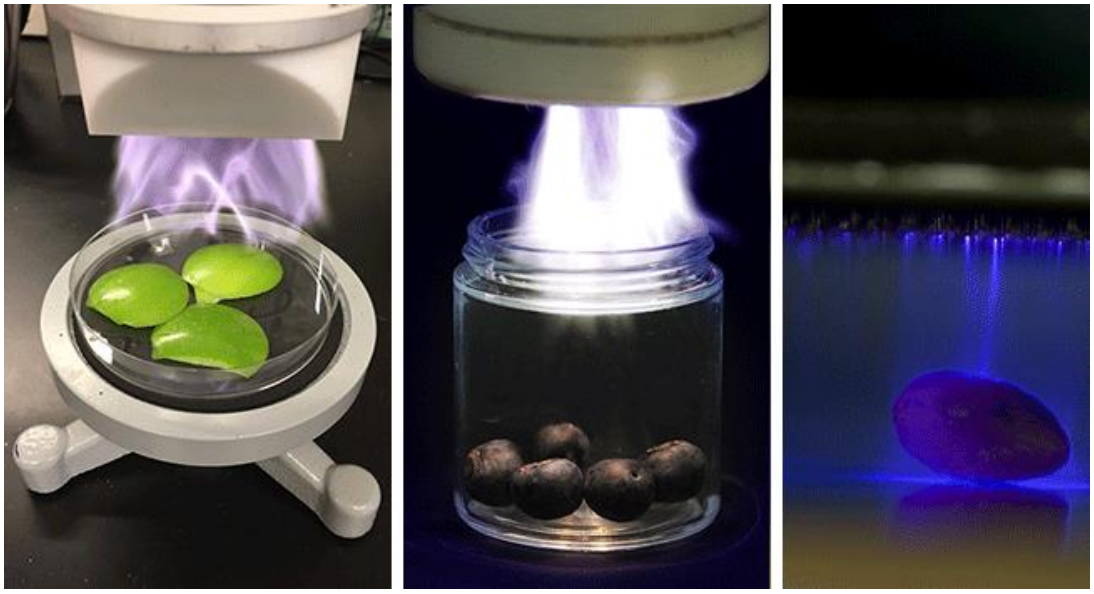
- Gombas, D., Luo, Y., Brennan, J., Shergill, g., Petran, R., Walsh, C., Khurana, K., Zomorodi, B., Rosen, J., Varley, R., Deng, K. 2017. Guidelines to validate control of cross-contamination during washing of fresh-cut leafy vegetables. Journal of Food Protection. 80(2):312-330.
- Bornhorst, E, R., Luo, Y., Park, E., Vinyard, B. T., Nou, X., Zhou, B., Turner, E, and Millner, P. D. 2018. Immersion-free, single-pass, commercial fresh-cut produce washing system: an alternative to traditional flume processing. Postharvest Biology and Technology. 146: 124-133.
- Teng, Z., Van Haute, S., Zhou, B., Hapeman, C., Millner, P. D., Wang, Q., and Luo, Y. 2018. Impacts and interactions of organic compounds with chlorine sanitizer in recirculated and reused produce processing water. PLOS One. 1-15.
- Luo, Y., Zou, B., Van Haute, S., Nou, X., Zhang, B., Teng, Z., Turner, E., Wang, Q., and Millner, P.D. 2018. Association between bacterial survival and free chlorine concentration during commercial fresh-cut produce wash operation. Food Microbiology. 70:120-128.
- Van Haute, S., Luo, Y., Samplers, I., Lei, M., Teng, Z., Zhou, B., Bornhorst, E.R, Wang, Q., and Millner, P.D. 2018. Can UV absorbance rapidly estimate the chlorine demand in wash water during fresh-cut produce washing processes? Postharvest Biology and Technology. 142:19-27.
- Patel, J., Keelara, S., Green, J. 2018. Inactivation of Escherichia coli O157:H7 and Salmonella on fresh herbs by plant essential oils. Foodborne Pathogens and Diseases. 15: 1-7.
- Kulkarni, P., Raspanti, G.A., Bui, A.Q., Bradshaw, R.N., Kniel, K.E., Chiu, P.C., Sharma, M., Sapkota, A, Sapkota, A.R. 2019. Zerovalent iron-sand filtration can reduce the concentration of multiple antimicrobials in conventionally treated reclaimed water. Environmental Research. 172: 301-309.

Studies at ERRC, Wyndmoor, PA were conducted through four sister projects (Niemira, Fan, Geveke and Sommers) whose overarching goal was the development of postharvest intervention strategies. New and/or improved intervention technologies would be developed, validated and optimized. Studies would consist of biological (antagonist-based biocontrols), chemical (sanitizer formulations), physical (nonthermal processing), and innovative packaging approaches. These will be developed in an iterative approach, with advances in each serving as drivers for optimization of combinatorial intervention strategies.

The (Niemira) project objectives were to: Develop and evaluate novel antagonists (e.g. Bacteriovorax, Bdellovibrio and non-pectolytic Pseudomonas) for biological-based intervention strategies, and identify means of combining pathogen microbial ecology with effective chemical and physical interventions; (2) Develop and optimize chemical decontamination interventions (e.g. novel sanitizer formulations and advanced gas-phase antimicrobial treatments), making use of pathogen microbial ecology information generated under (1) and (3) Develop nonthermal technologies (e.g. cold plasma, high-intensity monochromatic light and irradiation) as effective, waterless physical treatments, and establish protocols for combination treatments with interventions developed in (1) and (2).

Examples of Accomplishments

- **Cold plasma as a chemical-free antimicrobial process.** Fresh produce continues to be a leading source of foodborne illness. ARS developed cold plasma as a waterless, chemical-free, nonthermal treatment to kill Escherichia coli O157:H7, Salmonella, norovirus, Cryptosporidium, and other pathogenic bacteria, viruses, and parasites.



- **Process optimization of cold plasma.** Cold plasma is one of the most important antimicrobial processes to emerge in recent years, but much is still unknown about optimum treatment applications when used singly or in combination with other food safety hurdle technologies. The introduction of simple water vaper into the cold plasma ionization stream significantly increased the kill efficiency through the creation of peroxides. The required contact time to achieve a 99.99% reduction of L. monocytogenes on apples was subsequently reduced from 45-minutes to less than 3-minutes. Studies achieved precision temperature control of the plasma stream via integration of vortex tube cooling, a small-footprint refrigeration technology that uses no electricity, pumps, coolant or moving parts.

- **Natural antimicrobials control E. coli O157:H7 and Salmonella.** Sophorolipids are biological surfactants with antimicrobial properties produced by microbial agents. Their biodegradable potential, wide functionality and non-toxic properties make them ideally suited for pathogen control. ARS established that L. monocytogenes, Salmonella and E. coli O157:H7 were reduced by up to 99.999% on tomatoes by treatment with sophorolipids. Higher levels of kill were achieved by greater concentrations of sophorolipids, or combination of sophorolipids with conventional sanitizers. After 24-hours of storage, the effects were more pronounced at 25C than at 5C.

- **Predator drones - Bdellovibrio biocontrol of E. coli O157:H7 and Salmonella.** Bdellovibrio is a natural predator of Gram-negative bacteria, but its application for decontamination of foodborne pathogens on postharvest produce is largely unexplored. Studies deployed the predator species Bdellovibrio bacteriovorus 109J (Bb109J) against Escherichia coli O157:H7 and Salmonella in a buffer solution, on carrots, and on tomatoes. In all test conditions, both pathogens were effectively killed, with reductions ranging up to 99.99%.

- **On-demand and in-package chlorine dioxide.** Effective antimicrobial treatments that can improve the safety of packaged fresh produce. ARS worked with collaborators to develop an innovative means of generating chlorine dioxide inside a package, on-demand. This antimicrobial gas is generated with two precursor compounds are mixed. By incorporating these precursors into separate layers stacked one on top another within a special label, the chlorine dioxide can be generated at the right time and concentration, simply pressing the label and driving the layers together.

- **Bringing the heat to produce safety.** Thermal treatment is a widely used food safety intervention, but application to fresh and fresh-cut produce can lead to unwanted sensory damage. ARS developed a suite of thermal treatments that can reduce Salmonella, L. monocytogenes, and E. coli O157:H7 on a range of produce commodities, while retaining or improving taste, color, and aroma. Using precisely temperature-controlled wash process, water at 76C inactivates pathogens on mung beans, cantaloupe and green tomatoes up to 99.999%. For mung beans, post-treatment sprouting, and sprout quality were unaffected. The ripening of green tomatoes was indistinguishable from the non-thermally treated tomatoes. When hot water dip-treated cantaloupe was

peeled and sliced, the resulting cut fruit had a significantly longer shelf life, with improved quality throughout refrigerated storage.

Outcomes and Impact

- Successfully applied the cold plasma process to a variety of produce items, including blueberries, apples, oranges, lettuce, tomatoes, and packaged cut salads. Depending on the treatment conditions, these pathogens have been reduced by up to 99.999%. Since the process uses only air and electricity as inputs, cold plasma will be a green, sustainable antimicrobial treatment suitable for improving the safety of fresh and fresh-cut fruits and vegetables.
- Demonstrated that pre-treatment with cold plasma significantly enhances the antimicrobial efficacy of chemical sanitizers. Together this suite of research efforts is paving the way for pre-commercialized pilot scale development of cold plasma as a sanitizing treatment for a wide range of foods and food contact surfaces.
- Established that various foodborne pathogens were reduced by up to 99.999% on tomatoes by treatment with sophorolipids, or combination of sophorolipids with conventional sanitizers. This naturally occurring biomolecule, alone or in combination with chemical sanitizers, can enhance the safety of fresh and fresh-cut produce.
- Determined that Bdellovibrio could be used as a biocontrol system to improve the safety of fresh and fresh-cut fruits and vegetables. Studies continue to further enhance the kill efficiency, to improve the effective range of treatment conditions, and to optimize their use as a commercial intervention.
- Developed an innovative means of generating chlorine dioxide inside a package, on-demand. This flexible, adaptable, and commercially scalable technology can be integrated into a wide range of packaging designs to improve the safety of foods.
- Developed a suite of thermal treatments that can reduce various foodborne pathogens on a range of produce commodities, while retaining or improving taste, color, and aroma. This adaptable, flexible, and inexpensive hot water dip treatment enhances the safety and shelf-life of fruit and vegetable commodities.

Examples of Relevant Publications

- Min, S., Roh, S., Niemira, B.A., Sites, J.E., Boyd, G., Lacombe, A. 2016. Dielectric barrier discharge atmospheric cold plasma inhibits Escherichia coli 0157:H7, Salmonella, Listeria monocytogenes, and Tulane virus in Romaine lettuce. International Journal of Food Microbiology. 237:114–120.
- Ukuku, D.O., Mukhopadhyay, S., Geveke, D.J., Olanya, O.M., Niemira, B.A. 2016. Minimal thermal treatments for reducing bacterial population on cantaloupe rind surfaces and transfer to fresh-cut pieces. Journal of Food Protection. doi: 10.4315/0362-028X.JFP-16-046.
- Lacombe, A.C., Niemira, B.A., Gurtler, J., Sites, J.E., Boyd, G., Kingsley, D.H., Li, X., Chen, H. 2017. Nonthermal inactivation of norovirus surrogates on blueberries using atmospheric cold plasma. Food Microbiology. 63:1-5.

- Berrios-Rodriguez, A., Olanya, O.M., Annous, B.A., Cassidy, J.M., Orellana, L., and Niemira, B.A. 2017. Survival of Salmonella Typhimurium on soybean sprouts following treatments with gaseous chlorine dioxide and biocontrol Pseudomonas bacteria. Food Sci. Biotechnol. 26(2):513-520.
- Rodriguez, A., Olanya, O.M., Annous, B.A., Cassidy, J.M., Orellana, L., Niemira, B.A. 2017. Survival of Salmonella Typhimurium on soybean sprouts after treatment with gaseous chlorine dioxide and biocontrol Pseudomonas bacteria. Food Science and Biotechnology. 26:513-520.
- Alicea, C., Annous, B.A., Mendez, D.P., Burke, A., and Orellana, L.E. 2018. Evaluation of hot water, gaseous chlorine dioxide, and chlorine treatments in combination with an edible coating for enhancing safety, quality, and shelf-life of fresh-cut cantaloupes. J. Food Prot. 81: 534-541.
- Kingsley, D.H., Perez, R., Niemira, B.A., Fan, X. 2018. Evaluation of gaseous chlorine dioxide for the inactivation of tulane virus on blueberries. International Journal of Food Microbiology. 273:23-32.
- Min, S.C., Roh, S., Niemira, B.A., Boyd, G., Sites, J.E., Fan, X., Sokorai, K.J., Jin, Z.T. 2018. In-Package atmospheric cold plasma treatment of bulk grape tomatoes for their microbiological safety and preservation. 108:378-386.
- Olanya, O.M., Ukuku, D.O., Solaiman, D., Ashby, R.D., Niemira, B.A., Mukhopadhyay, S. 2018. Effects of temperature and storage time on inactivation of Listeria monocytogenes, Salmonella enterica, and Escherichia coli 0157:H7 populations by sophorolipid and sanitizer in-vitro and on tomato. International Journal of Food Science and Technology. http://dx.doi.org/10.1111/ijfs.13711.
- Olanya, O.M., Ukuku, D.O., Solaiman, D., Ashby, R.D., Niemira, B.A., Mukhopadhyay, S. 2018. Effects of temperature and storage time on inactivation of Listeria monocytogenes, Salmonella enterica, and Escherichia coli 0157:H7 populations by sophorolipid and sanitizer in-vitro and on tomato. International Journal of Food Science and Technology. http://dx.doi.org/10.1111/ijfs.13711.
- Saade, C., Annous, B.A., Gualtieri, A.J., Schaich, K.M., Liu, L., and Yam, K.L. System feasibility: designing a chlorine dioxide self-generating package label to improve 2018. fresh produce safety part II: Solution Casting Approach. Inno. Food Sci. Emerg. Technol. 47:110-119. 2018. http://doi.org/10.1016/j.ifset.2018.02.003
- Ukuku, D.O., S. Mukhopadhyay, O.M. Olanya, and B.A. Niemira. 2019. Antimicrobial treatments and cold storage inactivates injured Salmonella bacteria on melon surfaces and fresh-cut pieces. J. Food Process. and Preservation Vol. e13943, 10pg. 2019. https://doi.org/10.1111/jfpp.13943

The (Fan) project objectives were to: (1) Develop and optimize single intervention technologies to reduce pathogen populations, maintain quality, and extend shelf-life of foods; and (2) Determine the synergistic/additive effectiveness of combining non-thermal processing, antimicrobial packaging and effective chemical interventions utilizing information generated from the first objective.

Examples of Accomplishments

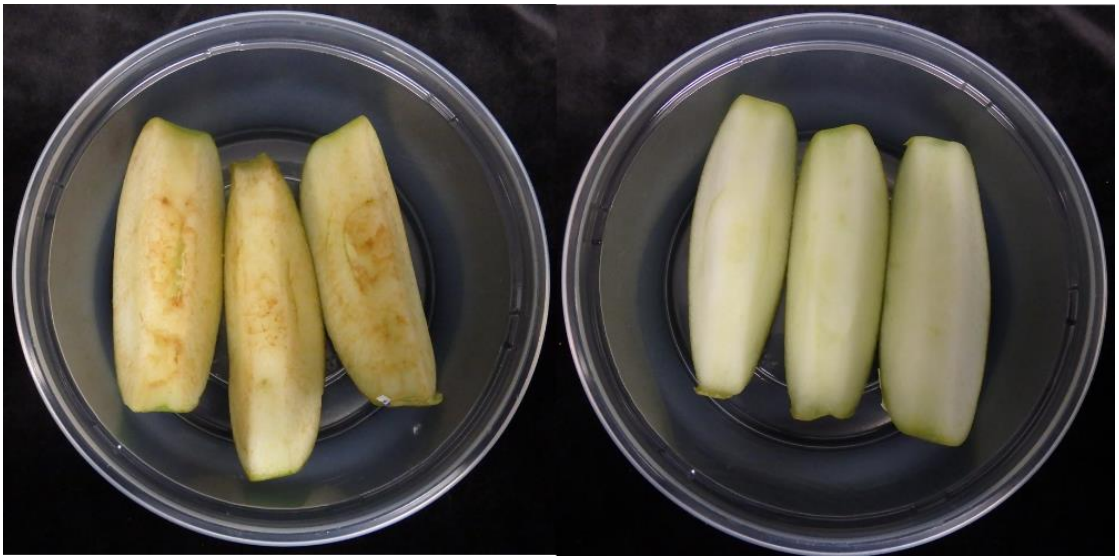
- **Food containers with antimicrobial surface.** It is critical to have a pathogen-free food containers. ARS developed methods and coating formulas to produce food containers with antimicrobial surface. Specifically, titanium dioxide nano-powders incorporated into zein or other polymers were used to form antimicrobial coating on the container surface. The surface coatings were activated by visible light to inactivate E. coli O157:H7 on the container surface, which achieved more than 99.7% reductions of the pathogen.

- **Novel antimicrobial phenolic fatty acids.** Currently used antimicrobials have limited effectiveness and antibiotic resistance of bacteria has become more common. ARS synthesized novel phenolic fatty acids from natural agricultural byproducts and evaluated their antimicrobial properties against both Gram-positive and Gram-negative bacteria. Studies showed that the compounds at low parts per million concentrations effectively inactivate Gram-positive bacteria. Further research indicated that the carboxylic group in the fatty acid moiety and the hydroxyl group on the phenol moiety were responsible for the antimicrobial efficacy.

- **Aerosolized antimicrobials to enhance microbial safety of fresh produce.** Washes with sanitizers such as chlorine have limited effectiveness against human pathogen on fresh produce, partially due to inability of the aqueous antimicrobials in reaching bacteria. Studies aerosolized several FDA-approved sanitizers and activated hydrogen peroxide aerosol using cold plasma to inactivate E. coli, Salmonella and Listeria inoculated onto leafy greens, apple, tomato, and cantaloupe. Studies showed that populations of bacteria on the surfaces of the fresh produce items could be reduced by more than 99.99% depending on types of inoculated bacteria and produce items.

- **Novel edible antimicrobial coatings for the reduction of foodborne pathogens.** Ready-to-eat foods, such as deli meat and fresh fruits, could be contaminated with foodborne pathogens. ARS developed edible antimicrobial coating to inactivate foodborne pathogens on deli meat and fresh strawberries, using the combination of high-pressure homogenization technology and bio-emulsifiers from plant byproduct. The coating treatments reduced 99% - 99.9% of Listeria, Salmonella and Escherichia coli O157:H7.

- **Novel antibrowning and antimicrobial formulation for cut apples.** There have been several recalls of cut apples due to contamination with Listeria monocytogenes. Studies evaluated the combinations of organic acids and various antioxidants for their anti-listerial and anti-browning properties. Results showed that formulations comprised of citric acid (a fruit acid), ascorbic acid (vitamin C), and N-acetyl-L-cysteine (an amino acid) were able to reduce populations of L. monocytogenes by more than 99.999% and at the same time, inhibited the surface browning of cut apples for at least 21 days.



- **Antimicrobial films used for in-package pasteurization.** Foodborne pathogens and spoilage fungi may reside in fresh produce after packaging. ARS developed antimicrobial films that released allyl isothiocyanate vapor (a natural flavor compound from mustard) into headspace of the container and inhibited E. coli and fungi growth in fresh tomatoes stored at 4 and 10 °C for 21 days. The treatment reduced the populations of bacteria and molds by 99 - 99.9% on tomatoes, and treated fruit had less changes in quality and nutritional values during storage than the non-treated samples.

- **Integrated interventions of processing and coating.** Currently the produce industry employs chlorine to avoid cross contamination during processing despite its limited ability to reduce pathogens, and potential of forming possible carcinogenic chlorine by-products in wash water. ARS developed a new chlorine-free decontamination technology which was safe and effective by combining a pulsed light treatment with a sanitizer wash. This integrated technology inactivated > 99.999% of pathogens such as E. coli O157:H7 in spinach. The combination treatment was also effective in controlling native microbial loads during 21 days of refrigerated storage. Furthermore, firmness and the visual appearance of spinach were not affected by the treatment.

Outcomes and Impacts

- Demonstrated that the developed methods and formula can be applied on different types of containers made of metal, wood, plastics, paperboard, etc., and for various foods, specially fruits and vegetables.
- Demonstrated that the novel class of phenolic fatty acids has potential for use as antimicrobials against Gram-positive bacteria. Two U.S. Patents were granted.
- Developed an aerosolization technology represents a novel and effective method to enhance microbial safety of fresh produce.
- Developed an edible antimicrobial coating to inactivate foodborne pathogens on ready-to-eat food strawberries.
- Developed formulations, if adopted by the fresh produce industry, may reduce the risk of Listeria contamination and maintain shelf life of cut apples.
- Developed antimicrobial film has the potential to enhance the safety and extend the shelf-life of perishable fresh produce.
- Developed a new integrated method of pulsed light treatment with the new formula sanitizer wash as a replacement for current chlorine-based industrial practice. Fortunately, pulsed light is an FDA approved technology while all components of the sanitizer are generally recognized safe compounds.
- Established CRADAs with TOMI Environmental Solution for application of ionized hydrogen peroxide aerosols to enhance microbial safety of fresh produce. Two MTRAs have been established with the University of Delaware and University of Minnesota on application of non-thermal food technologies.

Examples of Relevant Publications

- Ngo, H., K. Wagner, Z. Yan, A. Nuñez, W. Yee, X. Fan, R.T. Moreau. 2017. Synthesis, chemical characterization and economical feasibility of poly-phenolic-branched-chain fatty acids. European Journal of Lipid Science and Technology. Eur. J. Lipid Sci. Technol. 118, 1600380.
- Fan, X., Wagner, K., Sokorai, K.J.B., and Ngo. H. 2017. Inactivation of Gram-positive bacteria by novel phenolic branched-chain fatty acids. J. Food Protect 80(1):6-14.
- Guo, M., Yadav, M.P. and Jin, T.Z. 2017.Antimicrobial edible coatings and films from micro-emulsions and their food application. Internal Journal of Food Microbiology. 263, 9-16.
- Jiang, Y., Sokorai, K.J., Pyrgiotakis, G., Demokritou, P., Li, X., Jin, Z.T., Mukhopadhyay, S., Fan, X. 2017. Cold plasma-activated hydrogen peroxide aerosol inactivates Escherichia coli 0157:H7, Salmonella Typhimurium, and Listeria innocua and maintains quality of grape tomato, spinach and cantaloupe. International Journal of Food Microbiology. 249: 53-60.
- Gao, H., Wu, W, Chen, H., Qin, Y., Fang, X., Jin, T. Z. 2018. Microbial inactivation and quality improvement of tomatoes treated by package film with allyl isothiocyanate vapor. International Journal of Food Science and Technology. 53, 1983-1991.
- Guo, M., Jin, T.Z., Gurtler, J., Fan, X., Yadav, M.P. 2018. Inactivation of Escherichia coli O157: H7 and Salmonella and Native Microbiota on fresh strawberries by antimicrobial washing and coating. Journal of Food Protection. 81 (8), 1227-1235.

- Mukhopadhyay, S., Sokorai, K.J., Ukuku, D.O., Jin, Z.T., Fan, X., Olanya, O.M., Juneja, V.K. 2018. Inactivation of Salmonella in tomato stem scars by organic acid wash and chitosan-allyl isothiocyanate coating. International Journal of Food Microbiology. 266: 234-240.
- Fan, X., Sokorai, K.J.B., Phillips, J. 2018. Development of antibrowning and antimicrobial formulations to minimize Listeria monocytogenes contamination and inhibit browning of fresh-cut "Granny Smith" apples. Postharvest Biol. Technol. 143: 43–49.
- Mukhopadhyay, S., Sokorai, K.J., Ukuku, D.O., Fan, X., Olanya, O.M., Juneja, V.K. 2019. Effects of pulsed light and sanitizer wash combination on inactivation of Escherichia coli 0157:H7, microbial loads and apparent quality of spinach leaves. Food Microbiology, 82:127-134.

The (Geveke) project objectives were to: Further studies on the ARS-patented use of RFP for shell eggs through the development of pilot plant and commercial prototypes of continuous RFP equipment for multiple eggs; (2) Further studies on the use of innovative technologies to reduce microorganisms on fresh produce, and minimally preserved, brined, and fresh-cut refrigerated vegetables; and (3) Evaluate the use of biochars to reduce pathogens in manures, compost, and soils used for the production of fresh (both conventional and organic) produce.

Examples of Accomplishments

- **Novel antimicrobial solution.** Research developed a nisin-based organic acid sanitizer that reduces bacteria on fruits and vegetables to below detection. Further studies lead to and integrated approach with other nonthermal intervention technologies for solving and enhancing microbial food safety.

- **Browning reaction on fresh-cut fruits.** Browning leads to wastage, shortage of foods designated for consumers, and a loss of million dollars to the fresh-cut industry. ARS studies developed an antibrowning-antimicrobial solution that works as 2 for one step processing for the fresh-cut industry.

- **Selection of surrogate bacteria for biochar-amended soil studies.** Conducted studies to evaluate the survival of 22 strains of avirulent E. coli and Salmonella in crop soil with 10% fast-pyrolysis switchgrass biochar. The goal was to validate effective surrogate strains of bacteria to use in future studies. After screening 22 non-pathogenic strains of Salmonella and E. coli for survival in biochar-amended soils, three strains of E. coli were chosen as the best surrogates capable of surviving in biochar/soil matrices: strains E. coli TVS-354, TVS-355, and TVS 353.

- **Antimicrobial nature of biochar varies based on production temperature and age.** Studies were conducted to evaluate three fast-pyrolysis-generated biochars (FPBC) (pyrolyzed in-house at 450°C, 500°C and 600°C in a newly-designed pyrolysis

reactor) to determine their effects on the viability of four surrogate strains of Escherichia coli O157:H7 in soil. Additionally, a previously biocidal fast-pyrolysis biochar was aged two years and tested against E. coli to determine changes in antibacterial efficacy over time. While neither the 450° and 500°C FPBC from the new reactor proved antimicrobial, the 600°C biochar proved biocidal over 7 w of sampling with populations significantly reduced at the 3 percent and 3.5 percent concentration (5.34 and 5.84 log CFU/g, respectively) compared with concentrations of 0.0-2.0 percent. The aged 500°C biochar from the older reactor, previously shown to be antimicrobial, demonstrated a loss of efficacy after aging for two years.

- **Inactivation of E. coli O157:H7 in manure by supplementing with biochar.** Questions remained as to whether biochar could inactivate bacterial pathogens in dairy compost. Fresh dairy compost was supplemented with 10% walnut cyclone biochar from a collaborator. The no-biochar control compost maintained high levels of E. coli up to week 7 (8.51 log). However, the 10% biochar supplement inactivated 7.95 log of the pathogen within 7 days. No E. coli was detected in the biochar-supplemented compost throughout 7 weeks of storage which was attributed to high pH in compost.

Outcomes and Impacts

- Invention disclosures have been filed for both the antimicrobial and browning solutions. and work is in progress with an industry partner for possible licensing and Patent application.
- Demonstrate that the biocidal nature of FPBC varies based on production temperature and/or age of biochar.
- Demonstrated that biochar is antimicrobial to foodborne pathogens in crop soil and will provide guidance on the application of biochar added to compost or soil to inactivate foodborne pathogens, thus making produce production safer.

Examples of Publications

- Gurtler, J.B., M.P. Doyle, M.C. Erickson, X. Jiang, P. Millner, and M. Sharma. 2018. Aerobic Composting to Inactivate Foodborne Pathogens for Crop Soil Application: A Review. *J. Food Prot.* 81:1821-1837.
- Ukuku, D.O., Niemira, B.A., Uknalis, J. 2019. Nisin-based antimicrobial combination with cold plasma treatment inactivate Listeria monocytogenes on granny smith apples. LWT - Food Science and Technology. Volume 104, Pages 120-127. https://doi.org/10.1016/j.lwt.2018.12.049.
- Gurtler, J.B. X. Fan, T. Jin and B.A. Niemira. 2019. Effects of Antimicrobial Agents on the Thermal Sensitivity of Foodborne Pathogens: A Review. *J. Food Prot.* 82:628-644.
- Ukuku D. O. 2019.A Novel solution for Reducing Browning Reaction and Bacterial Populations On Fresh-cut Fruits. USDA Docket No 0052.19. August 14, 2019.

The fourth project (Sommers) was a new research direction that focussed on extraintestinal Escherichia coli (ExPEC). The objectives were to: (1) Develop and validate non-thermal and thermal intervention technologies to inactivate pathogens and spoilage microorganisms in raw and ready-to-eat foods and food contact surfaces; and (2) Examine any relationship between genotype (virulence factors) and pathogen resistance to interventions.

Examples of Accomplishments

- **Extraintestinal pathogenic E. coli that cause urinary tract infections, sepsis, and avian colibacillosis are in retail poultry meat.** Extraintestinal pathogenic E. coli (ExPEC) are associated with sepsis, urinary tract infections and sicken >10 million and kill >40,000 people per year in the U.S. at a cost of >$20 billion annually. They also cause avian colibacillosis, negatively affect the poultry industry, and may be present in retail poultry meat. Studies of retail poultry meat found that 12.6% of E. coli isolated from the skin of retail poultry parts were ExPEC, while over 10% of E. coli isolated from ground chicken were ExPEC, and over 75% of the ExPEC isolates were resistant to multiple antibiotics including extended beta-lactamases.

- **Inactivation of uropathogenic Escherichia coli (UPEC) in ground chicken or chicken purge by high pressure processing, gamma radiation, and ultraviolet light.** High pressure processing, gamma radiation, and ultraviolet light are sustainable food safety technologies that can kill harmful bacteria in meat and poultry. Uropathogenic E. coli are an emerging and common contaminant in poultry meat and are associated with urinary tract infections which affect over 10 million people, primarily women, each year including 23,000 deaths. Studies found that high pressure processing (500 MPa, 4 C, 4.43 min), gamma radiation (1.3 kGy at 4 C or 1.6 kGy at -20 C), or ultraviolet light (125 mJ/cm2) killed 99.999% of UPEC in chicken meat or chicken purge.

- **Various interventions to kill ExPEC.** ARS in collaboration with the Department of Veterans Affairs and the National Taiwan University determined: (a) The radiation resistance, an FDA approved process, for ExPEC suspended in ground chicken meat. Studies showed that a very modest radiation dose of 0.30 kGy kills 90% of UPEC and ExPEC in chicken meat. There was no correlation between ExPEC genotype and radiation resistance; (b) Cooking ground chicken meat for 1 min at 65C killed 99.999 % of ExPEC in ground chicken. Developed a mathematical equation to describe the process. Conducted WGS to determine the specific virulence factors that could affect their survival to heat; and (c) That 600 MPa of pressure to chicken meat for 5 min at 4C killed 99.999 % of ExPEC in ground chicken. From WGS found specific virulence factors; e.g. chuA, cnf1, sinH, papG, hlyA, vat, yncD) that could affect their survival to high pressure processing.

- **Staphylococcus saprophyticus, a foodborne pathogen killed by intervention technologies.** Staphylococcus saprophyticus is a foodborne pathogen responsible for ca. 600,000 urinary tracts in women annually. Studies found that cooking for 1 min at 65C, a gamma radiation dose of 3.1 kGy, or high-pressure processing at 600 MPa for 5 min killed 99.999% of S. saprophyticus suspended in ground chicken meat.

Outcomes and Impacts

- Determined that ExPEC were found in poultry meat with over 75% resistant to multiple antibiotics. Determined that high pressure processing, gamma radiation and ultraviolet light killed 99.999% of UPEC. Determined that heat, radiation, and high pressure killed 99.999% of ExPEC, and Staphylococcus saprophyticus. This data is critically important to regulatory agencies, industry and consumers, especially those who are immuno-compromised.

Examples of Relevant Publications

- Sommers, C.H., Scullen, O.J., Mackay, W. 2016. Inactivation of Staphylococcus saprophyticus in chicken meat and exudate using high pressure processing, gamma radiation, and ultraviolet light. Food Control. 75:78-82.
- Sommers, C.H., Gunther, N.W., Sheen, S. 2016. Inactivation of foodborne pathogens in chicken purge or skin using a 405-nm LED array. Food Microbiology. 64:135-138.
- Li, C., Hsu, H., Wang, Y., Cassidy, J.M., Sheen, S., Liu, S. 2017. Effects of heat treatment on antioxidative and anti-inflammatory properties of orange byproducts. The Royal Society of Chemistry. https://doi: 10.1039/C7FO00188F.
- Sheen, S., Huang, C., Ramos, R.V., Chien, S., Scullen, O.J., Sommers, C.H. 2018. Lethality prediction for Escherichia coli 0157:H7 and Uropathogenic E. coli in ground chicken treated with high pressure processing and transcinnamaldehyde. Journal of Food Science. 83(3):740-749.
- Xu, A., Hertrich, S.M., Needleman, D.S., Sheen, S., Sommers, C.H. 2018. Draft genome sequences of four uropathogenic Escherichia coli 04:H5 isolates (ATCC 700414,700415,700416 and 700417). Genome Announcements. 6(11):e00134-18.
- Xu, A., Johnson, J., Sheen, S., Needleman, D.S., Sommers, C.H. 2018. Draft genomic sequencing of six potential extraintestinal pathogenic Escherichia coli isolates from retail chicken meat. Genome Announcements. https://doi.org/10.1128/genomeA.00449-18.
- Xu, A., Johnson, J., Sheen, S., Sommers, C.H. 2018. Draft genome sequences of six neonatal meningitis-causing Escherichia coli isolates (SP-4, SP-5, SP-13, SP-16, SP-46, and SP-65). Genome Announcements. https://doi.org/10.1128/genomeA00091-18.
- Chien, S., Sheen, S., Sommers, C.H., Sheen, L. 2018. Effects of combined treatments of high- pressure processing, single-and multi-antimicrobial (Melissa officinalis extract) on the reduction of pathogenic Escherichia coli in ground beef. Food and Bioprocess Technology. 12:359-370. https://doi.org/10.1007/s11947-018-2211-5.
- Xu, A., Abdul Wakeel, A.Y., Gunther, N.W., Sommers, C.H. 2019. Draft genomic sequence of Campylobacter coli isolated from chicken carcasses. Microbiology Resource Announcements. 8(28):e00564-19. https://doi.org/10.1128/MRA.00564-19

- Xu, A., Mackay, W., Sommers, C.H. 2019. Draft genomic sequence of multi-drug resistant Klebsiella pneumoniae B8S35, isolated from retail chicken skin. Microbiology Resource Announcements. 8(28):e00502-19. https://doi.org/10.1128/MRA.00502-19
- Xu, A., Scullen, O.J., Sheen, S., Johnson, J., Sommers, C.H. 2019. Inactivation of extraintestinal pathogenic E. coli clinical and food isolates suspended in ground chicken meat by gamma radiation. Food Microbiology. https://doi.org/10.1016/j.fm.2019.103264.
- Xu, A., Mackay, W., Scullen, O.J., Ramos, R.V., Sheen, S., Sommers, C.H. 2019. Draft genomic sequence of Escherichia coli ST131 B7S75 isolated from retail chicken skin. Microbiology Resource Announcements. https://doi.org/10.1128/MRA.01533-18.
- Xu, A., Johnson, J., Scullen, O.J., Chuang, S., Sheen, L., Sheen, S., Sommers, C.H. 2019. Thermal inactivation kinetics of extraintestinal pathogenic Escherichia coli suspended in ground chicken meat. Food Control. 104:269-277. https://doi.org/10.1016/j.foodcont.2019.05.001.

Intervention and Control Strategies: Other Foods and Alternate Interventions

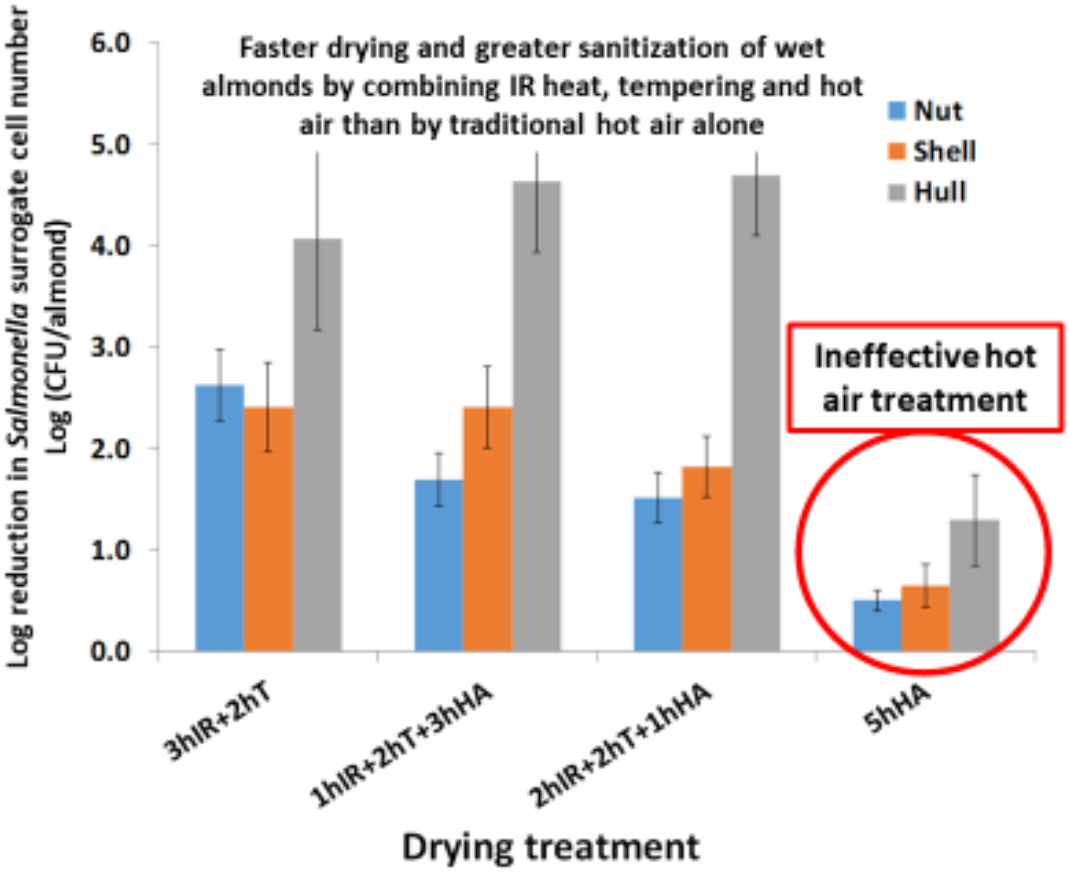
Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR); https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf *Pathogenic microbes exhibit a variety of responses to foods, the handling of foods before processing, the sequence of food processing steps, and the conditions under which foods are processed, prepared, and/or stored. Determining the ecological niches, persistence, and physiological responses that are initiated by pathogens under the conditions particular to foods and food processing, storage, and/or preparation is an essential prerequisite for the development of directed detection and intervention methods for human pathogens in foods. Considering existing and pending regulatory policies and the paucity of available literature, research is needed to better quantify the association and fate of select food borne pathogens in higher risk foods. Studies were aimed at increasing our understanding of where pathogens enter the food supply, and pathogen persistence in foods (which are both part of Problem Statement 1: Population Systems), and, in turn, developing and evaluating effective interventions to enhance the safety and security of our food supply: determining the prevalence, levels, types, and locations of pathogens at various points from production through to consumption of raw, further processed, and/or RTE foods, developing, optimizing, and validating processing technologies for eliminating pathogens; and to develop and/or validate strategies to deliver antimicrobials to raw and packaged foods from production through to consumption.*

NP108 assisted in research focusing on interventions for tree nuts. The work was conducted at WRRC, Albany, CA in association with NP306 [Product Quality and New Uses] (by Brandl in the Gorski project)

Examples of Accomplishments

- **An effective method to dry and decontaminate wet whole almonds.** California produces 80% of the world's almonds with a value of over $5.33 billion. Contamination of almonds with Salmonella has caused several large and expensive recalls by the industry and outbreaks of human illness. The occurrence of rain during the harvest season may result in the complete loss of an almond crop due to increased risk of microbial contamination and lack of adequate drying technology. ARS developed an effective and energy-saving new technology based on sequential infrared heat and hot air to simultaneously dry and decontaminate wet whole almonds.



- **Development of a simultaneous drying and decontamination technology for pistachios**. The U.S. is the second leading producer and exporter of pistachios in the world, producing 300,000 tons of pistachios in 2017, with a total economic output valued at $3.6 billion per year. Contamination of pistachios with Salmonella have caused large outbreaks of salmonellosis and expensive recalls, impacting public health and the pistachio industry. The current hot air drying of pistachios after their sorting in water tanks has low energy efficiency and drying rates and does not guarantee their microbial safety. ARS developed a novel technology based on sequential infrared heating and hot air that simultaneously dries and decontaminates pistachios with energy savings of 34% compared with traditional hot air drying alone.

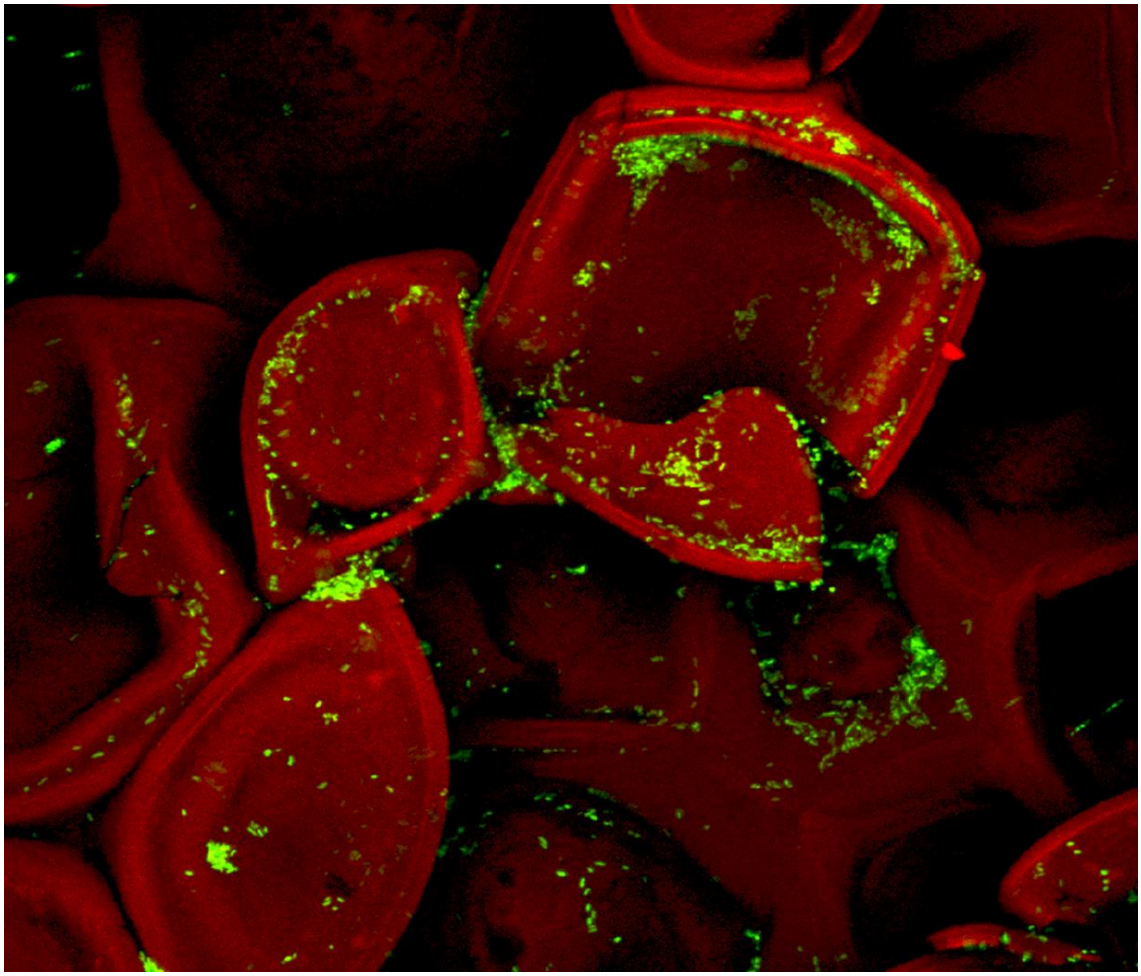


Image: Microscope image of Salmonella cells (shown in green) contaminating the skin of the almond kernel (in red). ARS scientists soaked whole almond fruit in a suspension of Salmonella in the laboratory to demonstrate that this foodborne pathogen can migrate all the way to the kernel when the almond fruit is wet. (Photo by Maria T. Brandl)

Outcomes and Impacts

- Provided the industry with an efficient and effective approach to produce high quality, safe tree nuts; and contributed to ARS receiving the 2018 Research and Development Award by the Institute of Food Technologists.

Examples of Relevant Publications

- Venkitasamy C, Brandl MT, Wang B, McHugh TH, Zhang R, Pan Z (2017) Drying and decontamination of raw pistachios with sequential infrared drying, tempering and hot air drying. *Intl J Food Microbiol* 246:85-91. https://doi:10.1016/j.ijfoodmicro.2017.02.005
- Venkitasamy C, Zhu C, Brandl MT, Niederholzer FJA, Zhang R, McHugh TH, Pan Z (2018) Feasibility of using sequential infrared and hot air for almond drying and inactivation of *Enterococcus faecium* NRRL B-2354. *LWT-Food Sci Technol* 95:123-128. https://doi:10.1016/j.lwt.2018.04.095

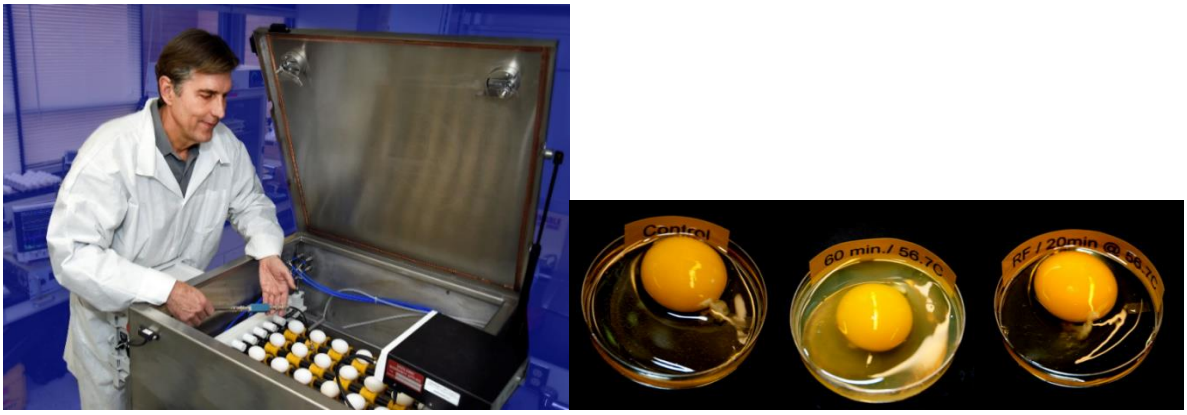
This research area was addressed by two projects (Geveke and Luchansky) conducted at ERRC, Wyndmoor, Pennsylvania.

The (Geveke) project continued research addressing development of a practical intervention for decontamination of shell eggs. Research had developed a novel shell egg radio frequency pasteurization (RFP) process that inactivates 5 logs of Salmonella, while maintaining the transparency of the egg white (a denaturation/quality indicator). The ARS process is now patented and under a CRADA with a company that manufactures egg processing equipment.

Examples of Accomplishments

- **RFP of shell eggs**. ARS and Princeton University showed and patented the process, that RFP can kill 99.999% of Salmonella subsequently producing safer eggs with exceptional quality. In this past cycle ARS designed and built an RFP unit that can simultaneously pasteurize many eggs. Three breakthroughs were achieved that will facilitate the industrial scale-up of RFP.

    - Modification of RFP to operate at 40.68 MHz, which is an international frequency reserved for industrial, scientific and medical purposes.
    - Showing that inexpensive ferrites to provide a match between the eggs and equipment. This will save $10 to $100K per RFP unit.
    - Reducing the commercial equipment costs. Designed and tested an inexpensive branch line splitter which divides the power by an acceptable 56:44. The cost savings of using branch line splitters in a commercial-scale RFP unit is estimated to be $100K.



Figures: (L) Geveke indicating the RFP processing equipment; (R) Shell egg processed by RFP

Outcomes and Impact

- Numerous companies expressed substantial interest in the RFP process. A funded CRADA was signed with Kuhl Corp. to scale up and commercialize RFP. In collaboration with Kuhl, an RFP unit that can simultaneously process 24 eggs was assembled and tested. This RFP unit was used to design a commercial prototype unit which is currently being assembled at Kuhl's facility. Pasteurization of all shell eggs in the U.S. would reduce *Salmonella* illnesses by an estimated 110,000 annually.

Examples of Relevant Publications

- Geveke, David J.; Bigley, Andrew B. W.; Brunkhorst, Christopher D.; Jones, Deana R., and Tilman, Eric. Improvement in the Radio Frequency Method to Pasteurize Shell Eggs by Automation and Cost Reduction. International Journal of Food Science and Technology. 2018a; 53:2500–2508.
- Geveke, David J.; Bigley, Andrew B. W., and Brunkhorst, Christopher D., inventors. Method and Apparatus for Pasteurizing Shell Eggs Using Radio Frequency. US 9883682. 2018b.
- Yang, Yishan; Geveke, David J.; Brunkhorst, Christopher D.; Sites, Joseph E.; Geveke, Noah J., and Tilman, Eric D. Optimization of the radio frequency power, time and cooling water temperature for pasteurization of *Salmonella* Typhimurium in shell eggs. Journal of Food Engineering. 2019; 247: 130-135.

The (Luchansky) project is unique in that it also addresses direct research needs for the FSIS and FDA. This project has unique facilities at ERRC for conducting for example, national surveys, and large-scale intervention studies. The base objectives of the project were to: (1) Determine the prevalence, levels, types, and locations of pathogens at various points from production through to consumption of raw, further processed, and/or RTE foods; (2) Develop, optimize, and validate processing technologies for eliminating pathogens; and (3) Develop and/or validate strategies to deliver antimicrobials to raw and packaged foods from production through to consumption.

Examples of Accomplishments

- **Surveys of raw meats and marinades for Shiga toxin-producing Escherichia coli (STEC) and Salmonella.** Undercooked and improperly handled red meat and poultry products are a common vehicle for foodborne illnesses. ARS conducted multi-institutional surveys of raw meats and marinades to gain insight on the true prevalence of STEC and Salmonella in these products. Samples of raw veal, beef, pork, meat marinades, and chicken livers were collected at retail establishments across the mid-Atlantic region of the U.S. over a 3-year period. The attendant recovery rates for STEC in raw veal (1,095 samples) and Salmonella in chicken livers (249 samples) being 7.0 and 59.4%, respectively, suggest that additional interventions and guidelines for processors are needed to further lower the prevalence and levels of these pathogens in raw veal and liver. In related studies, zero of 514 pork samples and zero of 115 spent/fresh marinade samples tested positive for STEC. Thus, the seven regulated serovars of STEC are apparently not common in retail raw pork or marinade samples in the Mid-Atlantic region of the U.S. Collectively, the findings were used to develop/populate risk assessments by regulatory agencies.

- **Control of Listeria monocytogenes in clean label/natural ready-to-eat (RTE) meats.** Consumers are looking for all-natural, minimally-processed, chemical-free, and healthier foods. However, manufacturing natural foods, such as RTE meats, without antimicrobials may pose a high safety risk because such products may support the outgrowth of L. monocytogenes (Lm) during extended storage. In collaboration with a CRADA partner, studies were conducted to validate the efficacy of natural/clean label acidulants, such as buffered vinegar (BV), to control Lm on several freshly-manufactured RTE meats such as mortadella and pork patties/sausage. Products were formulated with or without 0.6 to 2.4% of buffered vinegar, inoculated with Lm, and then stored at refrigerated or abusive temperatures for up to 120 days. At lower concentrations of BV, pathogen levels increased by <1000 cells, but when BV was used at high concentrations, growth of Lm was inhibited throughout storage. These data established BV as an effective antilisterial agent that also provides industry with options to manufacture so-called “all-natural”, “clean label” products. Inclusion of BV in RTE meats is not cost-prohibitive and/or does not cause untoward effects on the taste and/or sensory attributes of the finished product, while allowing meat processors to produce a safer product.

- **Control of Trichinella and Toxoplasma in cured meats.** Although trichinosis and toxoplasmosis from ingestion of raw or uncooked pork remains the most significant zoonotic foodborne diseases worldwide, little information is available regarding inactivation of Trichinella and Toxoplasma in cured meat products. ARS collaboratively validated the effects of salt, water activity, and pH, during fermentation and drying, on inactivation of Trichinella or Toxoplasma in a dried-cured pork sausage. Salt levels ≥1.3% and pH ≤5.2 inactivated cysts of trichinae or toxoplasmas during fermentation (>96% of inactivation) and drying (≤10 days; complete inactivation) of dried-cured sausage. These data validated a process to inactivate Trichinella and Toxoplasma in fermented/cured pork products. These data will be used to develop models for fermentation/drying that will lessen the need for costly product validation studies and, in turn, help industry/processors to meet regulatory requirements.

- **Perception of food safety risk at food retail markets.** Consumers perceive risks differently than food safety experts and/or develop opinions and habits that are not typically science/reality based and may thus be at higher risk from illness. To gain insight on perceptions, attitudes, and self-reported behaviors related to observed food safety hazards of consumers who shop at grocery stores, the source of <50% of food sales in the U.S, two studies were conducted. In phase I, to capture potential perceived and actual food safety risk situations while shopping, digital photographs (n = 119) were collected at retail establishments from CA, GA, MD, and CT over a 2-year period. Photographs were coded by use of qualitative content analysis techniques. The photographs confirmed the occurrence of risk factors for foodborne illnesses, including contaminated equipment resulting in cross contamination, poor personal hygiene, and improper temperature control. In phase II, a nationally-representative survey (n =1,041) evaluated food safety risks depicted in selected photographs and allowed consumers to self-report their perceptions, attitudes, and behaviors. These findings were used as a real-world teaching tool to better inform and engage a positive food safety culture among shoppers and employees at grocery stores. The digital photographs provided a set of learning materials that the retail food industry can use as examples of what shoppers may see if they are focused on food safety, and in turn, will assist in developing and implementing interventions that affect behavior changes.

- **Reducing the potential risk of Shiga toxin-producing Escherichia coli (STEC) in ground beef - communicating the message to consumers.** In collaboration with researchers from academia, along with high school students and teachers and marketing professionals in the local community, a message for the masses related to food safety, STEC, and beef (aka the “160° is good” campaign) was conceptualized. The message was delivered via website, video advertisements, radio, press releases, broadcast interviews, and movie theater pre-roll advertisements. These efforts infused science into a consumer-relevant media campaign that in 3 months generated ca. 450,000 video impressions (views) via the website (www.160isgood.com), ca. 8.3 M radio impressions (listen), movie theaters ads, logos, and digital media [ca. 1.7 M digital spots (views)].

This campaign will be used by trade associations, regulatory agencies, consumer groups, schools, and universities to raise awareness and reduce the risks of STEC in beef by communicating proper use of a thermometer to measure doneness (internal temperature of 160°F) of a beef burger.

Outcomes and Impacts

- Confirmed that interventions and processes effective for Shiga toxin-producing Escherichia coli (STEC) O157:H7 were equally effective against the six additional serovars (O26, O45, O103, O111, O121, and O145) of STEC also considered adulterants.
- Validation that cells of all seven regulated serovars/types of STEC behave similarly in response to common interventions.
- Validated that industry did not have to modify existing technologies or to develop new interventions tailored to the six non-O157 regulated serovars to ensure the safety of beef products.
- Validated translocation of STEC into whole-muscle raw beef via needle tenderization, cubing, and/or chemical injection. Results used to support rulemaking that now requires tenderized meat products (U.S. and Canada) to be labeled. data were used by FSIS for declaring non-O157 STEC an adulterant in raw beef.
- Validated the effect of time, temperature, pH, and salt on fate of STEC, L. monocytogenes, Salmonella, trichinae, and toxoplasma in various red meat and poultry products. Data on pathogen viability following fermentation and/or cooking was used to update risk assessments for beef and pork.
- Optimized methods/technologies to deliver antimicrobial agents on meats. Use of electrostatic spray or sprayed lethality in container methods reduced processing times, installation costs, and volume of water used.
- Optimized/validated interventions to control STEC on veal carcasses. Findings used by veal industry for FSIS inspectors, to validate their HACCP Program, and to reduce the potential risk of illness from STEC associated with veal.
- Assisted small processors to validate their processes. Results related to dry-fermented meats used by FSIS in the 2018 revisions to the multi-hurdle guidelines, entitled “Overview of the safe production of multi-hurdle ready-to-eat shelf-stable fermented/ acidified, salt-cured, and dried meat and poultry products”.
- Conducted a comprehensive survey for L. monocytogenes in retail ready-to-eat (RTE) foods in the U.S. to establish the recovery rate of the pathogen in higher risk RTE foods. Data established the “true prevalence” of L. monocytogenes Reductions in occurrence and levels may be attributed, in part, to regulatory reforms, more effective and frequent monitoring efforts, and strategic improvements to processes/formulations and sanitary measures by Federal agencies and the food industry.

Examples of Relevant Publications

- Luchansky, J. B., S. G. Campano, B. A. Shoyer, and A. C. S. Porto-Fett. 2016. Viability of *Listeria monocytogenes* on boneless, water-added hams, commercially prepared with and without food grade chemicals, during extended storage at 4 and/or -2.2°C. *J. Food Prot*. 79:613-619.
- Sevart, N. J., N. Baumann, H. Thippareddi, T. A. Houser, J. B. Luchansky, A. C. S. Porto-Fett, D. B. Marx, G. R. Acuff, and R. K. Phebus. 2016. Evaluating the efficacy of three USDA-approved antimicrobial sprays for reducing Shiga toxin-producing *Escherichia coli* (STEC) surrogate populations on bob veal carcasses. *J. Food Prot*. 79:956-962.
- Luchansky, J. B., A. C. S. Porto-Fett, and B. Chapman. 2017. A shopper's eye view of food safety at retail stores: lessons from photographs taken while grocery shopping. *Food Prot. Trends* 37:30-42. *[Paper most-viewed Food Protection Trends in 2017]*
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- Hasty, J. D., J. A. Henson, G. R. Acuff, D. E. Burson, J. B. Luchansky, N. J. Sevart, R. K. Phebus, A. C. S. Porto-Fett, and H. Thippareddi. 2018. Validation of a sequential hide-on bob veal carcass antimicrobial intervention comprised of a hot water wash and lactic acid spray in combination with scalding to control Shiga toxin-producing *Escherichia coli* surrogates. *J. Food Prot*. 81:762-768.
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- Shane, L. E., A. C. S. Porto-Fett, B. A. Shoyer, R. K. Phebus, H. Thippareddi, A. Hallowell, K. Miller, L. Foster-Bey, S. G. Campano, P. J. Taormina, D. L. Glowski, R. B. Tompkin, and J. B. Luchansky. 2018. Evaluation of post-fermentation heating times and temperatures for controlling Shiga toxin-producing *Escherichia coli* cells in a non-dried, pepperoni-type sausage. *Italian J. Food Safety* 7:116-120.

- Luchansky, J. B., M. Mayhew, Y. J. Jung, A. Klinedinst, L. Harkins, L. E. Shane, M. Osoria, L. McGeary, Z. Trauger, B. A. Shoyer, B. Chapman, S. J. Cope, S. G. Campano, and A. C. S. Porto-Fett. 2019. Meat bars: a survey to assess consumer familiarity and preparation parameters and a challenge study to quantify viability of Shiga toxin-producing *Escherichia coli* cells during processing and storage. *J. Food Prot.* 82:1249-1264.
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- Jung, Y., A. C. S. Porto-Fett, B. A. Shoyer, L. E. Shane, E. Henry, M. Osoria, and J. B. Luchansky. 2019. Survey of intact and non-intact raw pork collected at retail stores in the mid-Atlantic region of the United States for the seven regulated serogroups of Shiga toxin-producing *Escherichia coli.* J. Food Prot. https://doi.10.4315/0362-028X.JFP-19-192.
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Problem Statement 6. Predictive Microbiology and Modeling; Data Acquisition and Storage; Genomics (Database)

Goals

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
The tenet of predictive microbiology is that the behavior of any microorganism is deterministic and able to be, within limits, predicted from knowledge of the microorganism itself and the microorganism's immediate environment. However, it has been stressed by stakeholder groups that research should also include a greater emphasis on probabilistic modeling to balance the deterministic approaches. This would benefit predicting the behavior of pathogens under stressed conditions (more relevant to the food industry) where growth/inactivation is stochastic.

Behavioral predictions and models are internationally accepted as an integral part of microbial risk assessment used to support food safety measures by both food safety regulatory bodies and industry. The Food Safety Program however, does not develop or conduct Risk Assessments (RA), where RA is defined as the determination of a quantitative or qualitative value of risk related to a specific situation and a recognized hazard. The Program does conduct research and provides data when requested by our regulatory stakeholders (FSIS, FDA) for their use in conducting risk assessments.

The Program develops various modeling programs including; the Pathogen Modeling Program (PMP), a package of models that can be used to predict the growth and inactivation of foodborne bacteria, primarily pathogens, under various environmental conditions. In addition, the Predictive Microbiology Information Portal (PMIP) is geared to assist food companies (large and small) in the use of predictive models, the appropriate application of models, and proper model interpretation. The vision is that the PMIP will be the highway for the most comprehensive websites that bring together large and small food companies in contact with the information needed to aid in the production of the safest foods. The PMIP links users to numerous and diverse resources associated with models (PMP), databases (ComBase), regulatory requirements, and food safety principles.

An alternate modeling approach known as the "Integrated Pathogen Modeling Program (IPMP)" will continue to be developed and evaluated. The premise of the Program is that predictive microbiology research relies heavily on laboratory-generated data for estimating kinetic parameters and follows a three-step process of primary (growth curves), secondary (intrinsic and extrinsic effects). After the kinetic parameters for both primary and secondary models are available, they can be used to predict the growth of microorganisms under different conditions in a spreadsheet or stand-alone software, using kinetic parameters in mathematical models to make predictions (tertiary model). It is argued that this approach accumulates and propagates errors in each step of data analysis and model development. Studies developed a new more advantageous approach to estimate the kinetic parameters by a one-step direct construction of both primary and secondary models using multiple growth curves obtained under different dynamic conditions. This approach needs a limited number of dynamic curves to obtain an accurate estimation of kinetic parameters, and therefore is less time-consuming and more

efficient. The core of the new approach is that the growth kinetics are directly based on differential equations, which are the implicit forms of primary models, that define and capture the dynamic changes in the bacterial population under complex dynamic environmental conditions.

All predictive models developed must be available for external examination, review and utility. If predictive models are developed for internationally accepted high priority pathogen-food combinations, then they could have a major impact for food companies in the U.S. and other countries producing and exporting food to the U.S. This will require significant interactions with risk assessors and involvement in international initiatives such as National Advisory Committee on Microbial Criteria for Foods (NACMCF) and Codex Alimentarius Commission (CODEX). Collaborations with our food and environment related stakeholders (FDA/FSIS/EPA) must be strengthened with regards to what research needs to be conducted to effectively utilize the inherent ARS expertise and modeling systems mechanisms.

Data acquisition and storage: ARS and the University of Tasmania (UTas), as well as associate members University of Querétaro, Mexico; Unilever Research, UK; Agricultural University of Athens, Greece; National Food Research Institute, Japan; Hokkaido University, Japan; and Rutgers University also developed and maintains a publicly available global food safety database, ComBase - a Combined data Base for predictive microbiology – which is the number-one web-based resource for quantitative and predictive food microbiology in the world. https://www.combase.cc/index.php/en/ Its components include a database of observed microbial responses to a variety of food-related environments and a collection of relevant predictive models. The purpose and goal of ComBase is to provide an electronic repository for food microbiology observations and to make such data and the generated predictive tools freely available and accessible to the entire food safety community. Data acquisition and use is an interdisciplinary research challenge that translates into safer products and improved public health.

Genomics as a functional and critical part of omic-technologies holds great promise for improving the early detection, prevention and control of current and emerging foodborne pathogens, thus contributing to improved food safety and consequently public health. Genomics has the potential as a partner or replacement for culture-based techniques. Food safety regulatory agencies, USDA and the FDA, have discussed and are planning to implement the increased use of genomics, in particular partial and/or WGS for both regulatory monitoring, attribution and potentially for revising risk assessments. Implementation of such a redirection requires developing a coordinated system of genomic sequencing technology for routine testing. Critical within this issue is the development of an ARS database from our national and international sequencing/annotation efforts. For this work, a common or representative core set of bacterial pathogens or surrogates will be available. Additional data from isolates studies obtained from national and international collaborations will be incorporated. Allied to the sequencing efforts will be meta-data descriptors. This research will be part of both a national initiative “Interagency Collaboration on Genomics for Food and Feed Safety (Gen-FS)” [agencies within the Department of Health and Human Services and the USDA] and an international initiative “Global Microbial Identifier (GMI) the latter a global, visionary taskforce

including more than 30 countries who share an aim of making novel genomic technologies and informatics tools available for improved global infectious disease diagnostics, surveillance and research, by developing needs and end-user based data exchange and analysis tools for characterization of all microbial organisms and microbial communities.

Predictive Microbiology and Modeling: Environmental

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
The presence of pathogenic microorganisms in irrigation waters is considered a critically important factor for the Produce Safety Rule as part of the FDA-Food Safety and Modernization Act (FSMA) signed into Law, January, 2011. The goal was to investigate using both field studies and model systems, any role that irrigation waters had as a potential source of on-farm contamination of fresh produce. The Rule itself had issues, specifically where, when, and how the irrigation water samples should be taken. This created a problem for surface water-based irrigation, since the microbial populations in surface waters are known to be highly variable in space and time. Water sampling without accounting for such variability would make any water sampling results highly uncertain and vulnerable to challenge. Thus, there was a need to design and evaluate monitoring strategies to accurately assess the microbial quality of irrigation waters, accounting for the spatial and temporal variability of microbial populations.

Research was conducted at BARC, Beltsville, MD by the (Pachepsky) project addressing the objectives to: (1) Elucidate spatial variability of indicator bacteria concentrations in surface waters (e.g., streams, ponds, reservoirs), and describe factors responsible for this variability; and (2) Elucidate temporal variability of indicator bacteria concentrations in watersheds as a function of land use and meteorological conditions and develop/validate predictive models.

Examples of Accomplishments

- **Using imagery from unmanned aerial vehicles (drones) to guide sampling for microbial water quality assessment in irrigation ponds.** Microbial quality of water in irrigation water sources must be assessed to prevent spread of microbes that can cause disease in humans because of the produce consumption. Microbial quality of irrigation water is evaluated based on concentrations of the indicator bacterium E. coli. No recommendations have existed so far on where the pond water samples should be taken for microbial analysis. ARS proposed and tested the method of using the drone-based imagery and artificial intelligence techniques to obtain representative water samples for E. coli enumeration across irrigation ponds. Reflectance in different parts of the spectra are combined to characterize E. coli habitat in water.

Figure: Using a drone for sampling
https://bluewaterbaltimore.org/blog/9-17-2019-intern-spotlight-matt-stocker/

- **Efficient sampling to assess microbial quality of surface waters used in irrigation.** Microbial quality of irrigation water must be assessed to prevent the spread of microbes that cause disease, especially with fresh produce consumption. Irrigation water is evaluated based on the concentration of the indicator bacterium E. coli. No recommendations have existed so far as to where and when irrigation water samples should be taken for microbial analysis. ARS validated a method that allows users to find a single representative sampling location that provides valid estimates of E. coli concentrations in other locations along creek and in ponds.

Figure: ARS designed, built and validated a GPS water sampling vehicle for irrigation water evaluation

- **Effect of microorganism population in bottom sediments on the microbial quality of irrigation and recreation waters.** Reliability of modeling microbial water quality in irrigation water sources depends on the accuracy of simulating microorganism exchange between bottom sediment and water column. ARS conducted both field experiments and modeling to demonstrate that substantial microorganism exchange between bottom sediment and water column occurs during the baseflow, or low-flow periods. The first method of measuring such exchange was developed.

- **Microbial database editor software to be used in predictive environmental microbiology models.** Reliability of modeling microbial water quality in irrigation water sources depends on the accuracy of simulating microorganism survival in major environmental media including soil, water, manure, stream bed sediment. ARS with the EPA developed the microbial database editor software that allows to enter, store, and modify properties related to microbial indicators and pathogens, and to populate metadata standards, so the properties are available for consumption by microbial source, fate, transport, and risk models.

Outcomes and Impacts

- Determined that drone-based imagery sampling provided the knowledge base for efficient microbial water quality sampling and indicate the novel direction of monitoring microbial water quality, thus contributing to the improvements in food safety, with a focus on produce production.
- Determined that the sampling location selection method provides the knowledge base for accurate representative microbial water quality sampling and can be used by water resource managers and consultants who design and implement water quality monitoring programs, with a focus on produce production.
- Determined that the novel data and methodology of assessing the role of bottom sediments in microbial water quality will lead to substantial improvements in the accuracy of microbial water quality models used for assessment and predictions for recreational and irrigation water sources.
- Data demonstrate the need to scrutinize and possible re-evaluate the role of indicator organisms as markers for grazing and manuring effects on the microbial water quality.
- Developed the microbial database editor software. It is intended to be used by water resource and water use managers and consultants who currently employ modeling to follow regulatory guidance on microbial water quality and to meet regulatory microbial quality standards of irrigation and recreation waters.

Examples of Relevant Publications

- Whelan, G., M. Pelton, M. Molina, R. Zepp, Ravenscroft, J., Pachepsky, Y Microbial Properties Database Editor Tutorial. US EPA Office of Research and Development, Washington, DC, EPA/600/B-15/275, 2015. Available at: https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NERL&dirEntryId=310639

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- Hong, E. M., Park, Y., Muirhead, R., Jeong, J., & Pachepsky, Y. 2018. Development and evaluation of the bacterial fate and transport module for the Agricultural Policy/Environmental eXtender (APEX) model. Science of the Total Environment 615: 47–58.
- Smith, J.E., Kiefer, L.A., Stocker, M.D., Blaustein, R.A., Ingram, S., Pachepsky, Y.A. 2019. Depth-dependent response of fecal indicator bacteria in sediments to changes in water column nutrient levels. Journal of Environmental Quality, 48:1074-1081.
- Jeon, D. J., Pachepsky, Y., Harriger, D., Coppock, C, Wells, E., Hong, E. M. 2019. Analysis of *Escherichia coli* and enterococci concentrations patterns in a Pennsylvania creek using empirical orthogonal functions. Journal of Environmental Quality 48(6) https://doi:10.2134/jeq2019.05.0191
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- Morgan, B. J., Stocker, M. D., Valdes-Abellan, J., Kim., M. S., Pachepsky, Y. 2020. Drone-based imaging to assess the microbial water quality in an irrigation pond: a pilot study. Drones (submitted).

Predictive Microbiology and Modeling: Regulatory/Industry

Introduction
As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Predictive microbiology is a discipline based on the premise that microbial growth, survival and inactivation kinetics can be quantified and expressed through mathematical equations, and that under a specific set of environmental conditions, microbial behavior is invariably reproducible. Models that describe the combined effects of multiple factors are developed to assist risk managers in addressing the impact of both pathogens and spoilage microorganisms on our food supply. In addition, food industries and associated regulatory agencies need methodologies to validate new and existing conditions so that they can be safely applied across food systems, and effectively managing food safety risks. The predictive models provide regulatory agencies and the food industry with an objective means of assessing the microbial risk of a food and ensuring that the public is not at risk of acquiring spoiled foods or coming down with foodborne illness.

This research was conducted at ERRC, Wyndmoor, PA by three projects: (Huang, Juneja) using different approaches; and by (Sommers) a new initiative.

The (Huang) project objectives were: (1) Development and validation of predictive models for growth of high priority pathogens in processed foods; (2) Dynamic simulation and probabilistic modeling of growth of foodborne pathogens in foods; (3) Develop an advanced decision support system and software for predictive microbiology and food safety regulations. 4: Further, expand where necessary the ARS curve-fitting (modeling) program also known as the "Integrated Pathogen Modeling Program (IPMP)".

Examples of Accomplishments

Developed new mathematical models
- Thermal inactivation of L. monocytogenes in 10% salted liquid egg yolk.
- Dynamic modeling of growth of Escherichia coli O157:H7 in raw ground beef under competition from background flora.
- Dynamic modeling of growth of Bacillus cereus in cooked rice.
- Thermal resistance of. L. monocytogenes, Salmonella, and E. coli O157:H7 in meat and poultry.
- Growth/No Growth boundary of Clostridium perfringens in cooked meat.
- Growth of Clostridium botulinum in cooked beef using a nontoxigenic strain.

- Growth models for Staphylococcus aureus in lettuce.
- Dynamic prediction of growth and survival of Salmonella Enteritidis in liquid egg whites.
- Growth and survival of Salmonella paratyphi A in roasted and marinated chicken.
- Monte Carlo analysis of growth of Clostridium perfringens in cooked turkey meat during cooling.
- Expanded the USDA IPMP-Global Fit, a one-step kinetic analysis tool for predictive modeling. https://www.ars.usda.gov/northeast-area/wyndmoor-pa/eastern-regional-research-center/docs/ipmp-global-fit/.
- Monte Carlo analysis of microwave-assisted pasteurization of packaged foods. Thermal inactivation of Listeria monocytogenes in salmon roe.
- In-situ generation of chlorine dioxide for surface decontamination of produce.

Outcomes and Impacts

- Built upon the USDA Integrated Pathogen Modeling Program (IPMP 2013), a new one-step data analysis tool (IPMP-Global Fit). This allows the development of predictive models that minimize residual errors from isothermal experiments, thus providing more accurate predictions.
- Developed a dynamic method that can analyze both microbial growth and survival in a one-step approach for even more accurate prediction throughout the supply chain.
- Developed a new, more sophisticated, highly accurate stochastic dynamic method based on Bayesian analysis and Marko Chain Monte Carlo simulation. This up-graded method has attained the accuracy of ± 0.25 log CFU/g for predicting the growth of Clostridium perfringens in cooked chicken during cooling, with the mean of prediction errors only 0.1 log CFU/g during validation.
- Models allow the food industry and regulatory agencies to more accurately predict the growth and survival of pathogens throughout the supply chain, thus enhancing risk assessment. Additionally, making the models accessible for public use.

Examples of Relevant Publications

- Huang, L. 2016. Mathematical Modeling and Validation of Growth of Salmonella Enteritidis and Background Microorganisms in Potato Salad – One-Step Kinetic Analysis and Model Development. Food Control, 60: 69-76.
- Huang, L. 2017. Dynamic kinetic analysis of growth of Listeria monocytogenes in a simulated comminuted, non-cured cooked pork product. Food Control, 71: 160-167.
- Li, M., Huang, L., and Yuan, Q. 2017. Growth and survival of Salmonella Paratyphi A in roasted marinated chicken during refrigerated storage: Effect of temperature abuse and computer simulation for cold chain management. Food Control, 74: 17-24.
- Huang, L., and Hwang, C.A. 2017. Dynamic analysis of growth of Salmonella Enteritidis in liquid egg whites. Food Control, 80: 125-130.
- Huang, L., Li, C., Hwang, C.A. 2017. Growth/no growth boundary of Clostridium perfringens from spores in cooked meat: A logistic analysis. International Journal of Food Microbiology, 266: 257-266.

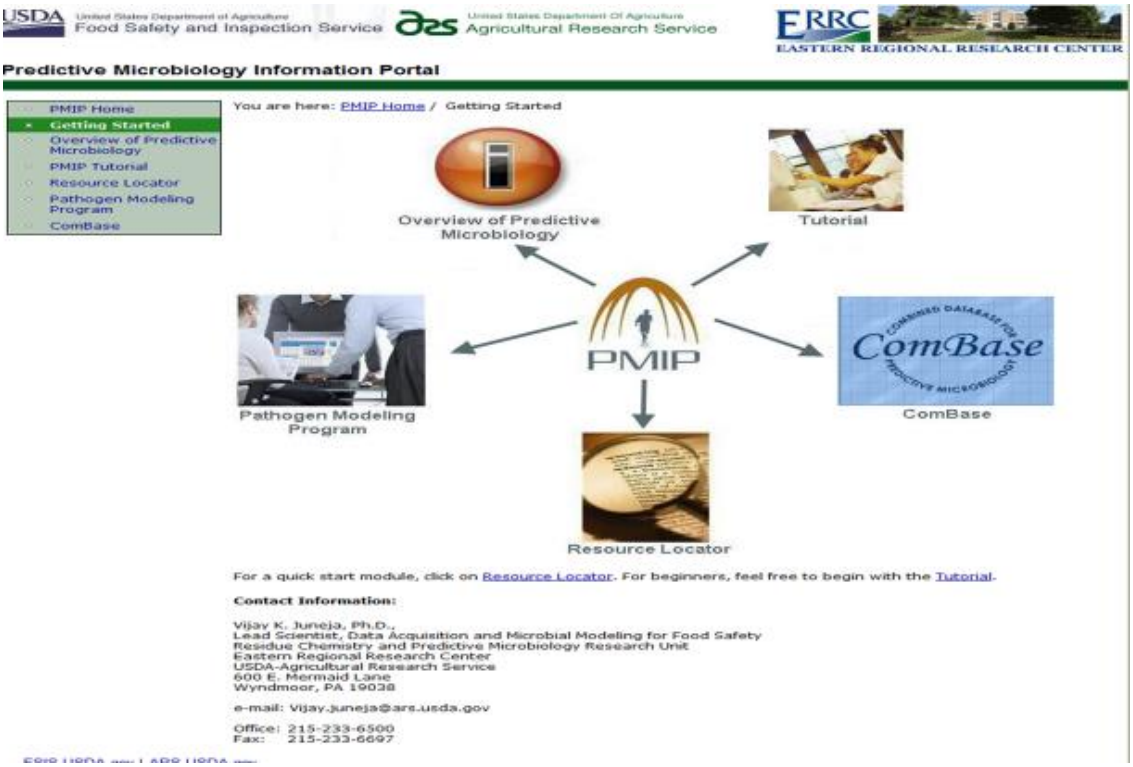
- Huang, L. 2017. IPMP Global Fit – A one-step direct data analysis tool for predictive microbiology. International Journal of Food Microbiology, 262: 38-48.
- Hwang, C.A., and Huang, L. 2018. Dynamic analysis of competitive growth of Escherichia coli O157:H7 in raw ground beef. Food Control, 93: 251-259.
- Huang, L. 2018. Growth of non-toxigenic Clostridium botulinum mutant LNT01 in cooked beef: One-step kinetic analysis and comparison with C. sporogenes and C. perfringens. Food Research International, 107: 248-256.
- Huang, Y., Hwang, C.A., Huang, L., and Wu, V.C.H. 2018. The risk of Vibrio parahaemolyticus infections associated with consumption of raw oysters as affected by processing and distribution conditions in Taiwan. Food Control, 86: 101-109.
- Huang, L., & Li, C.H., 2019. Growth of Clostridium perfringens in cooked chicken during cooling: One-step dynamic inverse analysis, sensitivity analysis, and Markov Chain Monte Carlo simulation. Food Microbiology, 8, 103285
- Hwang, C., & Huang, L. 2019. Growth and survival of Bacillus cereus from spores in cooked rice – One-step dynamic analysis and predictive modeling. Food Control, 96: 403-409.
- Huang, L., Hwang, C., & Fang, T. 2019. Improved estimation of thermal resistance of Escherichia coli O157:H7, Salmonella spp., and Listeria monocytogenes in meat and poultry: The effect of temperature and fat and A global analysis. Food Control, 96: 29-38.

The (Juneja) project objectives were to: (1) Develop and validate predictive models for behavior of stressed and unstressed pathogens in food with added antimicrobials. This includes development of validated dynamic models for spores and vegetative foodborne pathogens for evaluating heating and cooling process deviations; (2) Develop and validate process risk models for higher risk pathogen and food combinations; (3) Expand and maintain the ARS-Pathogen Modeling Program and Predictive Microbiology Information Portal: and continue to support the development and utilization of ComBase the database with our associated partner the University of Tasmania (UTas) as an international predictive microbiology data resource. https://www.ars.usda.gov/northeast-area/wyndmoor-pa/eastern-regional-research-center/residue-chemistry-and-predictive-microbiology-research/docs/pathogen-modeling-program/pathogen-modeling-program-models/; https://portal.errc.ars.usda.gov/PMP.aspx; and https://www.combase.cc/index.php/en/

Examples of Accomplishments

Developed new mathematical (predictive) models

- Thermal inactivation to estimate reduced heat treatment that may be employed to produce safe products with extended shelf life.
- For the safe cooling rate and to estimate the expected effectiveness of corrective actions because of deviations from a critical limit.
- Clostridium perfringens growth in sous vide cooked ground beef.
- Bacillus cereus growth in rice, beans, pasta and combination products.
- Salmonella and whole chickens to evaluate short-term and long-term effects of interventions.
- For oysters that predicts the rate and extent of physical removal of Vibrio from oysters by depuration.
- Salmonella, for flow-pack wrappers that predicts the risk to public health and considers how whole chickens sold in flow-pack wrappers are stored and handled by consumers.
- Salmonella for cooking predicting the time needed to kill different initial numbers of Salmonella in ground chicken during cooking.
- Salmonella for tomatoes that predicts growth of Salmonella on diced Roma tomatoes used in salads or tacos and held at different room temperatures.
- Salmonella in broth media that mimics food, demonstrating that AI based on ANN learning of patterns in big data has great potential.
- Salmonella model for cold storage.
- Furthering the USDA-ARS Pathogen Modeling (computer) Program (PMP) and the Predictive Microbiology Information Portal (PMIP).



ComBase, a microbial modeling database (https://www.combase.cc/index.php/en/) as an international data resource continues to grow the size and use. Advisory and Scientific groups oversee the growth of ComBase https://www.combase.cc/index.php/en/the-combase-team#advisory. During the past 5-years, approximately 1,000 new data records/year added. New features were added to the structure and function of the system including.

- Developed software indicating the number of times it has been viewed and downloaded.
- Developed YouTube videos/tutorials one of which has been viewed > 1,000 times https://www.youtube.com/channel/UCKyFWxGsbfazGXYD4kDJr1w/videos
- Developed a private data section to embargo data until publication.
- Developed new ComBase Predictor to 'Broth Models' in the menu so that it better aligns with the separate suite of 'Food Models'.
- Developed software allowing CB data to be (over-layed) on ComBase Predictor graphs.
- Updated Perfringens Predictor and inactivation models.
- Developed software to display all three kinetic parameters—lag, growth rate, MPD for ComBase Predictor growth model outputs.
- Developed a reset system for model default lag time.
- Integrated an API feature to link model predictions to 3rd party software.
- Developed social media accounts on Facebook, LinkedIn and Twitter.
- Conducted Webinar to assist regulatory agencies, industry (et al) to develop regulations & risk assessments, making foods safer for consumers.

Outcomes and Impacts

- The FSIS routinely uses the developed models to set priorities in relation to inspection efforts, and to establish FSIS regulations on performance standards for the production of processed meat and poultry products.
- The products continue to assist the FSIS and FDA in developing science-based risk assessment and associated policy options.
- Food processors can determine compliance with the stabilization (cooling) performance standards.
- Complex underlying mathematics of the predictive models were transformed into easy-to-use interfaces that can be successfully used by food microbiologists, regulatory staff members and industrial professionals to explore the predictions of these models on scenarios relevant to food processing operations.
- Small and very small food processors generally lack food safety resources, the models are particularly helpful to these producers to improve food safety of their products.
- New models were added to the online version of the PMPm while one of the existing models was removed from the desktop version of the PMP and Version 8 was released.
- ComBase assisted users in predicting and improving the microbiological safety of foods as well as in assessing microbiological risk in foods. In the past year alone, there were over 59,500 sessions among the 49,000 registered users, with the top 10 countries using ComBase being in order: Spain, USA, Italy, UK, Canada, Netherlands, Mexico, Denmark, Japan and Australia.

- ComBase saved the food industry millions of dollars each year by reducing the need for costly microbiological tests as well as helping to prevent recalls and foodborne illness.
- ComBase now contains >60,000 records of quantified microbial responses to the food environment that predict the growth and inactivation of microorganisms in food. It should be stressed that the data and models that underpin ComBase are the result of the generous time and support of numerous individuals, companies, and organizations.

Examples of Relevant Publications

- Oscar, T. P. Neural network models for growth of *Salmonella* serotypes in ground chicken subjected to temperature abuse during cold storage for application in HACCP and risk assessment. Int. J. Food Sci. Technol. 52:214-221. 2017.
- Oscar, T. P. Risk of salmonellosis from chicken parts prepared from whole chickens sold in flow pack wrappers and subjected to temperature abuse. J. Food Prot. 80:1496-1505. 2017.
- Lin, L., Cepeda, J., Subbiah, J., Froning, G., Juneja, V.K., and Thippareddi, H. Dynamic predictive model for growth of *Salmonella* spp. in scrambled egg mix. Food Microbiology. 64:39-46. 2017.
- Karyotis, D., Skandamis, P.N. and Juneja, V.K. Thermal inactivation of *Listeria monocytogenes* and *Salmonella* spp. in sous-vide processed marinated chicken breast. Food Research International. 100:894-898. 2017.
- Oscar, T. P. Development and validation of a neural network model for predicting growth of *Salmonella* Newport on diced Roma tomatoes during simulated salad preparation and serving: extrapolation to other serotypes. Int. J. Food Sci. Technol. 57:1789-1801. 2018.
- Juneja, V. K., Mishra, A. V. and Pradhan, A. K. Dynamic predictive model for growth of *Bacillus cereus* from spores in cooked beans. Journal of Food Protection. 81(2):308-315. 2018.
- Oscar, T. P. Short-term and long-term effects of pathogen reduction interventions on risk of salmonellosis from whole chickens. Food Sci. Nutr. 6:2515-2522. 2018.
- Brar, J. S., Waddell, J. N., Bailey, M., Corkran, S., Velasquez, C., Juneja, V. K. and Singh, M. Thermal inactivation of Shiga Toxin Producing *Escherichia coli* in ground beef with varying fat content. Journal of Food Protection. 81(6):986-992. 2018.
- Juneja, V. K., Mohr, T. B., Silverman, M. and Snyder, O. P. Influence of cooling rate on growth of *Bacillus cereus* from spore inocula in cooked rice, beans, pasta, and combination products containing meat or poultry. Journal of Food Protection. 81(3):430-436. 2018.
- Juneja, V. K., C. E. Golden, A. Mishra, M. A. Harrison, T. Mohr and M. Silverman. Predictive model for growth of *Bacillus cereus* during cooling of cooked rice. Intern. J. Food Microbiol. 290:49-58. 2019.
- Juneja, V. K., C. E. Golden, A. Mishra, M. A. Harrison, and T. Mohr. Predictive Model for Growth of *Bacillus cereus* at Temperatures Applicable to Cooling of Cooked Pasta. J. Food Sci. 84 (3): 590-598. https://doi: 10.1111/1750-3841.14448. 2019.
- Shen, X., Su, Y., Liu, C., Oscar, T. and DePaola, A. Efficacy of *Vibrio parahaemolyticus* depuration in oysters (*Crassostrea gigas*). Food Microbiol. 79:35-40. 2019.

The (Sommers) project was an entirely new research direction that focused on extraintestinal Escherichia coli (ExPEC) which includes Uropathogenic Escherichia coli (UPEC). The objective was to: (1) Develop and validate models to simulate pathogen behavior under both growth and inactivation conditions

Examples of Accomplishments

- **Growth kinetics for uropathogenic Escherichia coli in ground chicken meat.** Uropathogenic Escherichia coli (UPEC) are an emerging and common contaminant in poultry meat and are associated with urinary tract infections which affect over 10 million people, primarily women, each year including 23,000 deaths. ARS in collaboration with the National Taiwan University, completed a growth model to describe the ability of UPEC to grow in ground chicken. UPEC was unable to grow at proper refrigeration temperature (4C) but was able to grow significantly at the mild abuse temperature of 10C. Food processors and risk assessors will be able to provide safer ground poultry meat to consumers. Consumers, especially those who are immuno-compromised (e.g. women, cancer patients, diabetics, and the HIV/AIDS population) will benefit from having more information about foods treated with alternative processes which kill harmful bacteria such as the UPEC.

Outcomes and Impacts

- Developed a growth model in collaboration with the National Taiwan University that describes the ability of UPEC to grow in ground chicken. UPEC was unable to grow at proper refrigeration temperature (4C) but was able to grow significantly at the mild abuse temperature of 10C.

Examples of Relevant Publications

- Chien, S., Sheen, S., Sommers, C.H., Sheen, L. 2016. Modeling the inactivation of Escherichia coli 0157:H7 and uropathogenic E. coli in ground chicken by high pressure processing and thymol. Frontiers in Microbiology. 7(920):1-11.
- Chien, S., Sheen, S., Sommers, C.H., Sheen, L. 2016. Modeling the inactivating of Escherichia coli 0157:H7 and uropathogenic E. coli in ground beef by high pressure processing. Food Control. 73:672-680.
- Sommers, C.H., Huang, C., Sheen, L., Sheen, S., Huang, L. 2018. Growth modeling of uropathogenic Escherichia coli in ground chicken meat. Food Control. 86:397-402.

Predictive Microbiology and Modeling: Fermentation

Introduction

As outlined in the 2015 Retrospective Review Report (2015RRR); https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Consumption of refrigerated, fermented, and acidified pickled vegetable products is growing in popularity in the U.S. Consequently, ensuring the control of bacterial pathogens that may be present in these products remains an important issue for the FDA and industry, particularly as the FDA-Food Safety Modernization Act (FSMA) has now been implemented. Moreover, there are now a growing number of new, novel and imported fermented and acidified products being marketed which is concerning the FDA; since there are no acceptable methods available to assure complete removal of vegetative bacterial pathogens from fresh produce, and no guarantee that the fermentation process was performed correctly. Although FBI outbreaks have not occurred in correctly fermented vegetable products, both illnesses and deaths have occurred due to the consumption of juice products contaminated with Shiga-toxigenic E. coli (STEC's) that have similar pH values to fermented and acidified foods. Additionally, studies have shown that some bacterial pathogens (including STEC's) can survive in selected acidified vegetable products for extended periods of time, particularly under refrigerated conditions.

Research was conducted at Food Science Research, located at North Carolina State University by the (Breidt) project whose objectives were to: (1) Determining the safety of low and alternative salt fermentations, produced nationally and internationally; (2) Developing predictive models for 5-log reduction times for pathogenic Escherichia coli in fermented and acidified vegetable products; and (3) Enhance buffer capacity models for predicting pH changes in acidified foods with low acid ingredients.

Examples of Accomplishments

- **Identification of regulatory genes controlling acid resistance important for the survival of pathogenic Escherichia coli strains in acidified vegetables.** Studies examined disease causing bacterial strains of E. coli to determine how differences in acid resistance could be explained by differential gene regulation. There is a wide range of acid resistance among these bacteria, due to a regulatory mechanism involving short regulatory RNA molecules inside cells, that controls not only acid resistance, but also how the cells respond to their environment by making a fibrous network that can aid in cells attaching to surfaces.

- **Establishment of standards for challenge studies for processing cold-filled acidified food products.** To file a scheduled process for acidified foods producers must cite or carry out a scientific study to determine if the product meets federal food safety standards. ARS had a leading role in the development of a protocol (and a webinar) detailing the appropriate scientific methods for challenge studies for the assurance of safety of cold filled acidified foods that do not receive a heat process. A webinar was hosted by the 'Beverage and Acid/Acidified Foods Professional

Development Group' of the International Association for Food Protection. The protocol has been used by researchers, industry stakeholders, and supports FDAs need to assure that challenge studies are done with appropriate methodology.

- **Determining the presence of nitrate and nitrite in fermented and acidified vegetables.** The influence of nitrate and nitrite in foods on human health has been controversial, with literature citing both positive and negative health effects. Studies measured the concentration of these compounds in a wide variety of commercial acidified vegetable products, as well as some fermented foods currently available in the U.S. market. Studies found that nitrite was relatively rare in acidified vegetables but was present in some of the fermented foods tested. Nitrate, on the other hand, was found to be present at varying levels in many acidified products. These results provide new information to evaluating nitrate and nitrite content in pickled fruit and vegetable products in the U.S., to assess the potential health consequences of these compounds.

- **Vegetable fermentation safety.** Commercial fermentation for bulk preservation of cucumbers typically relies on natural microbiota and high salt sodium chloride brines. An alternative process utilizing low salt calcium chloride brines was previously developed to reduce or eliminate sodium chloride from commercial fermentation brines for reduced environmental impact. Studies found that pathogenic enteric bacteria such as E. coli survived longer in the traditional high salt brines compared to the new calcium brine process; and that addition of acetic acid as a pre-treatment to fermentation brines significantly enhances safety. Studies also identified bacteria involved in fermentation instability typical of low salt fermentations.

- **Development of hot-fill pasteurization of cucumber pickle spears as an alternative to tunnel pasteurization.** For commercial production of acidified vegetable products, a tunnel pasteurizer is typically used, resulting in high water use and energy costs. ARS developed a hot-fill method for pasteurization of common cucumber pickle products (cucumber spears). The method requires refilling jars multiple times with a hot brine (around 175°F). The data showed that for cucumber spears a hot fill method using off-the-shelf technology could achieve or exceed temperatures typically used for commercial pasteurization of pickle by most manufacturers and meet or exceed the conditions needed for safety. The in-jar pasteurization process meets current safety standards and saves significantly on the water usage and costs of currently used tunnel pasteurizers.

Outcomes and Impacts

- Determined that differences in acid resistance by E. coli strains could be explained by differential gene regulation.
- Developed a protocol detailing the appropriate scientific methods for challenge studies for the assurance of safety of cold filled acidified foods that do not receive a heat process. Protocol used by stakeholders, and supports FDA need to assure that challenge studies are done with appropriate methodology.

- Hosted webinar by the 'Beverage and Acid/Acidified Foods Professional Development Group' of IAFP.
- Determined that nitrite was relatively rare in acidified vegetables, but present in some fermented foods; alternately nitrate, was found in many acidified products. These results are critical to assess the potential health consequences (allergens) of these compounds.
- Developed previously a commercial fermentation process for bulk preservation utilizing low salt calcium chloride brines. Determined now that pathogenic survived shorter time in the calcium brine process and that addition of acetic acid as a pre-treatment to fermentation brines significantly enhances safety. This eco-friendly low salt fermentation technology is now being adopted by industry.
- Developed a hot-fill method for pasteurization of common cucumber pickle products that could achieve or exceed temperatures typically used for commercial pasteurization. The method meets current safety standards and saves significantly on the water usage and costs of currently used tunnel pasteurizers.

Examples of Relevant Publications

- Median-Pradas E, Perez-Diaz I, Breidt F, Hayes J, Franco W, Butz N, Azcarate-Peril A. 2016. Bacterial ecology of fermented cucumber rising pH spoilage as determined by non-culture-based methods. J. Food Sci. 81(1):M121-M129.
- Kim GH, Fratamico P, Breidt F, Oh DH. 2016. Survival and expression of acid resistance genes in Shiga toxin-producing *Escherichia coli* acid adapted in pineapple juice and exposed to synthetic gastric fluid. Appl Microbiol 121:1416-1426.
- Baranzoni GM, Fratamico PM, Reichenberger ER, Kim GH, Breidt F, Kay K, Oh DH. 2016. Complete genome sequences of *Escherichia coli* O157:H7 strains SRCC 1675 and 28RC that vary in acid resistance. Genome Announcements 4(4) e00743-16.
- Kay K, Breidt F, Fratamico PM, Baranzoni G, Kim G, Grunden A, Oh D. 2017. *Escherichia coli* O157:H7 acid sensitivity correlates with flocculation phenotype during nutrient limitation. Front Microbiol 8:1404.
- Breidt F, Andress E, Ingham B. 2018. Recommendations for designing and conducting cold-fill hold challenge studies for acidified food products. Food Protection Trends 38(5):322-328.
- Ding Z, Johanningsmeier SD, Price RE, Reynolds R, Truong VD, Conley Payton SB, Breidt F. 2018. Evaluation of nitrate and nitrite contents in pickled fruit and vegetable products. Food Control, 90:304-311.
- Yavuz N, Foster L, Sharma T, Patel K, Stoforos G, Sandeep KP, Planitkar P, Breidt F. 2018. Hot-fill pasteurization of cucumber pickle spears: An alternative to tunnel pasteurization. Food Protection Trends 38(4):258-265.
- Dupree D, Price RE, Burgess B, Andress E, Breidt F. 2019. Effects of sodium chloride or calcium chloride concentration on the growth and survival *Escherichia coli* O157:H7 in model vegetable fermentations. Journal Food Protection 82(4):570-578.
- McMurtrie EK, Johanningsmeier SD, Price RE, Breidt F. 2019. Effect of brine acidification on fermentation microbiota and texture quality of cucumbers fermented in calcium chloride brines. J Food Sci 84(5):1129-1137.

Predictive Microbiology and Modeling: FSRIO Database

Introduction

The USDA-ARS Food Safety Research Information Office (FSRIO) based at the National Agricultural Library was created by the Agricultural Extension and Education Reform act of 1998 and was formally launched in July 2001. FSRIO was established to support the research community, both nationally and internationally by collecting, organizing and disseminating food safety information through its web site. (https://www.nal.usda.gov/fsrio/about-fsrio-0)

FSRIO's mission is to provide to the research community and the general public information on publicly funded, and to the maximum extent practicable, privately funded food safety research initiatives for the purpose of preventing unintended duplication of food safety research and assisting the executive and legislative branches of the Federal Government and private research entities to assess food safety research needs and priorities. FSRIO works in cooperation with the National Institutes of Health, the Food and Drug Administration, the Centers for Disease Control and Prevention, public institutions, and, on a voluntary basis, private research interests. (https://www.nal.usda.gov/fsrio/about-fsrio-0)

The Office's website acts as central location to provide a publicly accessible and searchable research projects database that enables access to current food safety research efforts, outcomes, and the latest up-to-date scientific information. By providing these information tools and products, the FSRIO assists the research community to assess research needs and priorities; allows exploration of current research activities to prevent duplication of efforts allowing for efficient use of research dollars; fosters increased cooperation among individuals and agencies engaged in food safety research; and brings awareness of these services by presenting at scientific conferences and other food safety related meetings.

Examples of Accomplishments

FSRIO website resources

- Identified 53 new resources that were added to the FSRIO website on 8 topic pages including: 1. Chemical Contaminants, 2. Food Allergens, 3. Food Preparation and Handling, 4. Food Safety Modernization Act, 5. Predictive Microbiology, 6. Pathogenic Bacteria, 7. Sanitation and Quality Standards and 8. Viruses and Prions

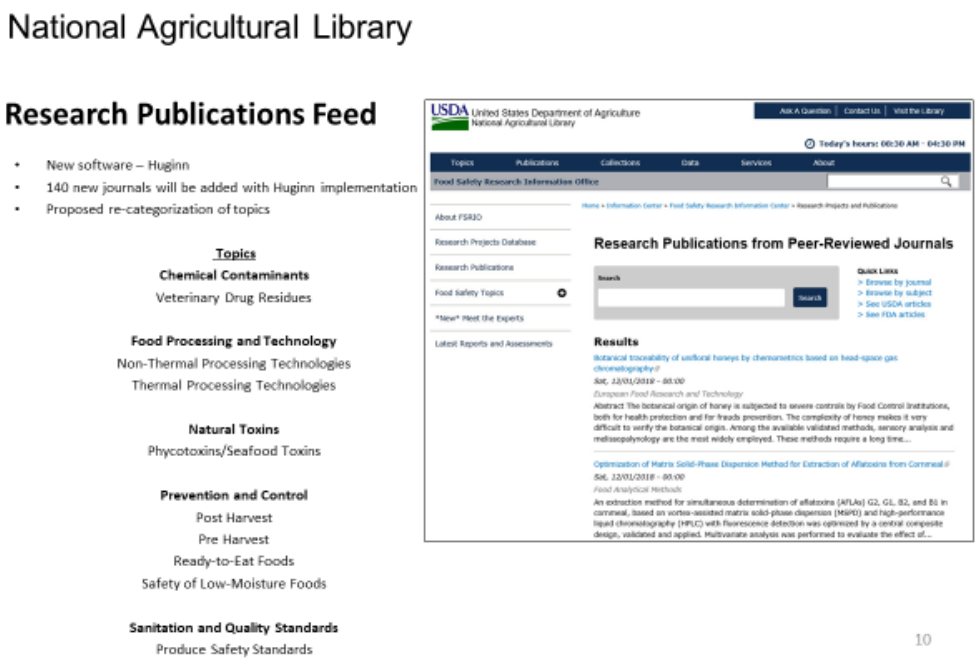
Literature reviews supporting food safety and antimicrobial resistance

- Conducted a comprehensive literature review on the Agricultural Antibiotic Resistance/Antimicrobial Resistance topic for ARS Office of National Programs. The review resulted in identifying 874 files on 7 lead categories (Antibiotic Alternatives, Antibiotic Resistance-Environment, Antibiotic Resistance-Food Safety, Antibiotic Resistance-Plants, Antibiotic Resistance-Animals, Antibiotic Resistance-General and Novel Antibiotics) and 37 subcategories to support ARS work in antimicrobial resistance.

- Updated two key resources on the Antimicrobial Resistance (AMR) page of the FSRIO website, including the overview description and AMR guidance

FSRIO research projects database and publications feed

- Created automated paths for collection of research projects and publications for the FSRIO website. These included programming with Huginn software and development of 25 agency xpaths.
- Identified 30 new food safety journals to add to the FSRIO Publications feed. The addition of new journals was based on emerging trends in food safety, as well as feedback received from National Program Leaders in food safety.
- Updated the RPD category and publications feed category lists with resources for ARS scientists to address the seven NP108 problem areas. These included; Systems Biology Microbial Populations; Detection and Characterization of Chemical and Biological Contaminants; Intervention and Control Strategies; Predictive Microbiology & Modeling, and Antimicrobial Resistance.
- Enhanced the food safety topics listed on the RPD and Publications feed of the website. Topic areas added to the category list included: Thermal and Non-Thermal Food Processing, Heavy Metals, Veterinary Residues, Produce Safety Standards, Safety of Low-Moisture Foods, and Seafood Toxins.



Meet the Experts

- Twenty-six (26) PowerPoint presentations from ARS scientists were reviewed for 508 compliance and prepared for posting to the FSRIO website. Presentations were obtained from NP108 research locations in Wyndmoor PA, Fargo ND, Athens GA, Albany CA, Peoria IL, New Orleans LA, Beltsville MD, College Station TX, and Clay Center NE. Topic areas covered in the presentations included: Genomics, Chemical Contaminants,

Detection Methods, Mycotoxins, Pre and Postharvest Interventions, Predictive Microbiology, Bacterial Pathogens and Produce.

- Eight videos of NP108 scientists were recorded and edited for production with the ARS Office of Communications video production team. Videos included interviews with scientists from ARS research facilities. The following topics were covered in the videos:
    - STEC biofilms within microbial communities in food.
    - Radio-frequency power for the pasteurization of Salmonella typhimurium in shell eggs.
    - Maintaining the quality and shelf-life of fresh produce.
    - Rapid inactivation of Taxoplasma gondi bradyzoites in dry cured sausage
    - Innovative Materials for use in Mycotoxin Detection.
    - Predictive model for growth of Clostridium Perfringens during cooling of uncooked beef.
    - Analytical methods for pesticide residues in fruits and vegetables.
    - An additional video of was added to the Meet the Experts page providing an update on drone technology used with/for farmers to monitor E. coli contamination in irrigation systems.

Website Analytics

- Total Page Views: ~ 80, 000 views per year on average, with a 5% increase per year
- Research Projects Database currently houses 13,624 active projects.
- Publications Feed currently tracks 97 active journals.

Outreach

- Exhibited at Annual International Association for Food Protection (IAFP) Conference in July, 2019. Distributed ~800 postcards to attendees. ~110 attendees signed up to receive FSRIO food safety email updates. Distributed FSRIO outreach materials.

FSRIO Working Group

- Conducted meetings with the FSRIO Working Group to gather feedback on program development, and topic areas to enhance FSRIO products. Members represent scientific expertise across USDA agencies: ARS, NIFA and FSIS.

Outcomes and Impacts

- By providing and updating these information tools and products, the FSRIO continues to assist the food safety research community, industry, general public and consumers nationally and internationally on relevant issues pertaining to the safety of the (global) food supply.

Predictive Microbiology and Modeling: Genomics (Database)

Introduction

The collection of genomics data from ARS food safety research was not formally combined into a specific Food Safety Program database, as was designed for ComBase, the FSRIO, or as for other National Programs; for example, the Human Nutrition (NP107) Nutrient Database.

The Program did however, undertake the following: (1) Developing a collection of bacterial pathogens (STEC's, Listeria, Campylobacter); including unique strains and serotypes which were examples that could be shared/used across the Food Safety Program for comparative studies. These isolates were deposited in the ARS Culture Collection (NRRL) housed at National Center for Agricultural Utilization Research (NCAUR), Peoria, Illinois; and (2) The Program became a Steering Committee member of the "Interagency Collaboration on Genomics for Food and Feed Safety" (Gen-FS) established to strengthen federal collaboration by addressing cross-cutting priorities for molecular sequencing of foodborne and other zoonotic pathogens causing human illness, for data collection and analysis, and for the use of these data in support of surveillance and outbreak investigation activities (as outlined at):

https://www.aphl.org/conferences/proceedings/Documents/2018/GenomeTrakr/Brown_2018GENFS_GTmeetingv1.pdf.

https://www.fsis.usda.gov/wps/wcm/connect/8bad2d05-e91c-4c8a-bf88-74dcd366ed70/WGS-Slides-Braden-102617.pdf?MOD=AJPERES

ARS through its various food safety genomics studies actively publishes sequence data and provides pathogen genome sequence (WGS) data to Gen-FS; and subsequently to Agencies within Gen-FS: CDC, FDA, National Center for Biotechnology Information (NCBI), National Library of Medicine (NLM); NIH, FSIS, and APHIS. Further, pathogen isolates related to data import or as needed (requested by agencies within Gen-FS for attribution analysis) were also sent to the FDA for inclusion in their culture collection.

Genomics research was conducted in various projects whose goal was to: (1) Characterize the genomes of production animal agriculture-related zoonotic bacteria including Salmonella enterica spp., Listeria monocytogenes, Shiga toxigenic E. coli, and Campylobacter spp. and contribute to the development of national databases of zoonotic bacteria sequences and annotations. Genomics data on fungal species; for example, Fusarium and Aspergillus were added to the NCBI.

Examples of Outputs:

- **Complete genome sequencing of STEC and Salmonella.** Genome sequence data are routinely being used to address an increasing number of problems in food microbiology- from outbreak detection and traceback, to comparative genomics to predict virulence and antimicrobial resistance phenotypes. However, because of the availability and reduced cost of short-read sequencing technologies, the majority (~93%) of the greater than 210,000 sequences available are incomplete (draft genome

sequences), as opposed to finished or complete genome sequences. Complete genome sequence data are needed for meaningful comparative, functional or forensic analyses. To help address this disparity regarding foodborne pathogens, complete genome sequences were generated for ~100 Salmonella strains, 370 STEC strains, 50 Campylobacter and Arcobacter strains and 7 Listeria strains. Over half of these sequences have been deposited into the NCBI database.

- **Genomics of Campylobacter and Arcobacter.** The majority of Campylobacter-related cases of human illness are caused by Campylobacter jejuni or C. coli. However, novel and/or emerging members of the Campylobacteraceae have been recently implicated in sporadic human illness and may be associated also with more severe illnesses, such as gastrointestinal cancer. To provide genomic data that could be used for epidemiological studies, development of improved typing and culturing methods, and research into host-association, virulence, antibiotic resistance, lateral gene transfer and novel metabolic pathways, genomes representing all validly-described Campylobacter and Arcobacter taxa (63 taxa in total) were sequenced to completion, assembled and annotated. Genomic data for all Campylobacter and Arcobacter type strains are now available for the first time. Moreover, for multiple species, several additional genomes were sequenced (up to 36 strains per species) to provide further insights into variation within some taxa. Putative virulence genes and plasmids were identified in many organisms, suggesting that several previously-uncharacterized taxa may be potential human or veterinary pathogens. Environmental- or host-associated genes and metabolic pathways were identified. The genomic data were used to develop new or improve existing Campylobacter/Arcobacter typing and species identification methods. These data were also critical in the characterization of novel campylobacters isolated from human clinical samples.

- **Defining diversity of Fusarium species that cause mycotoxin contamination in crops.** Many species of the fungus Fusarium are food and feed safety concerns because they contaminate crops with mycotoxins that are health hazards to humans, pets and livestock. An accurate understanding of the identity and diversity of species that cause mycotoxin contamination is an essential component of mycotoxin control strategies. However, there are significant knowledge gaps with respect to which species of Fusarium produce which mycotoxins, and there are potentially hundreds of unidentified species of the fungus that could be contributors to mycotoxin contamination around the world. Using genome sequencing and DNA-based phylogenetic analyses, ARS determined the phylogenetic relationships of over 350 isolates that represent the breath of diversity that exists within the genus Fusarium. Representative species from this phylogenetic study were also examined by mass spectrometry-based metabolomic analyses to assess their ability to produce all known Fusarium mycotoxins.

- **Software program for rapid searching of WGS data for protein biomarkers.** Software was developed to rapidly search WGS bacterial genomes for protein biomarkers that correspond to proteins analyzed by MALDI-TOF-TOF-MS/MS. Using this tool, a plasmid-borne protein was identified from a number STEC O113:H21 strains. The initial

version of this software tool was written as a macro in Microsoft Excel. Certain elements of this macro were incorporated into a re-designed, standalone version of the program written in Java for greater flexibility and accuracy with a user-friendly graphical user interface. This new and improved software resulted in the further identification of the colicin E3 immunity protein whose expression was induced by antibiotic exposure of STEC strains. This innovative program allows for rapid identification of proteins expressed from foodborne pathogens.

Outcomes and Impacts

- The availability of complete closed genomes for the scientific community to use in many different areas of research.
- Some of the proposed outcomes of Gen-FS include: (1) Coordinating research designed to incorporate cutting edge technology to improve identification and sequencing of pathogens obtained from clinical, food, feed, environmental, and animal sources; (2) Developing, documenting, and assessing protocols for comparing genomic sequences; and developing, sharing, and validating methods used to compare sequences and characterize pathogens; for example, single nucleotide polymorphisms (SNP) and whole genome multilocus sequence typing (wgMLST); (3) Sharing findings that support detection and response to outbreaks, regulatory actions, recalls, and other public health interventions, and research; and (4) Coordinating inter-agency efforts to define genomic determinants for antimicrobial resistance, define mechanisms for transmission within foodborne pathogens, and coordinating activities among agencies in support of the National Action Plan for Combating Antimicrobial Resistant Bacteria (CARB). [Extracted from the Gen-FS Charter].
- Determined the phylogenetic relationships of over 350 isolates that represent the breath of diversity that exists within the genus Fusarium. Representative species from this phylogenetic study were also examined by mass spectrometry-based metabolomic analyses to assess their ability to produce all known Fusarium mycotoxins. The DNA sequences characteristic of the novel species were integrated into online databases to determine the identities of Fusarium isolates that cause mycotoxin contamination. ARS is one of the biggest contributors of Fusarium genome sequences to the NCBI.

Examples of Relevant Publications (alphabetical order)

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Problem Statement 7. Antimicrobial Resistance

Goal

As outlined in the 2015 Retrospective Review Report (2015RRR);
https://www.ars.usda.gov/ARSUserFiles/np108/Final%20Accomplishment%20Report.pdf
Antibiotic use and the development of antibiotic resistant bacteria (ARB) represents a serious threat to both animal and human health and the economy. The concern for the development of has resulted in the development of both national and international strategies to address the issue. There are numerous areas of concern including: detecting, measuring, and assessing the amount of ARB within the production animal populations with an emphasis on foodborne pathogens; the genomics of antimicrobial resistance (AMR), and the transfer of antimicrobial resistant genes (ARG); developing alternatives to antibiotics; developing alternative strategies to minimize the use of antibiotics in production animals while maintaining and improving animal health. Research in this area is also covered under Problem Statement 1. Population Syatems.

Research incorporated omics-tools including WGS, metagenomics and microbiome analyses to identify the organisms that harbor the genetic elements associated with AMR, the effect of antimicrobials on the GI tract microbiome and the mechanisms pathogens used to acquire resistance. Studies investigated AMR across the entire spectrum of food safety and pathogen identification, identified novel organisms and resistances in ecological communities, determined reservoirs and amplifiers of resistance in animal production environments and identified factors that enhanced or reduced fitness characteristics of resistant and multidrug resistant microbial populations. Additional research was focused on identifying alternatives to antimicrobials to reduce the occurrence of ARB and to reduce the need for antimicrobials.

This area of research was conducted by numerous projects at various locations within the Program. Some objectives were part of a larger project not specifically directed to AMR, while others solely addressed AMR issues. The locations involved were as follows: WRRC, Albany, California; College Station, Texas; MARC, Clay Center Nebraska; NADC, Ames, Iowa; Fayetteville, Arkansas; U.S.NPRC, Athens, Georgia; and ERRC, Wyndmoor, Pennsylvania.

Research was conducted at WRRC, Albany, CA by the (Parker) project with the objective to: (1) Identify antimicrobial resistance gene reservoirs in the food production ecosystem and characterize the fitness and virulence of resistant pathogens.

Examples of Accomplishments

- **AMR in Campylobacters from farm to retail.** Campylobacter jejuni and C. coli are commensal organism of the intestinal tracts of poultry species and are leading causes of diarrheal disease in humans. Despite a reduction in the usage of antibiotics, these AMR Campylobacters are often recovered from poultry farms. Knowledge gaps remain regarding AMR emergence, spread and resilience in Campylobacter populations. Determined the genomic sequences of AMR C. jejuni and C. coli strains from turkey

farms. The strains possessed tetracycline resistance plasmids (pTet), and also harbored a chromosomally associated gentamycin resistance mobile element.

Outcomes and Impacts

- Provided evidence that AMR of Campylobacters in poultry is multi-dimensional with multiple hosts including the poultry and house flies, and several genomic mechanisms including plasmids and mobile elements. Also, the results provide evidence to the poultry industry that AMR Campylobacters persist despite reduction in antibiotic usage.

Examples of Relevant Publications

- Miller, W.G., Huynh, S., Parker, C.T., Niedermeyer, J.A., Kathariou, S. 2016. Complete Genome Sequences of Multidrug-Resistant *Campylobacter jejuni* Strain 14980A (Turkey Feces) and *Campylobacter coli* Strain 14983A (Housefly from a Turkey Farm), Harboring a Novel Gentamicin Resistance Mobile Element. Genome Announc. 4(5): e 01175-16.

Research was conducted at College Station, TX by the (Anderson) project with the objective to: (1) Identify, develop, and test interventions, including exploring possible synergies of multiple interventions and alternatives to antibiotics that can kill pathogenic or antibiotic resistant foodborne pathogens or mitigate their virulence and resistance in the animal production environment.

Examples of Accomplishments

- **A new probiotic antimicrobial alternative for ruminants**. Collaborating with scientists at Texas A&M University, studies isolated a never-before described bacterium from the rumen of a cow and found that when grown with certain feedstuffs, this bacterium reduced methane production while concurrently reducing numbers of dangerous pathogenic bacteria such as enterohemorrhagic Escherichia coli and Campylobacter in the rumen environment. The work further established that this bacterium, when trained to be supernaturally capable of degrading nitrate and nitrite, can effectively detoxify these toxins that often accumulate to high levels in heat- or drought-stressed forages which, when consumed by cattle, can cause illness or death.

Outcomes and Impacts

- The patented bacterial strain has been licensed to commercial partner and is now undergoing further cooperative research and development with additional support of industry and the Small Business Administration to provide ranchers a new tool to more efficiently produce meat and milk at less cost while concurrently lessening agricultural impact on the environment.

Example of Relevant Publications

- Latham, E.A., Pinchak, W.E., Trachsel, J., Allen, H.K., Callaway, T.R., Nisbet, D.J., Anderson, R.C. 2019. *Paenibacillus* 79R4, a potential rumen probiotic to enhance nitrite detoxification and methane mitigation in nitrate-treated ruminants. Science of the Total Environment. 671:324-328. https://doi.org/10.1016/j.scitotenv.2019.03.390.
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Research was conducted by two projects (Bono and Bosilevac) at MARC, Clay Center, NE.

The (Bono) project objective was to: (1) Development and validation of various antimicrobial resistance detection methodologies including culture and genomic techniques, such asWGS.

Examples of Accomplishments

- **Land application of beef cattle manure minimally impacts soil antimicrobial resistance.** Concerns have been raised that using beef cattle manure to fertilize croplands increases the amount of antimicrobial resistant bacteria in these soils. If the increases persist until crops are planted this could increase food-animal and human exposures to antimicrobial resistant bacteria through feed and produce. At three farms in the U.S. Upper Midwest, croplands were fertilized with either: beef cattle manure, inorganic fertilizer, or were not fertilized (control). Manure did not change the levels of all 8 antimicrobial resistance bacteria measured. Manure did not increase levels for 8 of the 10 antimicrobial resistance genes measured. For the other two antimicrobial resistance genes, AMR increases in manure fertilized croplands only occurred at one location, were transient, and generally were within the abundance ranges observed for control croplands. Thus, we conclude that the common practice in U.S. Upper Midwest of region of land applying beef cattle manure likely has minimal impact on environmental antimicrobial resistance levels, feed safety, food safety, animal health, and human health.

Outcomes and Impacts

- Demonstrated that current beef manure management in the Upper Midwest region does not increase environmental AMR. Season had a greater impact on soil AMR levels than manure application.

Examples of Relevant Publications

- Miller, E., Spiehs, M., Arthur, T.M., Woodbury, B., Cortus, E., Chatterjee, A., Rahman, S., and Schmidt, J.W. Cropland amendment with beef cattle manure minimally impacts antimicrobial resistance. J. Environ. Qual. Accepted 16 August 2019. JEQ-2019-02-0042-TR.R2. 2019. https://doi: 10.2134/jeq2019.02.0042

The (Bosilevac) project objective was to: (1) Identify environmental and management practices that influence AMR, colonization of lymph nodes, and colonization rates of cattle, veal, and swine.

Example of Accomplishments

- **ARB populations and ARG are found in environments impacted municipal waste as well as livestock**. ARB in livestock waste runoff is a topic of public concern. ARB and the presence of ARG were compared within samples cattle feedlot runoff catchment ponds, swine waste lagoons, municipal wastewater treatment facilities, and environments considered low impact (a municipal lake and a prairie). The studies showed that prevalence's and concentrations of ARB were similar among the livestock and municipal sample sources, but there were differences among the ARG found in agricultural, environmental, and municipal samples, with municipal samples harboring the highest number of ARG.

- **Feeding cattle preventative antimicrobials improves animal health and is a judicious use of antimicrobials.** Antimicrobial use in livestock production is under intense scrutiny in the U.S. due to potential contributions to AMR. The effect of a one-time, five-day in-feed chlortetracycline (an antimicrobial not considered critically important for human medicine) regimen as preventative treatment for bovine respiratory disease was evaluated. Over 25% of the animals that did not receive the in-feed treatment (control group) developed illnesses requiring therapeutic treatment with antimicrobials critically important to human medicine. None of the cattle that received the in-feed treatment developed pneumonia. No differences in ARG were seen between treated and non-treated in-feed animals. Further, no differences between treated and control groups was found in the levels of 10 ARGs at any time from 5- to 117 days following a 5-day in-feed chlortetracycline regimen.

- **AMR in mature beef cows is not correlated to antimicrobial use.** Studies have shown that there is an increase in ARB following an antimicrobial treatment of feedlot cattle. The ARB returns to pre-treatment levels approximately 14 to 36 days after treatment. Due to their longevity, beef cows are more likely to receive antimicrobial treatments than feedlot cattle, albeit spread over a longer period. Occurrences of ARB from beef cows for which complete antimicrobial treatment records were available were compared. Approximately half of the cows sampled for this study were treated with antimicrobials for the treatment of disease, while the other half did not receive any antimicrobial treatments over their lifetime. The occurrence of ARB was not associated

with prior history of antimicrobial treatments or duration of time since the last antimicrobial treatment.

- **Raising livestock without antibiotics has little influence on antimicrobial resistance found in animals or their meat.** The levels of 67 different ARGs were compared between U.S. beef cattle produced conventionally (CONV), with no restrictions on antibiotic use other than regulatory compliance, and beef cattle raised without antibiotics (RWA). Fifty of the 67 ARG levels were not different between production systems. Seventeen ARG were increased in CONV animals but the increases were so small they are considered biologically insignificant. Beef and pork products produced from CONV and RWA cattle and swine were compared. The studies demonstrated CONV and RWA ground beef products contained similar levels of 13 ARG, with one ARG level higher in CONV ground beef and two ARG levels higher in RWA ground beef. For CONV and RWA pork chops, similar levels of all 16 ARG assessed were found. Regardless of antibiotic use claims, AMR is similar in animals and ground beef and pork.

Outcomes and Impacts

- Demonstrated that AMR is a very widespread phenomenon and that a higher diversity of AMR can be found in human waste streams than in cattle and swine waste streams.
- Demonstrated that prophylactic in-feed treatment of chlortetracycline administered for five days to calves entering feedlots is judicious as this therapy reduced animal illnesses, reduced the use of antimicrobials more critical to human health, and had no long-term impact on the occurrence of AMR.
- Demonstrated the occurrence of AMR in beef cows was not associated with antimicrobial use indicating that other factors more strongly influenced the observed levels of ARB in beef cows.
- Demonstrated that beef cattle production without any antibiotics should not be expected to reduce the amount of AMR contributed to the environment compared to conventional beef production. Cattle records show cattle raised without antibiotics grow slower and must be fed 50 days longer than conventionally raised cattle. Further calculations showed this longer time on feed results in a 31% increase in amount of manure from cattle raised without antibiotics which more than offsets the small reduction in ARG and may actually increase total ARG in the environment.
- Determined that claims of detrimental impacts of antibiotic use during cattle and swine production on human health from eating beef or pork are without merit.

Examples of Relevant Publications

- Agga, G.E., Schmidt, J.W., Arthur, T.M. 2016. Effects of in-feed chlortetracycline prophylaxis of beef cattle on animal health and antimicrobial-resistant Escherichia coli. Applied and Environmental Microbiology. 82(24):7197-7204. https://doi:10.1128/AEM.01928-16.
- Agga, G.E., Schmidt, J.W., Arthur, T.M. 2016. Antimicrobial-resistant fecal bacteria from ceftiofur-treated and nonantimicrobial-treated comingled beef cows at a cow-calf operation. Microbial Drug Resistance. 22(7):598-608. https://doi:10.1089/mdr.2015.0259.
- Vikram, A., Rovira, P., Agga, G.E., Arthur, T.M., Bosilevac, J.M., Wheeler, T.L., Morley, P., Belk, K., Schmidt, J.W. 2017. Impact of "raised without antibiotics" beef cattle production practices on occurrences of antimicrobial resistance. Applied and Environmental Microbiology. 83:e01682-17. https://doi.org/10.1128/AEM.01682-17.
- Arthur, T.M., Schmidt, J.W. 2018. Effects of in-feed Chlortetracycline prophylaxis in beef cattle on antimicrobial resistance genes. Foodborne Pathogens and Disease. 15(1):689-697. https://doi.org/10.1089/fpd.2018.2475.
- Vikram, A., Parker, E., Arthur, T.M., Bosilevac, J.M., Wheeler, T.L., and Schmidt, J.W. 2018. Similar levels of antimicrobial resistance in U.S. foodservice ground beef products with and without a "raised without antibiotics" claim. J. Food Prot. 81:2007-2018. https://doi: 10.4315/0362-028X.
- Vikram, A., Schmidt, J.W. 2018. Functional blaKPC-2 sequences are present in U.S. beef cattle feces regardless of antibiotic use. Foodborne Pathogens and Disease. 15(7):444-448. https://doi.org/10.1089/fpd.2017.2406.
- Vikram, A., Parker, E., Arthur, T.M., Bosilevac, J.M., Wheeler, T.L., and Schmidt, J.W. 2019. Foodservice pork chops from three United States regions harbor similar levels of antimicrobial resistance regardless of antibiotic use claims. J. Food Prot. Accepted 3 June 2019. JFP-19-139.

Research was conducted by two projects at NADC, Ames IA (Bearson and Allen).

The (Bearson) project objectives were to: (1) Investigate the impact of antibiotic usage on influencing Salmonella virulence mechanisms and enhancing antibiotic resistance; (2) Develop novel non-antibiotic intervention strategies such as beneficial microbes and vaccines to limit Salmonella colonization, persistence and shedding; and (3) Evaluate immune networks and identify porcine genes for their relationship with the host microbiota to reduce Salmonella colonization, persistence, and shedding.

Examples of Accomplishments (Bearson)

- **Reducing Salmonella in swine by enhancing their innate immune defenses.** Controlling asymptomatic Salmonella carriage in food animals is impeded by the existence of >2,600 Salmonella serovars but is critical due to the possibility of farm-to-fork contamination. Development of intervention strategies that are effective against diverse Salmonella serovars are desired to limit cases of foodborne illness. Neutrophils

are cells of the immune system that play an important role in combatting bacterial infection, and the immune protein that induces neutrophil production in the animal is called granulocyte-colony stimulating factor (G-CSF). Studies demonstrated that pigs receiving an engineered vector expressing the porcine G-CSF (Ad5-G-CSF) had significantly reduced Salmonella fecal shedding and tissue colonization, and less Salmonella-induced disturbance of their gastrointestinal bacteria (microbiota) compared to the pigs that were exposed to Salmonella but not Ad5-G-CSF. Collectively, these data suggest that delivery of a targeted immunostimulant to enhance innate immunity may be a strategy to reduce Salmonella colonization, potentially during periods of immunological and animal production stress such as farrowing, weaning, and transportation.

- **Salmonella DIVA vaccine**. Salmonella is a major foodborne pathogen that often resides in the gastrointestinal tract of food animals including pigs, poultry and cattle without causing disease, but can cause significant disease in humans when products from food animals are consumed. A live-attenuated Salmonella vaccine was developed by ARS for food-producing livestock and poultry. Genetic mutations in the bacterial genome limited Salmonella serotype-specific immunity (greater than 2,600 Salmonella serotypes exist) and instead, induced an immune response that was cross-protective against diverse Salmonella serotypes. The vaccine effectively reduced Salmonella disease, colonization, and fecal shedding in vaccinated swine and turkeys. Furthermore, the vaccine was designed to differentiate infected from vaccinated animals (i.e. DIVA). Pre-harvest control of Salmonella in food-producing animals can protect animal health, limit antibiotic usage, decrease environmental contamination, reduce Salmonella carriage into the human food chain, and diminish the cost of meat product recalls to producers.

- **Antibiotic exposure of multi-drug resistant (MDR) Salmonella induces expression of disease-associated genes and enhances tonsil colonization in pigs.** Chlortetracycline is an antibiotic commonly used in veterinary medicine for respiratory and gastrointestinal infections, but many MDR Salmonella isolates are resistant to chlortetracycline. Therefore, chlortetracycline treatment for an infection may have unintended consequences in an animal unknowingly colonized with MDR Salmonella. Studies revealed that exposure of MDR Salmonella isolates to chlortetracycline changed the expression levels of over 50 percent of Salmonella genes, including genes involved in the ability of Salmonella to move (motility), invade host cells (invasion), and survive and replicate inside host cells (virulence). Furthermore, pigs exposed to MDR Salmonella and given a therapeutic dose of chlortetracycline had higher levels of Salmonella in the oral cavity (tonsils) and feces when compared to pigs that did not receive chlortetracycline. These data suggest that consideration for Salmonella status may be important when administering therapeutic antibiotics because animals that are unsuspectingly colonized with MDR Salmonella and receive therapeutic chlortetracycline for an unrelated infection may have prolonged host colonization and fecal shedding of the human foodborne pathogen, thereby increasing environmental or pork product contamination and posing a risk to public health.

Outcomes and Impacts

- Demonstrated an engineered vector expressing the porcine G-CSF significantly reduced Salmonella fecal shedding; tissue colonization, and less -induced disturbance of their gastrointestinal bacteria. This suggest that delivery of a targeted immunostimulant to enhance innate immunity may be a strategy to reduce Salmonella colonization, potentially during periods of immunological and animal production stress such as farrowing, weaning, and transportation.
- Developed, patented, and licensed to an industry partner a live vaccine that: (1) is attenuated in swine and turkeys; (2) provides cross-protection against various Salmonella serovars, and (3) is a DIVA vaccine (Differentiation of Infected from Vaccinated Animals). This vaccine can be used for pre-harvest control of Salmonella in food-producing animals; protecting animal health, limiting antibiotic usage, decreasing environmental contamination, reducing Salmonella carriage into the human food chain, and diminishing the cost of meat product recalls. The technology received an Excellence in Technology Transfer Award, Midwest Region from the Federal Laboratory Consortium in 2017.
- Determined that chlortetracycline exposure induces a 50% increase in gene expression in multi-drug resistant (MDR) Salmonella, particularly for motility, invasion, replication and virulence.
- Determined that pigs exposed to MDR Salmonella and given a therapeutic dose of chlortetracycline had higher levels of Salmonella in the oral cavity (tonsils) and feces, suggesting that Salmonella status may be critically important when administering therapeutic antibiotics.

Examples of Relevant Publications:

- Brunelle, B.W., Bearson, B.L., Bearson, S.M. 2015. Chloramphenicol and tetracycline decrease motility and increase invasion and attachment gene expression in specific isolates of multidrug-resistant *Salmonella enterica* serovar Typhimurium. Frontiers in Microbiology. 5(801). https://doi:10.3389/fmicb.2014.00801.
- Bearson, B.L., Bearson, S.M., Kich, J.D. 2016. A DIVA vaccine for cross-protection against *Salmonella*. Vaccine. 34:1241-1246. https://doi:10.1016/j.vaccine.2016.01.036.
- Bearson, S.M.D., Bearson, B.L., Loving, C.L., Allen, H.K., Lee, I., Madson, D., Kehrli, M.E. Jr. 2016. Prophylactic administration of vector-encoded porcine granulocyte-colony stimulating factor reduces *Salmonella* shedding, tonsil colonization, and microbiota alterations of the gastrointestinal tract in *Salmonella*-challenged swine. Frontiers in Veterinary Science. 3(66).
- Brunelle, B.W., Bearson, B.L., Bearson, S.M., Casey, T. 2017. Multidrug-resistant *Salmonella enterica* serovar Typhimurium isolates are resistant to antibiotics that influence their swimming and swarming motility. mSphere. 2:e00306-17. https://doi:10.1128/mSphere.00306-17.
- Bearson, B.L., Bearson, S.M., Brunelle, B.W., Bayles, D.O., Lee, I., Kich, J.D. 2017. *Salmonella* DIVA vaccine reduces disease, colonization and shedding due to virulent *S.* Typhimurium infection in swine. Journal of Medical Microbiology. 66:651-661. https://https://doi:10.1099/jmm.0.000482.

- Holman, D., Bearson, S.M., Bearson, B.L., Brunelle, B.W. 2018. Chlortetracycline and florfenicol induce expression of genes associated with pathogenicity in multidrug-resistant *Salmonella enterica* serovar Typhimurium. Gut Pathogens. 10:10. https://doi:10.1186/s13099-018-0236-y.
- Bearson, S.M., Bearson, B.L., Sylte, M.J., Looft, T.P., Kogut, M.H., Cai, G. 2019. Cross-protective *Salmonella* vaccine reduces cecal and splenic colonization of multidrug-resistant *Salmonella enterica* serovar Heidelberg. Vaccine. 37:1255-59. https://doi:10.1016/j.vaccine.2018.12.058.
- Holman, D., Bearson, B.L., Allen, H.K., Shippy, D.C., Loving, C.L., Kerr, B.J., Bearson, S.M., Brunelle, B.W. 2019. Chlortetracycline enhances tonsil colonization and fecal shedding of multidrug-resistant *Salmonella enterica* serovar Typhimurium DT104 without Major Alterations to the Porcine Tonsillar and Intestinal Microbiota. Applied and Environmental Microbiology. 6:e02354-18. https://doi:10.1128/AEM.02354-18.

The (Allen) project addressed objective to: (1) Characterize the microbiome of swine and turkeys and investigate the effects of antibiotics and non-antibiotic feed additives on the expression and transmission of virulence, fitness or ARB in intestinal microbial populations; and (2) Assess the interaction of the intestinal immune system and commensal bacteria in swine and turkeys to determine how the microbiota or foodborne pathogens affect tissue innate immunity and acquired immunity and evaluate non-antibiotic feed additives as an effective strategy to control colonization by foodborne pathogens.

Examples of Accomplishments

- **Collateral effects of in-feed antibiotics: Carbadox induces bacteriophage and antimicrobial resistance gene transfer in pig gut.** The antibiotic Carbadox is fed to US swine to improve feed efficiency and control swine dysentery. Carbadox induces phages in swine gut bacteria and Salmonella enterica serovar Typhimurium, a foodborne pathogen. Phages are viruses that infect bacteria and are important because they kill bacteria, but also because they transfer genetic material between cells. Within two days of in-feed Carbadox administration, gut bacteria in Carbadox-fed pigs expressed different genes than the gut bacteria of non-medicated pigs. Gut bacteria in the Carbadox-fed pigs were not multiplying or metabolizing carbohydrates as they normally would, and phages were being induced in the gut microbiota. Phage genetic material encoded antibiotic resistance genes that could provide resistance to antibiotics that are important in human medicine, indicating that human-relevant antibiotic resistance genes are mobile between bacteria via phages. This research demonstrates that Carbadox can cause dissemination of antibiotic resistance genes between bacteria via bacteriophage. Collateral effects of in-feed antibiotics are important since antibiotic administration may unintentionally affect antimicrobial resistance gene diversity, horizontal gene transfer, and bacterial diversity.

- **The in-feed antibiotic bacitracin methylene disalicylate (BMD) alters turkey intestinal microbiota structure and metabolome.** Concern for antibiotic resistance and restrictions on agriculture antibiotic use has heightened the need to identify the mechanism of action of antibiotics. BMD is an in-feed antibiotic with label use for feed efficiency (low dose) and therapeutic (high dose) applications in poultry production, with no human medicine applications. BMD reduced the number of bacterial members immediately after treatment, a trend that lasted after the BMD was removed from feed. BMD induced functional shifts among hundreds of metabolites in the turkey microbiome, including an increase of beneficial metabolites likely related to the feed efficiency applications of BMD. Bacteria associated with metabolic functional shifts in turkeys offer promising targets for non-antibiotic methods to enhance poultry gut health.

- **Non-antibiotic, in-feed product alters gut microbiota and immune status in nursery pigs**: Butyrate is a gut bacterial fermentation product with benefits for host tissues and resistance to pathogen colonization. Stimulating gut butyrate production in pigs is a potential method for supporting animal health and thereby reducing the need for antibiotic usage. A high-throughput amplicon-sequencing based strategy was developed to detect bacterial genes responsible for one important pathway of butyrate production in gut ecosystems. The method allows scientists to detect which bacterial members are expressing the genes for butyrate production across hundreds of gut microbiome samples simultaneously. Using this assay, bacterial members responding to an in-feed non-antibiotic treatment (resistant potato starch, RPS) were identified in pigs. RPS intake altered the composition of the gut microbiome, including the butyrate-producing bacteria, and microbiome changes correlated with increased production of butyrate. RPS-induced microbial changes also correlated with beneficial shifts in pig immune cell populations and gene expression. RPS intake in weaned piglets correlated with enhanced markers of gut health and may be one effective alternative to antibiotics for supporting swine health in the nursery phase. In total, this work represents progress towards developing non-antibiotic interventions with identified modes of action to support swine health.

Outcomes and Impacts

- Demonstrated that Carbadox can cause dissemination of antibiotic resistance genes between bacteria via bacteriophage. The Journal mBio (American Society for Microbiology publication) published an invited commentary by the director of the Antibiotic Resistance Action Center, George Washington University to discuss the study findings. Collateral effects of in-feed antibiotics are important since they may unintentionally affect antimicrobial resistance gene diversity, horizontal gene transfer, and bacterial diversity.
- Determined that application of BMD a poultry in-feed antibiotic reduced the number of bacterial members immediately after treatment, a trend that lasted after the BMD was removed from feed. BMD induced functional shifts in the turkey microbiome and an increase of beneficial metabolites. Bacteria associated with metabolic functional shifts offer promising targets for non-antibiotic methods to enhance poultry gut health.

- Developed a high-throughput amplicon-sequencing based strategy (HTA) to detect bacterial genes responsible for butyrate production in gut ecosystems. The allows determination of which bacterial members are expressing the genes for butyrate production across hundreds of gut microbiome samples simultaneously.
- Determined using the HTA assay which bacteria respond to in-feed non-antibiotic (resistance potato starch: RPS) treatment. RPS alters or is correlated with the composition of the gut microbiome; changes in butyrate-producing bacteria; beneficial shifts in pig immune cell populations and gene expression; and markers of gut health. RPS use may be one effective alternative to antibiotics for supporting swine health in the nursery phase.

Examples of Relevant Publications

- Trachsel J, Bayles DO, Looft T, Levine UY, Allen HK. Appl Environ Microbiol. 2016. Function and Phylogeny of Bacterial Butyryl Coenzyme A:Acetate Transferases and Their Diversity in the Proximal Colon of Swine. *Applied Environmental Microbiology*.82(22):6788-6798. https://doi.org/0.1128/AEM.02307-16
- Johnson, T.A., Looft, T.P., Severin, A.J., Bayles, D.O., Nasko, D.J., Wommack, E., Howe, A., Allen, H.K. 2017. The in-feed antibiotic carbadox induces phage gene transcription in the swine gut microbiome. *mBio*. 8(4):e00709-17. https://doi.org/10.1128/mBio.00709-17.
- Trachsel, J., Humphrey, S.B., Allen, H.K. 2018. Butyricicoccus porcorum sp. nov. a butyrate-producing bacterium from swine intestinal tract. *International Journal of Systematic and Evolutionary Microbiology*. 68:1737-1742. https://doi.org/10.1099/ijsem.0.002738.
- Hansen, R.L., Duenas, M.E., Looft, T., Lee, Y.J. 2018. Nanoparticle microarray for high-throughput microbiome metabolomics using matrix-assisted laser desorption ionization mass spectrometry. *Analytical and Bioanalytical Chemistry*. 411(1):147-156. https://doi.org/10.1007/s00216-018-1436-5.
- Trachsel, J., Briggs, C., Gabler, N.K., Allen, H.K., Loving, C.L. 2019. Dietary resistant potato starch alters intestinal microbial communities and their metabolites and markers of immune regulation and barrier function in swine. *Frontiers in Immunology*. 10:1381. https://doi.org/10.3389/fimmu.2019.01381
- Johnson, T., Sylte, M.J., Looft, T.P. 2019. In-feed bacitracin methylene disalicylate modulates the turkey microbiota and metabolome in a dose-dependent manner. *Scientific Reports*. 9:8212. https://doi.org/10.1038/s41598-019-44338-5.

Studies at Fayetteville, AR were conducted by the (Donoghue) project where the objectives were to: (1) Investigate the use of selected probiotics, natural plant compounds, and bacteriophage, as potential alternatives to antibiotics and mechanisms to reduce the levels of Salmonella and Campylobacter in poultry. Evaluate these products in multiple production/ processing systems including conventional, pasture raised, and organic systems; and (2) Develop innovative strategies for increasing disease resistance and improving immunity to foodborne pathogens of poultry using egg shell membrane technology.

Examples of Accomplishments

- **Elucidating the mechanisms of action of plant-derived antimicrobials against the food borne pathogen Campylobacter.** Campylobacter is one of the most commonly reported pathogens causing food borne infections in the U.S. and epidemiological evidence has implicated raw poultry products as a significant source of human infection. Therefore, it is very important to develop effective strategies for controlling this pathogen in poultry. ARS developed several phenotypic assays, cell culture and gene expression analysis protocols for rapid screening of phytochemicals for efficacy against Campylobacter. Using these assays, we have identified plant-derived antimicrobials with significant anti-Campylobacter efficacy. These compounds (trans-cinnamaldehyde, derived from cinnamon bark; carvacrol, an antimicrobial ingredient in oregano oil; and eugenol, the active ingredient in the oil from cloves) are effective in reducing Campylobacter colonization in chickens and survival on poultry products (chicken skin, wings). In addition, using proteomic analysis, successfully delineated the potential mechanism of action of these compounds. These plant phytochemicals can potentially provide the poultry industry (both conventional and organic) with economical, effective, and control strategies.

- **Developed novel carrier systems for controlling environmental persistence of foodborne pathogens.** Salmonella and Campylobacter jejuni can colonize the poultry gut thereby leading to contamination of the processing environment and carcass during slaughter. Both pathogens can form biofilms that facilitates their greater survival in the processing environment with increased resistance to antimicrobials and disinfectants. Studies developed a safe and effective strategy for controlling Salmonella and C. jejuni biofilms by combining the antimicrobial efficacy of Generally Recognized as Safe (GRAS) status phytochemicals with nanotechnology to develop natural disinfectants with significant antibiofilm efficacy against Salmonella and C. jejuni. Studies tested several phytochemical nano-emulsions and demonstrated that they are effective in inhibiting the biofilm formation and inactivating mature biofilms on common food contact surfaces. In addition, phytochemicals modulated critical genes and proteins required for C. jejuni biofilm formation. Since C. jejuni can form biofilms in the processing environment leading to contamination of products, phytochemicals could potentially be used for controlling biofilms thereby reducing the risk of human infections.

- **A method to study the mechanism of the changes in intestinal permeability.** Abnormal changes in intestinal permeability can lead to gastrointestinal (GI) problems since it can permeate toxins, antigens, pathogens, and parasites into the system creating problems such as inflammation, autoimmunity, and enteritis. With the restrictions in the antibiotic usage, the chances of poultry flocks prone to GI problems has significantly increased. Hence it is imperative to understand the mechanism of the disease and its therapy. ARS developed a method to culture intestinal epithelial cells (enterocytes) and used it to study intestinal permeability problems. Using a chemical named phorbol myristate acetate (PMA) that is found in croton oil, studies showed that this chemical profoundly affects the cell's health that resembles the change in intestinal permeability. Studies determined how this chemical affects the proteomics of the cells that may be relevant to their dystrophy and found that PMA affects the energy metabolism and nuclear activities of the enterocytes that may explain why these cells become cachectic. Further, studies used the enterocyte culture to screen what other factors may affect intestinal cells and make them amenable to gastrointestinal problems or may improve intestinal health. This cell culture system can be used to study intestinal problems in poultry in a cost effective and sustainable manner particularly for screening assays. Observations from this system has led to the development of chicken intestinal organoids, a 3D culture model, to study similar phenomenon such as intestinal disease pathogenesis, host pathogen interaction, and antibiotic alternative screening which is novel to poultry research.

Outcomes and Impacts

- Developed phenotypic assays, cell culture and gene expression analysis protocols for identification and rapid screening of plant derived phytochemicals for efficacy against Campylobacter. The phytochemicals can potentially provide the poultry industry (both conventional and organic) with economical, effective, and control strategies.
- Developed a method to culture intestinal epithelial cells examine intestinal permeability. This led to the development of chicken intestinal organoids, a 3D culture model, to study phenomenon such as disease pathogenesis, host pathogen interaction, and antibiotic alternative screening which is novel to poultry research.

Examples of Relevant Publications

- Shrestha, S., Arsi, K., Wagle, B.R., Donoghue, A.M., Donoghue, D.J. 2017. The Ability of Select Probiotics to Reduce Enteric Campylobacter Colonization in Broiler Chickens. International Journal of Poultry Science. 16:37-42.
- Upadhyay, A., Arsi, K., Wagle, B.R., Upadhyaya, I., Shrestha, S., Donoghue, A.M., Donoghue, D.J. 2017. Trans-cinnamaldehyde, carvacrol, and eugenol reduce Campylobacter jejuni colonization factors and expression of virulence genes in vitro. Frontiers in Microbiology. 8:713.

- Wagle, B. R., Upadhyay, A., Arsi, K., Shrestha, S., Venkitanarayanan, K., Donoghue, A.M. Donoghue, D. J. 2017. Application of β-resorcylic acid as potential antimicrobial feed additive to reduce Campylobacter colonization in broiler chickens. Frontiers in Microbiology. 8:599.
- Wagle, B. R., Arsi, K., Upadhyay, A., Shrestha, S., Venkitanarayanan, K., Donoghue, A.M., Donoghue, D.J. 2017. β-resorcylic acid, a phytophenolic compound, reduces Campylobacter jejuni in post-harvest poultry. Journal of Food Protection. 80(8):1243-1251.
- Rath, N.C., Liyanage, R., Gupta, A., Packialakshmi, B., Lay, J. 2018. A method to culture chicken enterocytes and their characterization. Poultry Science. 0:1-8. https://doi.org/10.3382/ps/pey248.
- Woo-Ming, A., Arsi, K., Wagle, B.R., Shrestha, S., Donoghue, A.M., Donoghue, D.J. 2018. Probiotic Cultures of *Lactobacillus* Spp. Isolates Reduce the Foodborne Pathogen, *Campylobacter jejuni* on Post-Harvest Chicken. *International Journal of Advances in Science Engineering and Technology*. 6(2):40-44.
- Wagle, B.R., Upadhyay, A., Shrestha, S., Arsi, K., Upadhyaya, I., Donoghue, A.M., Donoghue, D.J. 2019. Pectin or chitosan coating fortified with eugenol reduces Campylobacter jejuni on chicken wingettes and modulates expression of critical survival genes. Poultry Science. 98:1461-1471. https://doi.org/10.3382/ps/pey505.
- Shrestha, S., Wagle, B.R., Upadhyay, A., Arsi, K., Donoghue, D.J., Donoghue, A.M. 2019. Carvacrol antimicrobial wash treatments reduce Campylobacter jejuni and aerobic bacteria on broiler chicken skin. Poultry Science. 0:1-11. https://doi.org/10.3382/ps/pez198.
- Shrestha, S., Wagle, B.R., Upadhyay, A., Arsi, K., Upadhyaya, I., Donoghue, D.J., Donoghue, A.M. 2019. Edible coatings fortified with carvacrol reduce Campylobacter jejuni on chicken wingettes and modulate expression of select virulence genes. Frontiers in Microbiology. 10:583. https://doi.org/10.3389/fmicb.2019.00583.
- Arsi, K., A. M. Donoghue, J. H. Metcalf and D. J. Donoghue. 2019. Effect of Dietary Supplementation of Essential Oils, Eugenol or Trans-cinnamaldehyde, on Enteric Colonization of Campylobacter in Broiler Chickens. International Journal of Advances in Science, Engineering and Technology (IJASEAT). 7(1), Spl. Iss-2:30-32.
- Rath, N.C., Gupta, A., Liyanage, R., Lay, J. 2019. Phorbol 12-myristate 13-acetate induced changes in chicken enterocytes. Proteomics Insights. 10:1-13. https://doi.org/10.1177/1178641819840369.
- Wagle, B. R., Upadhyay, A., Upadhyaya, I., Shrestha, S., Arsi, K., Venkitanarayanan, K., Donoghue, A.M., Donoghue, D.J. 2019. *Trans*-cinnamaldehyde, eugenol and carvacrol reduce *Campylobacter jejuni* biofilms and modulate expression of select genes and proteins. *Frontiers in Microbiology*. 10:1837.

Research was conducted at NPRC, Athens, GA by several projects (Jackson, Line, and Cook/Oladeinde).

The Jackson project objective was to: (1) Provide data and characterize pathogen prevalence, unique characteristics and trends on AMR, subtyping and molecular characterization of foodborne pathogens in food animals.

Examples of Accomplishments

- **Finding the colistin gene.** Colistin is an antibiotic classified by the U.N. World Health Organization as being of critical importance to human health. In 2015, a gene (mcr-1) for colistin resistance was found that increases the ease and rate at which resistance can spread to different bacteria. In 2016, ARS researchers were the first in the U.S. to find this bacterial gene in the stomach contents of livestock. From over 2,000 cecal samples, two isolates of Escherichia coli were found that carried the mcr-1 gene. Isolates were characterized by WGS, resistance profiling and plasmid mobility studies. The total genomic DNA of the isolates were sequenced, and it was found that the U.S. isolates descended from isolates found in China but they were substantially different so there was not a direct link. At this time, it is unknown how the resistance gene traveled from China to the U.S.

- **Expansion of the Comprehensive Antibiotic Resistance Database.** The ability to detect and analyze ARG is highly dependent on using a complete and carefully annotated database of known resistance genes. Researchers from Ontario, Canada, and ARS have developed the Comprehensive Antibiotic Resistance Database (CARD; http://arpcard.mcmaster.ca). CARD is a manually curated database containing high quality reference AMR genetic data including genes and proteins. Its design allowed the development of novel genome analysis tools, such as the Resistance Gene Identifier (RGI) for prediction of resistance genes from whole genome sequences. CARD's RGI has been used to analyze thousands of bacterial genomes from hundreds of research and clinical studies, resulting in AMR data used in a wide variety of research.

- **Biocide assay for Salmonella.** Antimicrobial interventions, known as biocides, can be used to reduce bacterial contamination during processing of retail meat; however, little is known about how bacteria may develop resistance to these compounds and there is no systematic way to detect changes in susceptibility to these compounds that may indicate the development of resistance. A high throughput panel of 17 common household and commercially-used biocides was developed to determine the minimum inhibitory concentration of Salmonella for these compounds and to determine if resistance to biocides correlates with resistance to antibiotics. Multi-drug resistant Salmonella isolates were tested on the panel and the minimum inhibitory concentration of Salmonella to each of the chemicals was calculated. Isolates were resistant to cetylpyridinium chloride, hexadecyltrimethylammonium bromide, citric acid, acidified sodium chlorite, chlorhexidine, arsenite, and arsenate. No correlation was detected between susceptibility of Salmonella to the biocides and their antibiotic resistance. This biocide assay is under development for use at U.S. federal regulatory agencies.

- **Omics of streptogramin resistance**. Resistance to the streptogramin antimicrobials, quinupristin/dalfopristin and virginiamycin, has been found in enterococci and staphylococci due to the use of these antimicrobials in both human and veterinary medicine, respectively. Genomic sequencing and comparison of the resistome of resistant enterococci from food animals and the environment and a human-associated streptogramin and methicillin-resistant Staphylococcus aureus was done. Numerous streptogramin-resistance genes were identified in both bacterial genera along with resistance genes to several other classes of antibiotics used in treatment of human infections. Streptogramin resistance genes commonly found in staphylococci, but rare in enterococci, were identified suggesting acquisition of the genes from staphylococci to enterococci via a mobile genetic element. The long use of virginiamycin in food animals may also have contributed to streptogramin resistance in enterococci from those sources by providing selective pressure.

- **Pathogenic and AMR Escherichia coli in surface water.** Surface waters are important sources of water for drinking, industrial, agricultural, and recreational uses; hence, contamination of water by fecal bacteria, such as Escherichia coli, is a major environmental and public health concern. To address the limited data available on pathogenic or AMR E. coli in surface water, water samples were collected quarterly from 2015 to 2017 from the Upper Oconee River Watershed, Athens, Georgia. E. coli counts to assess the water quality were occasionally above the U.S. EPA threshold for recreational water. Pathogenic E. coli were detected in the water including those which cause diarrhea in humans. AMR and MDR in E. coli to antibiotics used to treat human infections was also detected. Results from this study demonstrated that E. coli is prevalent in high levels in the Upper Oconee Watershed, indicating possible widespread fecal contamination within the water. The study emphasized the role of environmental water as a reservoir of resistant and pathogenic E. coli that may be transferred to humans through drinking and recreational activities.

Outcomes and Impact

- Discovered the colistin gene (mcr-1) for the first time in the US. Genomic analysis determined that the U.S. isolates descended from China but there was no direct link, or known mechanism of transfer.
- Developed through a consortium with researchers from Canada a manually curated database containing high quality reference antimicrobial resistance genetic data including genes and proteins (Comprehensive Antibiotic Resistance Database (CARD; http://arpcard.mcmaster.ca). Its design allowed the development of novel genome analysis tools, such as the Resistance Gene Identifier (RGI) for prediction of resistance genes from whole genome sequences.
- Developed a high-throughput panel of biocides to determine the minimum inhibitory concentration for these compounds and to determine if resistance to biocides correlates with resistance to antibiotics. No correlation was detected between pathogen susceptibility to the biocides and their antibiotic resistance. This biocide assay is under development for use at U.S. Federal regulatory agencies.

- Determined that the resistance to streptogramin antimicrobials, quinupristin/ dalfopristin and virginiamycin, was via a mobile genetic element. Further, the use of virginiamycin in food animals may also have contributed to streptogramin resistance in enterococci through selective pressure.
- Emphasized the role of environmental water as a reservoir of resistant pathogens that may be transferred to humans through drinking and recreational activities.

Examples of Relevant Publications

- Meinersmann RJ, Ladely SR, Plumblee JR, Hall MC, Simpson SA, Ballard LL, Scheffler BE, Genzlinger LL, Cook KL. 2016. Colistin Resistance mcr-1-Gene-Bearing Escherichia coli Strain from the United States. Genome Announc. 4(5): e00898-16. https://doi: 10.1128/genomeA.00898-16.
- Jia, B., Raphenya, A., Alcock, B., Waglechner, N., Guo, P., Tsang, K., Lago, B., Dave, B., Pereira, S., Sharma, A., Doshi, S., Courtot, M., Lo, R., Williams, L., Frye, J.G. 2016. CARD 2017: expansion and model-centric curation of the Comprehensive Antibiotic Resistance Database. Nucleic Acids Research. https://doi:10.1093/nar/gkw1004.
- Meinersmann RJ, Ladely SR, Bono JL, Plumblee JR, Hall MC, Genzlinger LL, Cook KL. Complete Sequence of a Colistin Resistance Gene (mcr-1) Bearing Isolate of Escherichia coli from the United States. Genome Announc. 4(6):e01283-16.
- Meinersmann RJ, Ladely SR, Plumblee JR, Cook KL, Thacker E. 2017. Prevalence of mcr-1 in US Food-Animal Cecal Contents of Food Animals. Antimicrobial Agents Chemotherapy, 61(2):E02244-16
- Sharma P., Gupta, S.K., Barrett, J.B., Hiott, L.M., House, S.L., Woodley, T.A., Frye, J.G., Jackson, C.R. 2017. Draft genome sequences of eight streptogramin-resistant *Enterococcus* species isolated from animal and environmental sources in the US. Genome Announcements. 5(46): e01287-17.
- Humayoun, S.B., L.M. Hiott, SK. Gupta, J.B. Barrett, T.A. Woodley, J.J. Johnston, C.R. Jackson, and J.G. Frye. 2018. An assay for determining the susceptibility of Salmonella isolates to commercial and household biocides. PLoS ONE, Dec 20;13(12):e0209072. https://doi: 10.1371/journal.pone.0209072. eCollection.
- Cho, S., L.M. Hiott, J.B. Barrett, E.A. McMillan, S.L. House, S.B. Humayoun, E.S. Adams, C.R. Jackson, J.G. Frye. 2018. Prevalence and characterization of Escherichia coli isolated from the Upper Oconee Watershed in Northeast Georgia. PLoS One. 2018 May 8;13(5):e0197005. https://doi: 10.1371/journal.pone.0197005.
- Gupta, S., Sharma, P., Barrett, J.B., Hiott, L.M., Woodley, T.A., Frye, J.G., Jackson, C.R. 2019. Draft genome sequence of human-associated streptogramin resistant Staphylococcus aureus. Journal of Global Antimicrobial Resistance. https://doi.org/10.1016/j.jgar.2018.11.021.

The (Line) project objective was to: (1) Develop and evaluate potential alternatives to antimicrobials and other intervention products and strategies to control and reduce foodborne pathogens in poultry and swine.

Examples of Accomplishments

- **Using mass spectrometry-based proteomics in conjunction with chicken sera to map the epitope of the Salmonella enterica FlgK protein.** The Salmonella flagellum has a complex structure. The FlgK protein (also called hook associated protein is important because FlgK is required for proper formation of flagella and has been regarded as an important virulence determinant for colonization and invasion. Studies applied immunoprecipitation and mass spectrometry to identify the immunodominant epitopes in FlgK. These epitopes also recognized by sera collected from the field.

- **Using peptide microarrays to assess broiler humoral immune activity.** One of challenges is to control infectious diseases during poultry production. Humoral immune response to various microorganisms plays critical factors to protect hosts from infectious diseases. ARS in collaboration with Arizona State University, applied 330,000 random-sequenced peptide microarrays to assess broiler immune response to the peptides from week 1- to 6. Ten peptides and 6-peptides were identified to have strongest reaction and a decrease in reaction, respectively, as broilers grew up to week six. These preliminary results provide a rationale for further evaluating whether this array can be used for disease diagnostics and/or vaccination monitoring.

- **Preliminary test of Salmonella recombinant proteins in broilers.** Studies evaluated seven recombinant proteins together for immunization of 2-week-old broilers twice. The broilers were challenged with Salmonella orally 1-week after the second immunization. Cecal samples were collected. The results show that three out of four chickens harbored Salmonella in their ceca in the non-immunized group, while no Salmonella was detected in the birds immunized with 7-proteins, suggesting that these proteins may be able to induce broilers to generate antibodies to reduce the level of Salmonella colonization in cecae.

- **In vitro inhibition of Campylobacters by selected antimicrobial peptides.** Novel alternatives to traditional antibiotics are urgently needed for food-animal production. Antimicrobial peptides (AMP) have been found in most every class of living organism where they have evolved as a host defense mechanism against invading microorganisms. Natural and synthetic AMP exhibit great potential as new therapeutic agents because of their unique mechanisms of antimicrobial activity, coupled with the difficulty of bacteria to develop resistance to them. Studies selected a set of 38 AMP which were chemically synthesized and evaluated for the ability to inhibit growth of two strains of Campylobacter jejuni. Sixteen of the AMP tested produced zones of inhibition on lawns of C. jejuni in a traditional spot-on-lawn assay. These 16 AMP were subsequently tested for ability to produce zones of inhibition against 15 different Campylobacters including C. jejuni, C. coli and C. lari strains and minimum inhibitory

concentrations (MIC) were determined. The three most active AMP included RL-37, a 37-residue AMP of the cathelicidin family from Rhesus monkey bone marrow; the synthetic octyl-acyl-lysyl (OAK) denoted C12K-2β12; and the de novo AMP Syn-GNU7, shortened for enhanced protease resistance and fused with a lipopolysaccharide-targeting peptide. Further studies demonstrated that AMPs such as those identified in this work can significantly inhibit Campylobacter growth in vitro. Ongoing research is determining if AMP can be produced economically in yeast and evaluated for efficacy under simulated commercial conditions.

Outcomes and Impacts

- Provided a rationale for further evaluation for utilization as subunit vaccines for chicken immunization.
- Developing a peptide microarray may be a method to measure the humoral immune response that protects hosts from infectious diseases. Preliminary studies indicated that 6 randomly-synthesized generated peptides provide a positive rationale to further array development for disease diagnostics and/or vaccination monitoring.
- Studies evaluated seven recombinant proteins together for immunization of two-week-old broilers twice against Salmonella. No Salmonella was found in immunized birds suggesting that these proteins may be able to induce broilers to generate antibodies to reduce the level of Salmonella colonization in cecae.
- Developed AMPs, and demonstrated that 16 AMP's identified could significantly inhibit Campylobacter growth in vitro. Ongoing work is determining if AMP's may be produced economically in yeast and then evaluated under simulated commercial conditions.

Examples of Relevant Publications:

- Yeh, H., Serrano, K.V., Acosta, A.S., Buhr, R.J. 2016. Production of recombinant *Salmonella* flagellar protein, FlgK, and its uses in detection of anti-*Salmonella* antibodies in chickens by automated capillary immunoassay. Journal of Microbiological Methods 122: 27-32.
- Yeh, H., Telli, A.E., Jagne, J.F., Benson, II, C.L., Hiett, K.L., Line, J.E. 2016. Epitope mapping of *Campylobacter jejuni* flagellar capping protein (FliD) by chicken (*Gallus gallus domesticus*) sera. Comparative Immunology, Microbiology and Infectious Diseases 49: 76-81.
- Yeh, H., Line, J.E., Hinton, A. 2018. Molecular analysis, biochemical characterization, antimicrobial activity, and immunological analysis of *Proteus mirabilis* isolated from broilers. Journal of Food Science. 83: 770-779.
- Yeh, H., Kojima, K., Mobley, J.A. 2018. Epitope mapping of *Salmonella* flagellar hook-associated protein, FlgK, with mass spectrometry-based immuno-capture proteomics using chicken (*Gallus gallus domescticus*) sera. Veterinary Immunology Immunopathology. 201: 20-25.

The (Cook/Oladeinde) project objectives were to: (1) Characterize the AMR of pathogens and commensals from agroecosystems and identify factors that contribute to their spread; and (2) Optimize methods for detecting bacteria with AMR and develop tools to assess biological and environmental factors that contribute to their persistence and transmission in agroecosystems.

Examples of Accomplishments

- **Persistence of antibiotic resistant Salmonella Heidelberg in poultry litter** The bedding material/litter used for growing broilers has a significant impact on the developmental process of the chicken gut and its eventual microbiome. Preharvest interventions that can reduce the population of MDR Salmonella and commensals in litter will have a better potential to limit their transfer to the chicken gut. Reused broiler litter was inoculated with two strains of Salmonella Heidelberg and monitored for 14 days. Strains surviving in broiler litter were collected and characterized by sequencing their genomes. In addition, the bacteria population present in the broiler litter used were also identified.

- **Reused litter decreases the transfer frequency of MDR plasmids from the broiler gut microbiota to Salmonella Heidelberg** Horizontal gene transfer plays an important role in the spread of AMR and virulence genes to food-borne bacterial pathogens. The notion that ARG's are horizontally transferred from commensal bacteria residing in the animal gut or environment to food-borne pathogens is becoming more recognized. ARS completed an in vivo broiler study evaluating the interactions between broiler gut microbiome, litter age and AMR acquisition in Salmonella Heidelberg. Studies showed that litter reuse resulted in a lower transfer of MDR plasmids (transfer frequency = < 1 %) from the broiler gut microbiome to inoculated S. Heidelberg strain than broilers raised on fresh litter (transfer frequency = ~ 15 %) after 2 weeks of grow-out. Further, Bifidobacterium spp. was the major bacterial genera significantly higher in reused litter versus fresh litter.

- **Detection of pathogens and commensals present in broiler litter.** ARS developed a method to quantify and detect Salmonella enterica (serovars Heidelberg, Typhimurium, Enteritidis and Kentucky), Campylobacter (jejuni and coli), E. coli and Enterococcus (faecalis, faecium, hirae and Santins) from broiler litter. Based on this method, the limit of quantification for viable and culturable Salmonella, Campylobacter, E. coli and Enterococcus in 1 g (~ 90 % dry weight) of broiler litter was determined to be ~ 30, 70, 20 and 140 colony forming units (CFU), respectively. Their corresponding limit of detection was 5, 130, 6 and 2 CFU/g dry weight. The result shows that this method is useful for the quantification and detection of low levels of pathogens and commensals present in broiler litter.

- **Dynamics between horizontal gene transfer and acquired antibiotic resistance in Salmonella Heidelberg following in vitro incubation in chicken ceca.** Acquisition of new genetic material from microbial flora in the gastrointestinal tract of food animals, including chickens, may contribute to increased fitness of pathogens like Salmonella Heidelberg and may increase their level of antibiotic tolerance. Therefore, it is critical to gain a better understanding on the dynamic interactions that occur between important pathogens and the commensals present in the animal gut and other agroecosystems. Broiler ceca collected from a commercial processing plant was used as a model to investigate antibiotic resistance genes that can be transferred in vitro from ceca flora to S. Heidelberg.

Outcomes and Impacts

- Provided new information on the fitness and drug resistance associated with the acquisition of Col-like plasmids by S. Heidelberg strains. Revealed that the litter microbiome present in reused broiler litter may competitively exclude some S. Heidelberg strains.
- Determined that consecutive reuse of litter promotes an "unfavorable" microbiome for E. coli and Salmonella carrying multidrug resistant plasmids, therefore limiting their potential for transfer. Further studies are underway that will characterize and test these Bifidobacterium spp. in vitro and in vivo.
- Developed a method to quantify and detect S. enterica, Campylobacter, E. coli, and Enterococcus from broiler litter, with limits of quantification of < 140 CFU, and a limit of detection of <130 CFU.
- Determined that the native flora in the chicken ceca could transfer a plasmid producing beta-lactamase. In vitro, this plasmid was transferrable between E. coli and S. Heidelberg strains, but transfer was unsuccessful between S. Heidelberg strains. This confirms that antibiotic resistance can be transferred between some food-borne pathogen and other bacteria within the chicken gut microbiome.

Examples of Relevant Publications

- Oladeinde A, Cook K, Orlek A, Zock G, Herrington K, Cox N, Lawrence JP, Hall C. Hotspot mutations and ColE1 plasmids contribute to the fitness of Salmonella Heidelberg in poultry litter. PloS one. 2018 Aug 31;13(8):e0202286. https://doi.org/10.1371/journal.pone.0202286
- Oladeinde, A., Cook, K., Lakin, S.M., Abdo, Z., Looft, T., Herrington, K., Zock, G., Lawrence, J.P., Thomas, J.C., Beaudry, M.S. and Glenn, T., 2019. Horizontal gene transfer and acquired antibiotic resistance in S. Heidelberg following in vitro incubation in broiler ceca. *Applied and Environmental Microbiology*. (in press) bioRxiv https://doi.org/10.1101/684787

Studies at BARC were conducted by the (Van Kessel) project to address the following objectives to: (1) Examine the relationship between gut bacteria and the bovine host to determine factors that contribute to observed age-related differences in colonization by AMR bacteria; (2) Examine and determine if resistance determinants in bacteria are linked to specific genomic characteristics that influence bacterial colonization capacity in the young dairy calf; and (3) Compare and contrast interactions between bovine host cells and Salmonella enterica to identify factors that contribute to differences between Salmonella serotypes that behave as commensal inhabitants of the dairy cow gut and serotypes that are transient in the cow or cause systemic infections.

Examples of Accomplishments

- **Prevalence of bacterial pathogens in U.S. bulk tank milk.** Dairy farms are known reservoirs of bacterial pathogens and numerous foodborne illness outbreaks have been associated with consumption of unpasteurized milk and products made from unpasteurized milk. As part of the APHIS-National Animal Health Monitoring System (NAHMS) Dairy 2014 study, bulk tank milk and milk filters were collected from 234 dairy operations in 17 major dairy states and analyzed for the presence of Salmonella enterica, Campylobacter spp., Shiga-toxigenic Escherichia coli, and Listeria monocytogenes. Campylobacter spp. was detected in samples from 25% of operations; most of the isolates were C. jejuni and 13% were resistant to ciproflozacin and naldixic acid. Salmonella enterica was isolated from 18% of operations and the most common serotypes were Cerro, Montevideo, and Newport. Most of the isolates were pan-susceptible but resistance to antibiotics was detected in a few strains.

- **Antimicrobial resistance of Salmonella enterica from dairy animals.** Dairy animals and their environments are reservoirs for Salmonella and, while Salmonella can cause severe disease in the cows and calves, many Salmonella serotypes have been commonly isolated from animals that exhibit no signs of disease. However, asymptomatic carriage remains concerning due to the potential for contamination of food products or the environment. AMR in zoonotic bacteria exacerbates the potential risk to human health. In several studies of dairy animals, it was determined that, even when significant MDR was identified in the fecal E. coli population, salmonellae isolated from the same population were pan-susceptible. Resistance is often observed in serotypes that are known to cause disease (i.e. Newport, Dublin, Typhimurium) in cattle. In a study of 488 milk and milk filters collect from 234 commercial dairy operations in 17 states, Salmonella Newport, Muenchen, Dublin, and Give were sporadically detected and were all MDR. However, the asymptomatic, persistent, or transient serotypes such as Cerro, Kentucky, Montevideo are almost entirely susceptible to all antimicrobials tested. Salmonella was isolated from 67% of farms in an 80-farm study in Pennsylvania. Cerro, Montevideo, and Kentucky were the most frequently identified serovars, all of which were pan-susceptible.

- **Diversity of Salmonella Kentucky from poultry and dairy**. Salmonella Kentucky is the most frequently isolated serovar from non-human, non-clinical cases in the U.S. and is frequently isolated from dairy and poultry and human clinical cases are reported each year. This serovar generally does not cause clinical disease in cattle, but both transient and persistent, long-term carriage of S. Kentucky has been identified in dairy cattle. In a genomic comparison of 119 isolates, bovine and poultry S. Kentucky isolates were phylogenetically distinct and harbored different suites of plasmids. In the U.S. and globally, most human S. Kentucky infections are associated with sequence type (ST) 198 but most of the isolates from cattle were ST152. In a collaborative study with the Maryland Department of Health, ARS determined that most human S. Kentucky infections in the state of Maryland were ST198 and that most cases were associated with international travel outside the U.S. Pinpointing the genomic differences between persistent and transient S. Kentucky is a critical step towards mitigating long-term, Salmonella infections in cattle.

- **Distribution and diversity of AMR Escherichia coli in dairy herds.** Agricultural operations have often been implicated as a source of antibiotic-resistant bacteria, but AMR in bacteria from dairy animals has not been well characterized. Along with collaborators from Pennsylvania State University, ARS isolated fecal E. coli from four animal groups (pre-weaned calves, post-weaned calves, dry cows, and lactating cows) on each of 80 commercial dairy herds and tested the isolates for resistance to 15 antimicrobials. MDR E. coli (resistant to >3 antimicrobial classes) were isolated from 83% of farms and were more likely to be isolated from pre-weaned calves than the other three groups. Third generation cephalosporin (3GC)-resistant strains were isolated from both calves and cows on the same farms although more frequently from calves. Significant genomic diversity was observed in the population of 3GC-resistant E. coli, but clonal strains were identified in animals of different age groups on the same farms.

- **Diversity and virulence of Listeria monocytogenes from bulk tank milk on U.S. dairy farms.** Dairy farm environments are known reservoirs of L. monocytogenes and unpasteurized milk and milk products have been implicated in listeriosis outbreaks and sporadic cases of listeriosis. A genomic analysis was conducted on 121 L. monocytogenes isolates that were recovered from bulk tank milk, milk filters, and milking equipment from dairy farms in 19 states between 2002 and 2014. Based on a whole-genome phylogenetic analysis, multi-virulence locus sequence typing (MVLST), and multilocus sequence typing (MLST) analysis, significant diversity was observed in the dairy-associated L. monocytogenes; 59 Virulence Types (VTs) and 56 Clonal Complexes (CCs) were identified. Listeria pathogenicity islands 1, 3, and 4, known to be involved in severe and sometimes fatal infections, and stress survival island 1 were distributed differently between L. monocytogenes lineages. This study established that L. monocytogenes isolated from dairy farms across the U.S. are highly heterogeneous. Results also indicated that a significant portion (25%) of the isolates are the same CCs and virulence types (VTs) as those that are frequently isolated from human clinical cases and outbreaks on a global scale, and that many of those strains encode virulence factors implicated in severe disease, fatal infections, and miscarriages.

- **AMR in fecal E. coli from veal calves.** Many of the male calves from commercial dairy farms are sold from the herds and raised for the veal market. The diet of these calves is different from those being raised as herd replacements and generally consists of milk, or a milk replacer, and a grain mix throughout the growing period. Along with collaborators from Pennsylvania State University, ARS characterized the resistance of E. coli isolated from the feces of calves on 12 veal farms shortly after the calves arrived at the farm and just prior to marketing. MDR E. coli (resistant to >3 antimicrobial classes) were isolated from 91 and 100% of the fecal samples collected from the younger calves and the calves prior to market, respectively. E. coli isolates from the calves just prior to marketing were resistant to more antimicrobial classes than isolates from the younger calves. Extended-spectrum cephalosporin resistance is an increasing human and animal health concern and isolates harboring genes conferring cephalosporin resistance (bla_{CTX-M} β-lactamases) were obtained from 15% of the fecal samples and at least one isolate was recovered from each farm. A metagenomic analysis of a subset of the samples showed a clear difference in both the community structure and the resistome in the feces of the young calves and older calves. The resistance patterns and the presence of bla_{CTX-M}-E. coli on most of the farms suggest that these veal production systems are a reservoir for resistant genetic materials that may pose a risk to human health.

Outcomes and Impacts

- Collected as part of the APHIS-NAHMS Dairy 2014 study, bulk tank milk and milk filters from dairy operations in 17 major dairy states and analyzed for the presence of food safety pathogens. The study confirmed the appreciable prevalence of bacterial pathogens and reinforces the potential public health risk in consuming non-pasteurized milk or dairy products.
- Determined from dairy animal studies that antimicrobial resistance among the Salmonella populations in dairy cows is vertically transmitted through Salmonella cell replication and rarely transmitted from resistant E. coli to Salmonella.
- Developed and validated a genomic system to determine where S. Kentucky isolates from food or human infections originated. Utilized the system to determine that most human S. Kentucky infections were mostly associated with international travel.
- Determined that pinpointing the genomic differences between persistent and transient S. Kentucky is a critical step towards mitigating long-term, Salmonella infections in cattle.
- Conducted an 80 herd dairy cattle fecal E. coli survey to determine the type and range of antimicrobial resistance. Significant genomic diversity was observed in the population of 3GC-resistant E. coli, but clonal strains were identified in animals of different age groups on the same farms suggest that mitigation efforts directed at pre-weaned calves have the highest potential for control of resistance in dairy herds.
- Conducted a genomic analysis on L. monocytogenes isolates recovered from bulk tank milk, milk filters, and milking equipment from dairy farms in 19 states. Established that isolates are highly heterogeneous; and that a significant portion are the same complex and virulence types as those frequently isolated from human clinical cases and outbreaks.

- Characterized the resistance patterns of E. coli isolated from the feces of calves on veal farms after arrival and prior to marketing showing resistant to >3 antimicrobial classes from 91% and 100% respectively. Conducted a metagenomic analysis of a subset of the samples showing a clear difference in both the community structure and the resistome. Resistance patterns suggest that veal production systems are a reservoir for resistant genetic material.

Examples of Relevant Publications

- Haley, B., M. Allard, E. Brown, E. Hovingh, J. S. Karns, and J. S. Van Kessel. 2015. Molecular detection of the index case of a subclinical *Salmonella* Kentucky epidemic on a dairy farm. Epidemiol. and Infect. 143:682-686.
- Haley, B. J., S. W. Kim, J. Pettengill, J. S. Karns, and J. S. Van Kessel. 2016. Genomic and evolutionary analysis of two *Salmonella enterica* serovar Kentucky sequence types isolated from bovine and poultry sources in North America. PLoS ONE 11 (10): e0161225.
- Kim, S. *W.*, B. J. Haley, D. Roberson, M. Allard, T. S. Hammack, E. W. Brown, and J. S. Van Kessel. 2017. Genome sequences of four nonhuman/nonclinical *Salmonella enterica* serovar Kentucky ST198 isolates recovered between 1972 and 1973. Genome Announc. 5: e01699-16.
- Del Collo, L. P., J. S. Karns, D. Biswas, J. E. Lombard, B. J. Haley, R. C. Kristensen, C. A. Kopral, C. P. Fossler, and J. S. Van Kessel. 2017. Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter* spp. in bulk tank milk and milk filters from US dairies. J. Dairy Sci. 100:3470-3479.
- Kim, S. W., J. Haendiges, E. N. Keller, R. Myers, A. Kim, J. E. Lombard, J. S. Karns, J. S. Van Kessel, and B. J. Haley. 2018. Genetic diversity and virulence profiles of *Listeria monocytogenes* recovered from bulk tank milk, milk filters, and milking equipment from dairies in the United States (2002 to 2014). PLoS ONE. 13(5): e0197053.
- Sonnier, J. L., J. S. Karns, J. E. Lombard, C. A. Kopral, B. J. Haley, S. W. Kim, and J. S. Van Kessel. 2018. Prevalence of *Salmonella enterica*, *Listeria monocytogenes*, and pathogenic *Escherichia coli* in bulk tank milk and milk filters from US dairy operations in the National Animal Health Monitoring System Dairy 2014 study. J. Dairy Sci. 101:1-14.
- Cao, H., A. Pradhan, J. S. Karns, D. Wolfgang, B. Vinyard, S. W. Kim, S. Salaheen, B. J. Haley, E. Hovingh, and J. S. Van Kessel. 2019. Age-associated distribution of antimicrobial resistant *Salmonella enterica* and *Escherichia coli* isolated from dairy herds in Pennsylvania, 2013-2015. Foodborne Pathog. Dis. 16:60-67.
- Haley, B. J., *S. W. Kim,* J. Haendiges, E. Keller, D. Torpey, A. Kim, K. Crocker, R. A. Myers, and J. S. Van Kessel. 2019. *Salmonella enterica* serovar Kentucky recovered from human clinical cases in Maryland, USA (2011 to 2015). Zoonoses Public Health. 66:382-392.
- Salaheen, S., *H. Cao,* J. L. Sonnier, S. W. Kim, L. P. Del Collo, E. Hovingh, J. S. Karns, B. J. Haley, and J. S. Van Kessel. 2019. Diversity of extended-spectrum cephalosporin-resistant *Escherichia coli* in feces from calves and cows on Pennsylvania dairy farms. Foodborne Pathog. Dis. 16:368-370.

- Salaheen, S., S. W. Kim, *H. Cao*, D. R. Wolfgang, J. S. Karns, B. J. Haley, E. Hovingh, and J. S. Van Kessel. 2019. Antimicrobial resistance among *Escherichia coli* isolated from veal calf operations in Pennsylvania. Foodborne Pathog. Dis. 16:74-80.

A project at ERRC, Wyndmoor, PA (Paoli) assists in collaborative research which has direct implication for the National Antimicrobial Resistance Monitoring System (NARMS). The objective is a collaboration with projects at the U.S. NPRC Athens, to; (1) Identify several large conjugative plasmids from MDR Salmonella enterica and E. coli isolates collected from the NARMS and other programs.

Examples of Accomplishments

- **Small plasmids contribute to antibiotic resistance in Salmonella.** Antibiotic resistance in bacterial pathogens is a major concern in both food production and medicine. While the role of large mobile DNA molecules (plasmids) in the expression and spread multi-drug resistance in pathogens has been conclusively demonstrated, small plasmids carrying antibiotic resistance genes are often over-looked. Studies had previously developed a method to screen for the presence of small plasmids in MDR isolates of the pathogenic bacteria Salmonella. These isolates, collected by the 2005 National Antibiotic Resistance Monitoring System (NARMS), were further characterized by using molecular techniques and DNA sequence analysis. A follow-up study was conducted using NARMS isolates collected during the 2010-2011 period, resulting in the isolation and characterization of additional novel small plasmids. The results indicate that these small plasmids are widespread in pathogens Salmonella (and pathogenic E. coli) isolated from animals raised for food, that their overall population is changing over time, and underscores the important roles they may play in the harboring and transmission of antibiotic resistance genes between pathogenic bacteria.

Outcomes and Impacts

- Examined and characterized small plasmids collected by NARMS. These plasmids are widespread in Salmonella and E. coli isolated from animals and underscores the important roles they may play in the harboring and transmission of antibiotic resistance genes between pathogenic bacteria.

Example of Relevant Publication

- Chen, C., Strobaugh Jr, T.P., Nguyen, L.T., Abley, M.J., Lindsey, R.L., Jackson, C.R. 2018. Isolation and characterization of two novel groups of Kanamycin-resistance ColE1-like plasmids in *Salmonella enterica* serotypes from food animals. PLoS One. https://doi.org/10.1371/journal.pone.0193435.

Additional Research

A special project at WRRC, Albany, CA was conducted at the request of FSIS through the (Cheng) project by (He).

- **Development of novel antibodies and detection assay to screen food samples for colistin-resistant bacteria.** The recent discovery and rapid spread of mobile colistin-resistant gene, mcr-1, in bacteria is undermining our ability to treat bacterial infections and threatening human health and safety. ARS developed novel polyclonal and monoclonal antibodies against MCR-1 and MCR-2. An enzyme-linked immunosorbent assay (ELISA) established using these antibodies was able to detect 0.01 nanogram per milliliter of MCR-1 in buffer and 0.4 colony forming units per gram of meat, including ground chicken, pork and beef, demonstrating strong tolerance to complex food matrices. This new ELISA along with a real-time PCR method were used to test samples from the environment and commercial foods for the presence of colistin resistance in a collaborative study with FSIS.

- He, X., Mavrici, D., Patfield, S.A., Rubio, F. 2018. Development of novel antibodies for detection of mobile colistin-resistant bacteria contaminated in meats. Scientific Reports. 8:16744.

Examples of Externally Funded Projects

Multi-Institutional Collaborative Study

Project Title:	Characterization and mitigation of bacterial pathogens in the fresh produce production and processing continuum
Support:	USDA ARS Environmental Microbial and Food Safety Laboratory (8042-32420-006-00D)
Project Title:	Persistence of enteric pathogens in manure-amended soils in Northeast U.S. produce-growing environments
Support:	Food and Drug Administration (IAA 224-11-2046)
Project Title:	Survival of fecal organisms and indicators in agricultural soils amended with raw manure
Support:	University of Vermont (SCA 58-1245-4-110)

The FDA-Food Safety and Modernization Act (FSMA), passed in 2011, stipulates that there should be an interval between the application of animal manure, used as fertilizer, and the harvest of fruits and vegetables, to limit contamination of these crops and prevent human illness. The FDA "Produce Safety Rule" in FSMA states that it currently has objection to the interval between manure application and harvest as the USDA National Organic Program (NOP), which is 90 days for edible portions of the plant that do not touch the ground (soil), or 120 days for crops which have an edible portion that touches the soil.

Animal manure can contain bacterial pathogens that can cause human illness, but in most cases, the levels of these pathogens in manure declines over time. The appropriate length of this interval is highly dependent on factors like climate, soil type, manure type and application method, and the type of crops grown in a specific region. The collaborative study investigated all the factors noted above to determine how long pathogens can survive in manure-amended soils. The project conducted (2014-2017) determined what the appropriate duration was between the application of dairy cattle manure and the harvest of leafy green or radish crops that are commonly grown in Vermont.

In summary, E. coli survival in dairy-manure amended soils in Vermont was shorter than in the Mid-Atlantic regions, most likely due to lower soil temperature and water potential characteristics. Poultry litter-based composts supported longer durations of E. coli survival than vermi-composts or dairy-manure based composts in amended soils. The data collected here will not only aid FDA but also Vermont growers and farmers who commonly utilize dairy manure as fertilizer.

The following paper was one outcome of this study:

Neher, D., Cutler, A., Weicht, T., Sharma, M., Millner P.D. 2019. Composts of poultry litter or dairy manure differentially affect survival of enteric bacteria in fields with spinach. *J Appl. Microbiol.* 126:1910-1922. https://doi.org/10.1111/jam.14268

The following poster presented at the 2017 IAFP meeting was another outcome from the study.

Effect of Soil Management on the Persistence of *E. coli* and *Listeria sp.* in Manure-Amended Soils in the Northeast United States

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Introduction

Biological soil amendments of animal origin (BSAAOs), including manure and compost, play an important role in conventional and organic agriculture. The use of these amendments can provide nutrients to soils, improving soil fertility and crop production. However, foodborne illness outbreaks associated with fresh fruits and vegetables focused attention on agricultural inputs used to grow fresh produce commodities. In response to these outbreaks, the U.S. Food and Drug Administration (FDA) issued "Standards for the Growing, Harvesting, Packing and Holding of Produce for Human Consumption". These standards, currently known as the Produce Safety Rule (PSR), have specific criteria for use of BSAAOs of animals intended for growing fresh produce for human consumption, including that raw manure must not come into contact with produce. Currently, FDA does not take exception to the USDA National Organic Program (NOP) 90- or 120-day interval between application of manure to the field and the harvest of crops while further data is collected to complete a risk assessment. While the USDA NOP does stipulate the 90/120-day interval for production of organic fruits and vegetables , this standard was not supported with data on the survival of enteric pathogens in manure-amended soils. Data are needed specifically for the Northeast region of the U.S.

Objective

Compare population dynamics of non-pathogenic *E. coli* (*gEc*) and *Listeria spp.* in surface and tilled plots in two soil types amended with untreated dairy manure in the Northeastern U.S (Vermont).

Methods

Field plots (2m x 1m) of soils (Table 1) located in Burlington VT were either unamended or amended with dairy manure solids (DMS) at a rate equivalent to 11.2mt/ha (5 T/ ac).

Experimental design: A randomized complete block was used with 4 replicates/treatment. Treatments included plots that were tilled after manure application, or manure left on the surface: Tilled or surface field plots were either 1) amended with dairy manure solids (DMS) and inoculated with *rE. coli*; 2) unamended and inoculated with *rE. coli*; 3) amended with DMS and not inoculated with *rE. coli*; 4) unamended and uninoculated.

Manure application: DMS, 2.3 kg (from University of Vermont dairy herd) were manually distributed evenly onto the surface of each designated plot ($2m^2$). Prior to application, a sample of DMS was tested for presence of *Listeria* spp. and for indigenous non-rifampicin-resistant generic *E. coli* (*gEc*). After surface inoculation, plots were mechanically tilled to incorporate the manure to a depth of 15 cm. The equipment was surface sterilized with 10% bleach solution after each treatment to prevent cross contamination.

Inocula contained equal populations of three rifampicin (r)-resistant strains of non-pathogenic *E. coli* (*rEc*) (TVS 353, 354, 355, provided by Trevor Suslow, UC-Davis). Strains were cultured separately in sterile dairy manure extract and combined at equal cell densities (3.3 x 10^5 CFU/ml). One liter of inoculum (10^8 CFU/ml) was sprayed onto the surface of designated field plots using a backpack sprayer after application of dairy manure (see below).

Sampling: Surface (1-2cm) and core samples (10cm) were transferred to sterile Whirl-Pak® bags periodically from days 0 to 165 after manure and inoculum application; collection loci were marked to prevent re-sampling. Samples were transported to the lab in insulated coolers.

Sample preparation: Samples were hand-massaged for 30 sec; 30g from each sample was weighed into a Whirl-Pak® filter bag then manually homogenized in 120mL buffered peptone water (1:5 dilution).

Microbial analysis : For analysis of *rE. coli* from inoculated plots, volumes or serial dilutions of homogenized samples were plated onto Tryptone Bile X-glucuronide (TBX) agar with 80 mg/ml rifampicin (TBXr) to quantify *rE. coli*; plates were incubated at 42ºC overnight before counting. For analysis of indigenous *E. coli* (*gEc*)present in soils or dairy manure, homogenized samples from uninoculated plots were plated on TBX.

MPN assays for *E. coli*: When *rEc* populations were below the detection limit for direct plate counts, a mini-MPN assay was used. Homogenates (1:5 primary dilutions) were diluted 1:2 in 2X Tryptic Soy Broth supplemented with rifampicin (TSBr), and subsequently serially diluted in 1X TSBr in sterile, 48-well polypropylene blocks using 8 replicate wells/dilution. Blocks were sealed with Breathe-Easy plate film and incubated overnight at 42ºC. Dilutions of homogenates were then isolated on TBXr and incubated at 42ºC to determine the presence of *rEc*. For uninoculated homogenates , MPN dilutions were made in EC broth and *gEc* isolated on TBX without rifampicin.

Bag enrichment: If no *rEc* were recovered from homogenates using the MPN procedure, the entire sample (30 g in 120 ml BPW) was incubated (42ºC, 24 h) before isolation of homogenates on TBXr or TBX.

MPN assay for *Listeria sp.* : *Listeria sp.* were determined using an MPN method as described in Hitchins and Jinneman (2013), *Bacteriological Analytical Manual (BAM) Chapter 10*. Homogenates were diluted in 48-well polypropylene blocks with 4 replicate wells/dilution. Homogenates (1 ml) were initially added to 1.0 mL of 1x Buffered Listeria Enrichment broth (BLEB) containing 2mL/L acriflavin, 8 mL/L nalidixic acid, and 5 mL/L cyclohexamide. Subsequent serial dilutions were made in BLEB with antibiotics. Blocks were sealed with Breathe-Easy plate film and incubated for 48 h at 30ºC. All enriched wells with turbidity after 48h were streaked onto CHROMagar™ *Listeria* chromogenic media to confirm their presence.

Dry weight determination. Moisture content of 10g sub-samples was obtained after oven drying (90°C) to constant weight.

Data Management: Microbial populations of *E. coli* and *Listeria* spp. are expressed as mean CFU or MPN/gdw (gram dry weight of soil).

Results

Table 1. Soil characteristics of two experimental sites (A and B) used.

Site	Soil type	Slope
A	Hinesburg B Sandy Loam (Sand: 60%, Silt: 10%, Clay: 30%)	3-8%
B	Adams B Loamy Sand (Sand: 40%, Silt: 40%, Clay: 20%)	5-12%

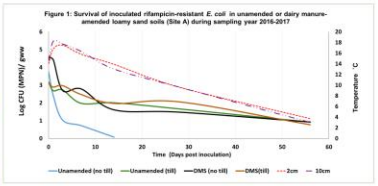


Figure 1: Survival of *rE. coli* at Farm site A, measured as log CFU(MPN)/gww. Temperature was measured for the duration of the study at 2cm and 10 cm depths. The average temperature is plotted on the secondary Y axis for each of the sampling days.

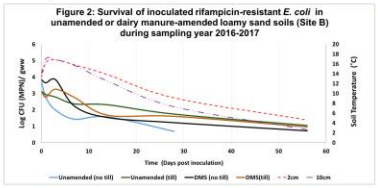
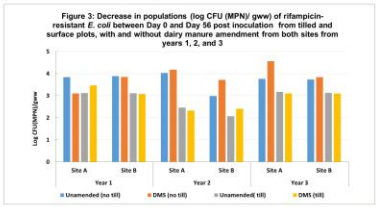
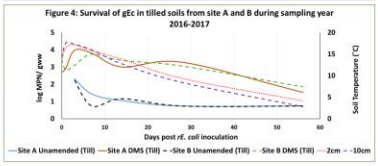
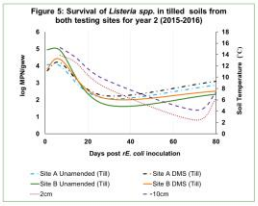
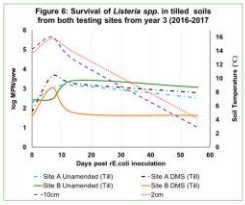


Figure 2: Survival of *rE. coli* at Farm site B, measured as log CFU(MPN)/gww. Temperature was measured for the duration of the study at 2 cm and 10 cm depths. The average temperature is plotted on the secondary Y axis for each of the sampling days.









- Populations of *rEc* declined by 2.1 – 2.3 log CFU(MPN)/gdw in tilled soils, while those in no till soils declined by 3.1-3.7 log CFU(MPN)/gdw over 60 days, regardless of DMS amendment or site (A or B). (Figures 1&2).
- No till soils supported larger *rE. coli* population declines (Site A: 3.7 log CFU/gdw, Site B: 3.1 log CFU/gdw) than tilled soils (Site A: 2.3 log CFU/gdw, Site B: 2.1 log CFU/gdw, regardless of DM amendment over 60 days (Figure 1&2).
- No detectable *rE. coli* were recovered from unamended, no-till sites after day 14 and 28 for site A and B respectively.
- *gEc* populations increased within the first 7 days in DMS plots compared to tilled unamended plots. Populations of *gEc* in unamended plots decreased by 0.8-1.4 log MPN /gww during the first 7 days of the study (Figure 4).
- *Listeria* spp. were present in both unamended tilled and DMS-amended soils in both years 2 and 3 of this study (Figure 5&6).
- Unlike *E. coli*, *Listeria* spp. prevalence was not as affected by fluctuations in soil temperature changes or the presence of dairy manure amendment (Figure 5&6).
- During the second year of the study *Listeria* spp. in both sites A and B, had larger declines in unamended tilled soils (1.2 – 2.5 log MPN/gdw) compared to in DMS-amended tilled soils (0.6 – 1.27 log MPN/gdw) over 80 days (Figure 5).
- During the third year of this study *Listeria sp.* levels increased initially (Day 7,: 0.8-1.4 log MPN/gww) with the exception of the DMS tilled plots (Site B) (Figure 6).
- Regardless of the initial populations of *Listeria* spp. on day 0 reached between 2.5-3.0 log MPN/gww in unamended plots and 2.7-3.0 log MPN/gww for DMS amended plots.
- During the duration of the study, *rEc* survived at higher populations for longer durations in tilled plots regardless of amendment with dairy manure (Figure 3).

Conclusions

- Tilling unamended soils or soils amended with DMS may slow the decline of *E. coli* populations in soils tested compared with soils that are untilled and not amended with DMS.
- *E. coli* populations decline at similar rates in tilled unamended soils as in tilled soils amended with DMS, indicating tilling may influence population dynamics of *E. coli* in soils as much manure amendment.
- Dairy manure amendments temporarily increased the populations of *E. coli* in soil; however populations decrease to levels identified at the start of the study.
- Tilling and amendment with dairy manure solids may affect the population dynamics of *E. coli* and *Listeria* spp. in soils differently.
- Data from this study suggests that dairy manure amendments and tilling can temporarily influence E. coli and Listeria spp. populations in soils.
- Data for this study indicates that *E. coli* levels tend to decrease while *Listeria* spp. populations increased as temperature decreases.

Acknowledgments

This study was funded through a Specific Cooperative Agreement (58-1245-4-110) between USDA–ARS and the University of Vermont, and through an Interagency Agreement (IAA 224-11-2046) between the U.S. Food And Drug Administration, Center for Food Safety and Applied Nutrition, Division of Produce Safety, Fresh Produce Branch, and USDA–ARS Environmental Microbial and Food Safety Laboratory

Multi-Institutional Collaborative Study

Project Title:	Characterization and mitigation of bacterial pathogens in the fresh produce production and processing continuum
Support:	USDA ARS Environmental Microbial and Food Safety Laboratory (8042-32420-006-00D)
Project Title:	Development and analysis of a protocol to assess survival of fecal organisms in agricultural soils amended with raw manure"
Support:	Food and Drug Administration (IAG 224011-2046)
Project Title:	Survival of fecal organisms and indicators in agricultural soils amended with raw manure
Support:	University of Maryland Eastern (SCA 58-1265-1-087)

The Produce Safety Rule within the FDA-Food Safety Modernization Act states that there are no objections to a 90- or 120-day interval between application of untreated biological soil amendments of animal origin and harvesting of crops. The aim of the no-objection is to minimize the transfer of pathogens to produce intended for human consumption, with the goal to limit potential cases of foodborne illness. However, this time interval has not been scientifically validated.

For amended soils in the Mid-Atlantic region, results from this multi-institutional collaboration over several seasons and twelve separate field trials determined the survival of Escherichia coli in soils amended with manure over time. Results indicated that spatiotemporal factors (site, year, and season) affect survival durations of E. coli in manure-amended soils more than agricultural factors (manure type, organic or conventional management of soils, and depth of application) or weather effects.

In summary, the study determined that poultry litter supported extended survival of E. coli in comparison to horse or dairy manure; however, spatiotemporal factors may have a greater influence than manure type in supporting survival beyond 90 days.

This work provided critical insight to vegetable and fruit growers to understand which types of raw animal manure present the most risk for produce contamination and provided primary data to the FDA for development of FSMA standards and rules for biological soil amendments.

The following extensive publication was a major accomplishment from the study.

- Sharma M, Millner PD, Hashem F, Vinyard BT, East CL, Handy ET, White K, Stonebraker R, Cotton CP. 2019. Survival of *Escherichia coli* in manure-amended soils is affected by spatiotemporal, agricultural, and weather factors in the Mid-Atlantic United States. Appl Environ Microbiol 85:e02392-18. https://doi.org/10.1128/AEM.02392-18.

Project title: Enhancing the Safety and Quality of Fresh Produce and Low-Moisture Foods by Waterless Nonthermal Technologies.
Support: USDA, National Institute of Food and Agriculture

The goal of this study is to develop an integrated project among academia, government, industry partners, and stakeholders to identify and develop effective waterless non-thermal processing technologies to provide consumers with safe, nutritious, high-quality produce and low moisture food (LMF), and facilitate the commercialization and dissemination of technologies and knowledge through education and outreach.

Gaseous ozone and chlorine dioxide have been studied for its efficacy in inactivating various microorganisms. However, there have been few studies dealing with quality, particularly nutrients, of fresh produce treated with gaseous ozone or chlorine dioxide to enhance microbial safety.

Results demonstrated that relatively low concentrations of gaseous chlorine dioxide and high concentrations of gaseous ozone significantly reduced Salmonella population on tomatoes. During storage, appearance, texture, color, odor, and lycopene and ascorbic acid contents of tomatoes were maintained after chlorine dioxide treatment while nutrients and deterioration in other quality attributes due to ozone treatment were observed. Therefore, gaseous chlorine dioxide can be used to enhance microbial safety of grape tomatoes without compromise in sensory and nutrient quality of the fruit.

Publications:
Wang, L., Sokorai, K., Wu, V.C.H., Fan, X. 2019. Gaseous chlorine dioxide maintained the sensory and nutritional quality of grape tomatoes and reduced populations of Salmonella enterica serovar Typhimurium. Food Control. 96: 299-309.

Wang, L., Fan, X., Sokorai, K. and Sites, J., 2019. Quality deterioration of grape tomato fruit during storage after treatments with gaseous ozone at conditions that significantly reduced populations of Salmonella on stem scar and smooth surface. Food Control, 103, 9-20.

Project Title: Ionized hydrogen peroxide to enhance safety and maintain quality of fresh produce
Support: TOMI Environmental Solutions

The objectives of the collaborative study are to evaluate the efficacy of hydrogen peroxide aerosol activated by cold plasma in inactivating human pathogen surrogates and extending shelf life of fresh fruits and vegetables.

Controlling and inactivating foodborne pathogenic microorganisms on fresh produce remains a formidable challenge. Aerosolized hydrogen peroxide was ionized by passing through a cold plasma arc, and then used to inactivate bacteria on four types of fresh produce items. Results showed that, compared to hydrogen peroxide alone, cold plasma significantly enhanced the efficacy of hydrogen peroxide against bacteria on fresh produce, resulting in more than 99.99% reduction of Salmonella and Listeria on apples, tomatoes, cantaloupe and leafy greens. The information will be of interest to the produce industry which is seeking more effective intervention technologies to enhance the microbial safety of fresh produce.

Cooperative Research and Development Agreement

Project Title:	Molecular Characterization of Foodborne Pathogen Responses to Stress (8072-42000-082-00D)
Support:	Molecular Characterization of Foodborne Pathogens Research Unit
Project Title:	Identification of E. coli sequences for diagnostic assay development (58-8072-9-008)
Support:	Life Technologies, Pennsylvania State University

The objectives of this 3-way CRADA were to (1) identify genetic markers in E. coli strains that cause human illness, particularly in Shiga toxin-producing E. coli (STEC) that cause severe disease; (2) develop methods to identify pathogenic E. coli strains based on DNA sequence and marker information; and (3) develop methods for molecular serotyping of E. coli and for detection of STEC and other pathogenic E. coli.

The genome sequences of over 80 standard reference strains (provided by the collaborator at the Pennsylvania State University and purchased by the Cooperator) of E. coli belonging to different O-groups was determined followed by analysis of the sequence data to determine the O-antigen gene cluster (OAGC) sequences of the different E. coli O-groups. PCR-based assays to identify the various E. coli o-groups were developed based on the sequence information.

The OAGC sequences were submitted to the NCBI GenBank database. A paper co-authored by ARS and collaborators on this work was recently published in the journal PLOS ONE. Currently, work is underway to develop a method for molecular serotyping of E. coli to determine the O- and H-antigens using the collaborator's AmpliSeq technology. The assay will be evaluated at ARS and the Pennsylvania State University.

The following paper was one outcome of this study:

DebRoy, C., Fratamico, PM, Yan, X, Baranzoni, GM, Liu, Y, Needleman, DS, et al. Comparison of O-Antigen Gene Clusters of All OSerogroups of Escherichia coli and Proposal for Adopting a New Nomenclature for O-Typing. 2016. PLoS ONE 11(1): e0147434. doi:10.1371/journal. pone.0147434.

The following poster presented at the 2019 IAFP meeting was another outcome from the study.

Development of a molecular serotyping assay for *Escherichia coli* via targeted sequencing of the O-antigen gene cluster

ThermoFisher SCIENTIFIC

OAK RIDGE INSTITUTE FOR SCIENCE AND EDUCATION

Jacob R. Elder, Pina M. Fratamico, Yanhong Liu*, Lori Bagi, Robert Tebbs, Adam Allred, Prasad Siddavatam, Haktan Suren, Krishna Reddy Gujjula, Chitrita DebRoy, Edward Dudley, Xianghe Yan and David Needleman

USDA United States Department of Agriculture Agricultural Research Service

PennState

ABSTRACT

Introduction: Antibody-based serotyping of *Escherichia coli* is laborious have limitations and while whole genome sequencing is becoming a routine method for subtyping, it produces large amounts of data that must be parsed to identify serotype information. A targeted, sequence-based assay and accompanying software for data analysis would be a great improvement over the currently available methods for serotyping.

Purpose: The purpose of this study was to develop a high-throughput, molecular serotyping method for *E. coli* based on the sequences of the O-antigen gene cluster (O-AGC) and flagellar subunit genes. We also sought to develop software for data analysis and serogroup identification.

Methods: Publicly available *E. coli* O-AGC and flagellar subunit sequences were analyzed, and those that shared >95% identity were grouped into clusters. Representative sequences from each cluster were selected and analyzed for unique signature regions. Primers were designed and checked for specificity, confirming that there was no off-target mapping to other serogroups or *Enterobacteriaceae* genomes. To validate the assay, we extracted genomic DNA from O- and H-serogroup standard strains, amplified the targeted flagellar subunit genes and O-AGC regions, prepared sequencing libraries from the amplified products, and sequenced the libraries on the Ion S5™ sequencer. The resulting sequence files were analyzed via the SeroTyper™ software for confirmation of serogroup.

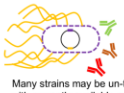
Results: The initial sequence analysis for primer design revealed a total of 168 clusters of *E. coli* (some serogroups share the same O-AGC sequence and group together) O-AGC sequences with unique signature regions in the *wzm*/*wzt* or *wzx*/*wzy* genes. Our primer design method allowed us to pool the 168 primer pairs into a single reaction and test >190 strains per sequencing run. Of the 176 O-serogroup standard strains tested, 173 (98%) were correctly identified by this assay. Three strains, representing serogroups O92, O106, and O126, were misidentified in one replicate.

Significance: The high-throughput, sequence-based method presented here is a reliable alternative to antisera-based serotyping methods for *E. coli*.

BACKGROUND

Issues encountered with current serotyping methods

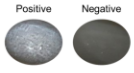
Traditional, sera-based serotyping



Many strains may be un-typable with currently available antisera.

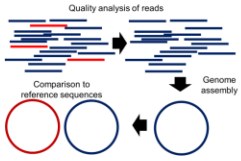
Antisera can cross-react leading to incorrect identification.

Antisera can vary in quality and be unreliable.



Expertise is required to correctly perform and interpret agglutination

Whole-genome sequencing (WGS)



Whole-genome sequencing requires sequence analysis and handling large amounts of data with each sample tested.

**For Research Use Only. Not for use in diagnostic procedures.

RESULTS

O-typing Results

Serotype	Call on SeroSeq	No. correct calls	Serotype	Call on SeroSeq	No. correct calls	Serotype	Call on SeroSeq	No. correct calls
O1	O1	3	O69	O69	2	O135	O13/O129/O135	2
O2	O2/O50	2	O70	O70	1	O136	O136	2
O3	O3	3	O71	O71	3	O137	O137	2
O4	O4	3	O73	O17/O44/O73/O77/O106	2	O138	O138	2
O5	O5	3	O74	O74	3	O139	O139	1
O6	O6	3	O75	O75	1	O140	O140	2
O7	O7	3	O76	O76	3	O141	O141	2
O8	O8	3	O77	O17/O44/O73/O77/O106	3	O142	O142	
O9	O104	0	O78	O78	3	O143	O143	
O10	O10	3	O79	O79	3	O144	O144	
O11	O11/OX19	2	O80	O80	3	O145	O145	
O12	O12	3	O81	O81	3	O146	O146	
O13	O13/O129/O135	3	O82	O82	3	O147	O147	
O15	O15	3	O83	O83	2	O148	O148	
O16	O16	3	O84	O84	3	O149	O149	
O17	O17/O44/O73/O77/O106	2	O85	O85	3	O150	O150	
O18	O18	3	O86	O86	3	O151	O118/O151	
O19	O19/OX43	3	O87	O87	2	O152	O152	
O20	O20	2	O88	O88	3	O153	O153	
O21	O21	3	O89	O89	3	O154	O154	
O22	O22	3	O90	O90/O127	2	O155	O155	
O23	O23	3	O91	O91	3	O156	O156	
O24	O24	2	O92	O92	3	O157	O157	
O25	O25	1	O93	O93	3	O158	O158	
O26	O26	1	O95	O95	0	O159	O159	
O27	O27	2	O96	O96	2	O160	O160	
O28ab	O28ab	1	O97	O97	3	O161	O161	
O28ac	O28ac/042	0	O98	O98	3	O162	O121/O162	
O29	O29	2	O99	O99	3	O163	O163/OX21	
O30	O30	3	O100	O100	2	O164	O164	
O32	O32	2	O101	O101/O162	2	O165	O165	
O33	O33	2	O102	O102	1	O166	O166	
O34	O34	2	O103	O103	2	O167	O167	
O35	O35	2	O104	O104	1	O168	O168/OX6	
O36	O36	3	O105	O105	1	O169	O169	
O37	O37	3	O106	O17/O44/O73/O77/O106	3	O170	O170	
O38	O38	3	O107	O107/O117	2	O171	O171	
O39	O39	3	O108	O108	2	O172	O172	
O40	O40	2	O109	O109	2	O173	O173	
O41	O41	3	O110	O110	1	O174	O174	
O42	O28ac/042	2	O111	O111	2	O175	O175	
O43	O43	2	O112ab	O112ab	2	O176	O176	
O44	O17/O44/O73/O77/O106	2	O112ac	O112ac	0	O177	O177	
O45	O45	3	O113	O113	2	O178	O178	
O46	O46/O134	3	O114	O114	2	O179	O179	
O48	O48	3	O115	O115	2	O180	O180	
O49	O49	3	O116	O116	2	O181	O181	
O50	O2/O50	1	O117	O107/O117	2	O182	O182	
O51	O51	2	O118	O118/O151	2	O183	O183	
O52	O52	3	O119	O119	2	O184	O184	
O53	O53	3	O120	O120	2	O185	O185	
O54	O54	1	O121	O121	2	O186	O123/O186	
O55	O55	3	O123	O123/O186	2	O187	O187	
O56	O56	3	O124	O124/O164	2	OX6	O168/OX6	
O58	O58	1	O125	O125	2	OX9	OX9	
O59	O59	1	O126	O126	1	OX13	OX13	
O60	O60	3	O127	O90/O127	2	OX19	OX19	
O61	O61	3	O128	O128/OX38	2	OX21	O163/OX21	
O62	O62/O66	3	O129	O13/O129/O135	2	OX25	OX25	
O63	O63	3	O130	O130	2	OX28	OX28	
O64	O64	3	O131	O131	2	OX38	O128/OX38	
O65	O65	3	O132	O132	2	OX43	OX43	
O66	O66	3	O133	O133	2			
O68	O62/O66	3	O134	O46/O134	2			

H-typing Results

Serotype	Call on SeroSeq	No. correct calls
H1	H1	3
H2	H2	3
H3	H3/H16	3
H4	H4	3
H5	H5	3
H6	H6	3
H7	H7	3
H8	H8	3
H9	H9	3
H10	H10	3
H11	H11	3
H12	H12	3
H14	H14	3
H15	H15	3
H16	H16	3
H17	H17/H4	3
H18	N/A	0
H19	H19	3
H20	H20	3
H21	H21	3
H23	H23	3
H24	N/A	0
H25	H25	3
H26	H26	3
H27	H27	3
H28	H28	3
H29	H29	3
H30	H30	3
H31	H31	3
H32	H32	3
H33	H33	3
H34	H34	0
H35	H35	3
H36	H21	0
H37	H37	3
H38	H38	3
H39	H39	3
H40	H40	3
H41	H41	3
H42	H42	3
H43	H43	2
H44	H44/H44	3
H45	H45	3
H46	H46	3
H47	H21/H47	2
H48	H48	2
H49	H49	3
H51	H51	0
H52	H40/H52	2
H53	H53	2
H54	H54/H54	3
H55	H55	0
H56	H56	3

Serotype is consistently correctly identified

Serotype has been mis-identified on one occasion

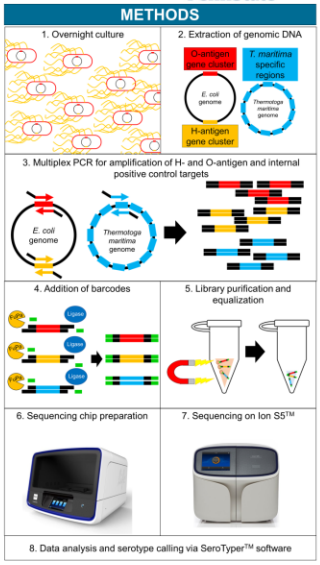
If the K9 antigen is present this serotype is called as O104

Multiple serotypes with indistinguishable O-AGC sequences but are consistently correctly identified

Strains with these serotypes possess multiple flagellar genes resulting in multiple serotypes called

Primer are still under development or undergoing validation

METHODS



E. coli strains were grown overnight (16-18hr) in tryptic soy broth. DNA was extracted with the DNeasy Blood and Tissue Kit™ (Qiagen). *Thermotoga maritima* DNA (10 pg) was added to each sample of *E. coli* DNA (30 ng) before amplification of O- and H-antigen targets, and internal positive control (*T. maritima*) targets via a single primer panel containing; 168 *E. coli* O-type primer pairs, 55 *E. coli* H-type primer pairs, and 13 internal positive control *T. maritima* primer pairs. The amplified targets from each sample were given a unique barcode and pooled. Pooled products were equalized and purified with the Ion Library Equalizer™ kit. Libraries were added to Ion S5™ chip using the Ion Chef™ and then sequenced on the IonS5™ instrument.

CONCLUSIONS

- The sequence-based serotyping assay presented here is an accurate alternative to traditional antisera based serotyping.
- Targeted sequencing of O- and H-serotype genes circumvents some of the data analysis required of WGS-based methods while increasing the capacity for multiplexing samples.

ACKNOWLEDGEMENTS

Funding

Project Title:	Ecology and detection of human pathogens in the produce production continuum
Support:	USDA ARS Produce Safety and Microbiology Laboratory (2030-42000-050-00D)
Project Title:	Effect of plant systemic resistance and role of type III secretion system in colonization of basil and lettuce by *Salmonella enterica*
Support:	US – Israel Binational Agricultural Research and Development Fund (2030-42000-050-04R)

The interaction of human enteric pathogens with the plant immune system has been the focus of much attention in produce safety research as it may provide genetic tools to inhibit plant colonization by foodborne pathogens. Taking a cue from bacterial plant pathogens which commonly interact very specifically with plant cells by injection of effectors via their type 3 secretion system to avoid plant defenses, several studies have suggested that this type of interaction may also have a role in the survival of *Salmonella* in plant tissue. This collaborative project with researchers at The Volcani Center and the University of Tel Aviv, Israel aimed at testing whether plant pathogen effectors, which cause macroscopic symptoms if transferred into plant cells, can be injected via the type 3 secretion apparatus of Salmonella. Using elegant molecular tools constructed during this project and used for the first time to test this hypothesis, the study provided new evidence that *Salmonella* cells do not interact directly with plants through their type 3 secretion system. However, in separate studies, the project also demonstrated that treatment with inducers of broad defense pathways for plant systemic resistance decreased *Salmonella* colonization of the internal leaf tissue of lettuce and basil plants. The results of this project provide important information by showing that plant breeding approaches targeting plant receptors of type 3 effectors will be unsuccessful in reducing *Salmonella* colonization of plants, but that other approaches making use of the plant basal immune response to *Salmonella* hold potential for mitigation strategies. These discoveries have importance at this time given that federal funding agencies and the produce industry have shown a new interest in plant breeding to enhance the microbial safety of crops.

The findings regarding the *Salmonella* type 3 secretion system were describe in the following publication:
Chalupowicz, L., Nissan, G., Brandl, M., McClelland, M., Sessa, G. Popov G., Barash, I., Manulis-Sasson, S. 2018. Assessing the ability of *Salmonella enterica* to translocate Type III effectors into plant cells. Molec. Plant-Microbe Interact. 31(2):233-239. https://doi.org/10.1094/MPMI-07-17-0166-R

The findings regarding the effect of systemic resistance inducers on plant colonization by *Salmonella* were presented at the following conferences attended by produce safety scientists, plant breeders, USDA NIFA program leaders, and produce industry members:
"Colonization of leafy vegetables by enteric pathogens: rare opportunities at various scales" Seminar Series. ARS Salinas, CA, April 2019 "

Multi-Institutional Collaborative Study

Project Title:	Ecology and detection of human pathogens in the produce production continuum
Support:	USDA ARS Produce Safety and Microbiology Laboratory (2030-42000-050-00D)
Project Title:	Genetics and Breeding of Lettuce, Spinach, Melon, and Related Species to Improve Production and Consumer-related Traits
Support:	USDA ARS Crop Improvement and Protection Laboratory (2038-21530-002-00-D)
Project Title:	Effect of lettuce genotype and phyllosphere microbiome from farm through storage on the colonization of fresh-cut lettuce by E. coli O157:H7
Support:	Food and Drug Administration (IAA 75F40119S20049)

Fresh-cut lettuce is the most common type of produce associated with outbreaks of Shiga toxin-producing *E. coli* (STEC) infection. Fresh-cut lettuce, which is stored and sold in Modified Atmosphere Packaging (MAP) has variable shelf-life depending on storage conditions and lettuce variety, but knowledge of the effect of leaf tissue deterioration in lettuce colonization by STEC is lacking. Also unknown is the role of the lettuce microbiome from farm through storage in the persistence of STEC in fresh-cut lettuce at various stages of shelf-life. The FDA has a major interest in characterizing the microbiome of produce and its role in the transfer of STEC throughout the farm to table continuum, as well as its potential to predict contamination of produce with enteric pathogens. ARS scientists in Salinas, California, have taken into account shelf-life traits in their lettuce breeding program to ensure that the industry has access to lettuce varieties of prolonged high physiological quality as a processed product but lacks data regarding their microbial quality.

The collaborative study was the first to investigate the microbiome of lettuce from farm through cold storage in MAP. Using lettuce varieties selected and grown by the ARS, Salinas, CA collaborator, the project determined that STEC survived at a higher rate at cold temperature in decaying lettuce tissue than in healthy tissue. The microbiome of lettuce at various production stages was characterized by the FDA, Laurel, MD collaborators. The microbiome in MAP lettuce of poor shelf-life shifted from that in lettuce of good shelf-life to being enriched in bacterial species that are closely related to STEC, providing the FDA and public health agencies with an important risk factor in the contamination of fresh-cut lettuce with enteric pathogens. Additionally, enhanced survival of STEC in MAP lettuce under cold storage correlated with high %CO2 in the bag atmosphere, which in turn repressed bacterial taxa known to have the ability to inhibit other bacterial species. These results suggest new approaches to inhibit STEC in fresh-cut lettuce stored in MAP and aid the lettuce industry in designing new MAP-based mitigations.

Presented at (1) ARS, Salinas, CA attended by plant breeders and lettuce industry members, (2) the 2019 IAFP meeting; and (3) at the FDA Produce Safety Research Consortium meeting in Laurel, MD.

Inter-Agency (IAG) Collaborative Study

Project Title:	Bacterial pathogens in regulated foods, processing technologies for their elimination.
Support:	USDA ARS ERRC Food Safety and Intervention Technologies (8072-41420-019-00D)
Project Title:	"Prevalence, levels and subtypes of Listeria monocytogenes in ready-to-eat retail foods."
Support:	DHHS, Food and Drug Administration, CFSAN (58-1935-0-078)
Project Title:	"Prevalence, levels and subtypes of Listeria monocytogenes in ready-to-eat retail foods."
Support:	USDA, Food Safety and Inspection Service (60-1935-0-033)

Listeria monocytogenes remains a major food borne pathogen, responsible for numerous and costly product recalls, and the etiological agent for a significant number of hospitalizations (1,575) and illnesses (1,591) each year in the U.S. Listeriosis is also among the top five leading causes of death (255 fatalities per year) due to the consumption of contaminated foods, with fatality rates approaching 30% for persons at elevated risk, namely the young, old, pregnant, and immune-compromised. The pathogen is widespread in nature and food processing environments and is commonly recovered from foods, including ready-to-eat (RTE) foods. Moreover, compared with most other food borne pathogens, L. monocytogenes is quite tolerant of food relevant levels/conditions of salt, cold, and heat, as well as high and low pH.

In the early 2000's, considerable efforts and associated resources were directed towards controlling L. monocytogenes in foods. As a key component of this strategic approach to better manage the threat of food borne listeriosis, several studies were conducted to elaborate the prevalence and levels of L. monocytogenes in higher volume, higher risk foods and to use these data to support the development of quantitative microbial risk assessments. Depending on the food analyzed, the recovery rates ranged from ca. 0.5 to 7.6%, with positive foods harboring the pathogen at levels of <0.03 MPN to 5.2 log CFU/g. In addition to estimating the prevalence and load of L. monocytogenes in RTE foods, Federal agencies issued regulations and guidance on science/risk-based control of the pathogen. For their part, the food industry both followed and developed various guidance documents and changed how RTE products were prepared, handled, and/or stored. As one metric to assess progress at lowering the public health risk of food borne listeriosis, ARS researchers at Wyndmoor, PA, were tasked to determine the "true" prevalence and levels of L. monocytogenes in the aftermath of corrective action taken by regulatory and industry food safety professionals.

With leveraged funding from USDA ARS, USDA FSIS, and DHHS FDA, ARS researchers in Wyndmoor, PA, conducted the L. monocytogenes market basket survey (Lm MBS) to quantify the recovery rate and levels of the pathogen among 16 RTE food categories from 1042 stores visited. In short, a total of 27,389 food samples purchased at chain supermarkets and

independent grocery stores across CA, MD, CT, and GA over a 100-week prior yielded 102 samples (average recovery rate = 0.37; range = 0 to 1.07%) from which a viable isolate of the pathogen was recovered: pathogen levels ranged from <0.036 MPN to 6.1 log CFU/g.

Impact: The interagency Lm MBS is the most comprehensive survey of L. monocytogenes in retail RTE foods in the U.S. since the early 2000's. The observed reductions in contamination may in part reflect regulatory reforms, monitoring efforts, directed modifications of food processes and formulations, and improved sanitation measures implemented by federal regulatory agencies and by the food industry. Data collected on pathogen prevalence, persistence, levels, and types, as well as attendant data on product formulation and handling, will be used to populate joint risk assessments by USDA FSIS and DDHS FDA to further lower the risk of food borne listeriosis.

Awards: Recipient of the Department of Health and Human Services (DHHS), Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition (CFSAN) Secretary's Honor Award "For exceptional collaboration in the development and implementation of research to characterize Listeria monocytogenes prevalence and levels in ready-to-eat foods." Team Leaders: John B. Luchansky and Anna C. S. Porto-Fett (USDA-ARS), Janell Kause and William Shaw (USDA-FSIS), and Sherri Dennis and Yuhuan Chen (DHHS-FDA).

The following paper reports the recovery rate and levels of L. monocytogenes among 27,316 RTE foods at retail:

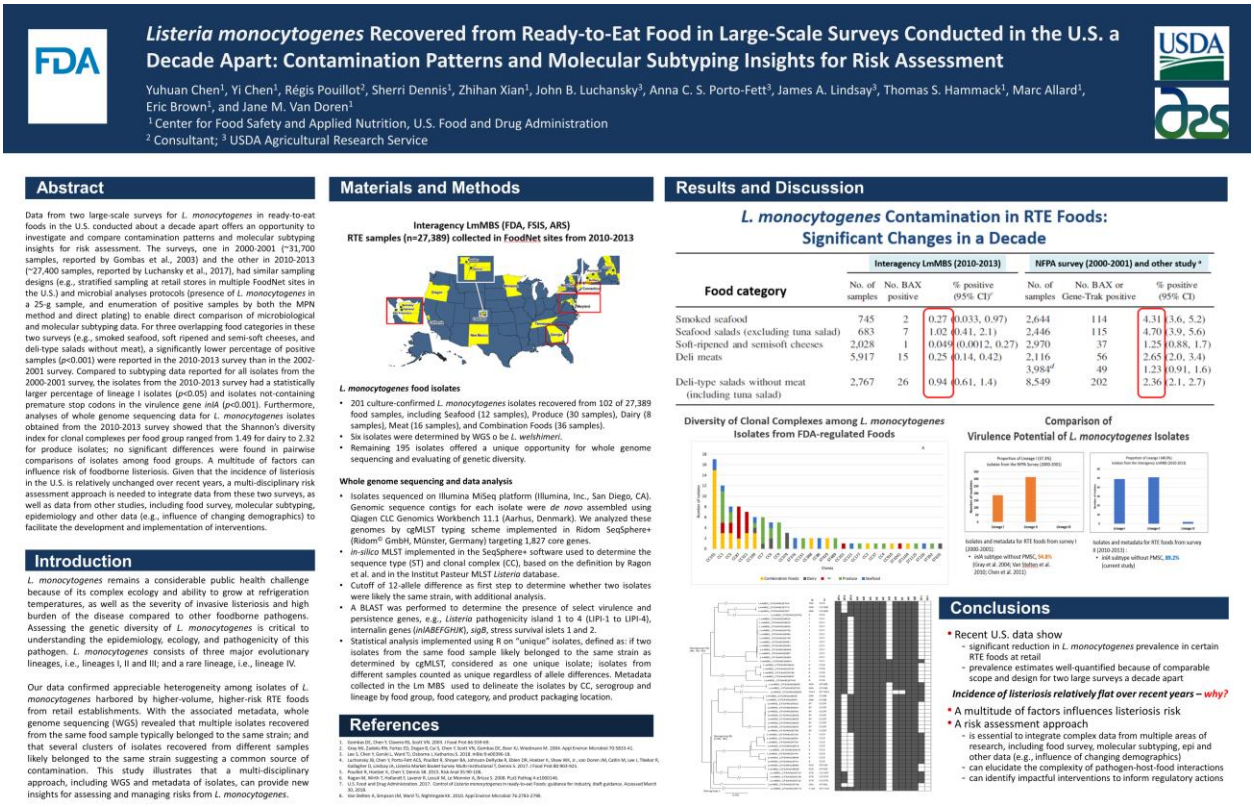
Luchansky, J. B., Y. Chen, A. C. S. Porto-Fett, R. Pouillot, B. A. Shoyer, R. Johnson-DeRycke, D. R. Eblen, K. Hoelzer, W. K. Shaw Jr., J. M. Van Doren, M. Caitlin, J. Lee, R. Tikekar, D. Gallagher, J. A. Lindsay, The *Listeria* Markey Basket Survey Multi-Institutional Team, and S. Dennis. 2017. Survey for *Listeria monocytogenes* in and on ready-to-eat foods from retail establishments in the United States (2010 – 2013): assessing potential changes of pathogen prevalence and levels in a decade. J. Food Prot. 80:903-921. [doi: http://dx.doi.org/10.4315/0362-028X.JFP-16-420]

The following paper reports the types of L. monocytogenes recovered from 0.37% of 27,316 RTE foods at retail:

Chen, Y., Y. Chen, R. Pouillot, S. Dennis, Z. Xian, J. B. Luchansky, A. C. S. Porto-Fett, J. Lindsay, M. Allard, E. Brown, and J. M. Van Doren. 2019. Genetic diversity and virulence gene profiles of *Listeria monocytogenes* isolates from the 2010-2013 interagency market basket survey. Appl. Environ. Microbiol., Under Review.

The following poster addresses the relatedness, types, and potential pathogenicity of L. monocytogenes recovered from 0.37% of 27,316 RTE foods at retail:

Chen, Y., Y. Chen, R. Pouillot, S. Dennis, Z. Xian, J. B. Luchansky, A. C. S. Porto-Fett, J. A. Lindsay, T. Hammack, M. Allard, E. Brown, and J. M. Van Doren. 2019. *Listeria monocytogenes* recovered from ready-to-eat food in large-scale surveys conducted in the U.S. a decade apart: contamination patterns and molecular subtyping insights for risk assessment. Proceedings of the 20th International Symposium on Problems of Listeria and Listeriosis (ISOPOL). Toronto, Canada; (#74), p99.



Multi-Institutional Collaborative Study

Project Title:	Bacterial pathogens in regulated foods, processing technologies for their elimination.
Support:	USDA ARS ERRC Food Safety and Intervention Technologies (8072-41420-019-00D)
Project Title:	"Shiga toxin-producing Escherichia coli in the beef chain: assessing and mitigating risk by translational science, education, and outreach"
Support:	USDA National Institute of Food and Agriculture (58-1935-2-314)

Shiga toxin-producing cells of Escherichia coli (STEC) have been a significant cause of foodborne illness in the U.S. since the early 1980's. Although STEC are readily recovered from various livestock and fabricated foods therefrom, as well as from leafy greens, certain fruits, and water, over the last 40 years beef products were arguably the most common vehicle of outbreaks and recalls. Based on epidemiological data, the seven serovars of STEC responsible for most human illnesses, that being O157:H7 and cells of the big six serovars of O26, O45, O103, O111, O121, and O145, are now considered adulterants in raw, ground/non-intact beef.

To address virulence, epidemiology, prevalence/levels, communication, and control of STEC associated with beef in a holistic and coordinated fashion, some 50 collaborators at 18 institutions across the U.S. were awarded a 5-year, $25M multi-institutional, multi-disciplinary grant focused on STEC and beef. This USDA National Institute of Food and Agriculture (USDA-NIFA) Coordinated Agricultural Project (CAP) was tasked to significantly reduce the occurrence and public health risks from STEC in beef. In addition to serving as a Co-Project Director, ARS researchers in Wyndmoor, PA, focused their efforts on collaboratively filling data voids related to the occurrence and persistence of STEC in beef and on elaborating interventions to lower its prevalence and levels in beef.

The ARS researchers in Wyndmoor, PA, validated processes and interventions such as fermentation, high pressure processing, bio-preservatives, food grade chemicals, and heat (cooking), alone or in combination, to inhibit/remove STEC and better manage pathogen presence, populations, and/or survival during manufacture and storage of target foods or on carcasses. The team optimized methods to deliver antimicrobials to food systems, including electrostatic spraying and various strategies to introduce interventions into/onto foods or food containers/packaging (e.g., SLIC®). Inclusion of food grade chemicals and/or post-fermentation cooking were effective for eliminating STEC within or on specialty and ethnic products such as chicken liver/pate, meat bars, and çiğ köfte. For non-intact beef, STEC were distributed throughout the whole muscle, but most cells resided in the top-most 1 cm. For thermal inactivation studies, no differences in lethality were observed among STEC serovars following cooking of tenderized/injected steaks, veal cutlets, meatballs, prime rib, or cubed steaks. As one outcome from these efforts, a highly successful national campaign was launched to deliver a message to the masses about proper cooking of burgers. Additionally, the findings revealed a two- to four-fold greater risk for tenderized and injected steaks compared to intact steaks. Lastly, our survey data confirmed that the recovery rate for STEC is higher for ground/non-intact veal than ground/non-intact beef. The significance and uniqueness of this research is the

use of actual beef products (e.g., ground beef patties and whole muscle subprimals) rather than simulated restructured beef products, inoculation with pathogenic strains of E. coli rather than surrogates, use of pilot scale processing equipment rather than a bench top apparatus, and use of an industry-relevant experimental matrix. The ensuing results will assist manufacturers in meeting current regulatory guidelines and assist regulators in making science-based policy decisions, thereby enhancing the safety of our Nation's food supply.

Impact: Intervention data established that existing technologies for O157:H7 STEC are equally effective against the other 6 regulated serovars of STEC in beef, thus saving appreciable resources from being directed towards process validation or intervention development/optimization specific for the big six STEC serovars. Inoculated package challenge studies greatly assisted small processors to validate their processes, with a focus on specialty/ethnic products which, in turn, enhanced safety/quality and allowed several companies to remain in business. Findings related to non-intact beef filled key data gaps for determining risk from tenderization/injection, updated risk assessments for raw beef, used for declaring non-O157 STEC an adulterant, and supported adoption rules for labeling meat products as non-intact (U.S. and Canada). Understanding and controlling STEC bacteria throughout the entire beef system will lead to additional improvements in beef safety and public health.

Awards:
1) Recipient of the U.S. Department of Agriculture (USDA), National Institute of Food and Agriculture (NIFA) Partnership Award for Mission Integration of Research, Education, and Extension. "For reducing public health risks while preserving a sustainable beef industry." (2017).
2) Luchansky, J. B., A. C. S. Porto-Fett, and B. Chapman. 2017. A shopper's eye view of food safety at retail stores: lessons from photographs taken while grocery shopping. *Food Prot. Trends* 37:30-42. [NOTE: International Association for Food Protection Award for most-viewed Food Protection Trends publication in 2017]

Manuscripts:
The following paper highlights our research on thermal inactivation of STEC in beef:
Porto-Fett, A. C. S., M. Oliver, M. Daniel, B. A. Shoyer, L. J. Stahler, L. E. Shane, L. S. Kassama, A. Jackson-Davis, and J. B. Luchansky. 2016. The effect of deep frying or conventional oven cooking on inactivation of Shiga toxin-producing cells of *Escherichia coli* (STEC) in meatballs. *J. Food Prot.* 79:723-731.

The following paper details validation of chemicals to reduce STEC on veal carcasses:
Hasty, J. D., J. A. Henson, G. R. Acuff, D. E. Burson, J. B. Luchansky, N. J. Sevart, R. K. Phebus, A. C. S. Porto-Fett, and H. Thippareddi. 2018. Validation of a sequential hide-on bob veal carcass antimicrobial intervention comprised of a hot water wash and lactic acid spray in combination with scalding to control Shiga toxin-producing *Escherichia coli* surrogates. *J. Food Prot.* 81:762-768.

Outreach:
The following logo and webpage were developed to communicate proper use of a thermometer to determine doneness of burgers, that being when an internal temperature of 160°F is achieved: Chapman, B., J. Hochstein, A. Porto-Fett, K. Longacre, and J. Luchansky. 2019. Creation and implementation of a social marketing campaign for beef food safety. Abstracts of the Annual Meeting of the International Association for Food Protection. Supplement A to J. Food Prot. (P1-94, p106).

Theater Ads

Animated spot played in the 51 theaters/screens in Fayetteville, NC. Check it out at:

https://160isgood.com/

www.160isgood.com

Interagency Reimbursable Agreement (Study) July 2018-Sept. 2020
Project Title: Trichinella Testing Laboratory
Support: USDA-APHIS (8042-32420-07-31-1)

Abstracted from research proposal (see Rosenthal)

For details refer to: https://www.ars.usda.gov/research/project/?accnNo=435156

Trichinella is a nematode parasite that can infect a wide range of animals including pigs. Humans become infected by eating raw or undercooked meat containing infective stages of Trichinella. Trichinellosis was common in humans in the 1800's and early 1900's (15% of the U.S. population infected) due in large part to the practice of feeding raw garbage to pigs. Since the mid-20th century, Trichinella infection in pigs has declined to a point where it is essentially absent in commercial (confinement-reared) pigs.

The U.S. pork industry wanted to promote the safety of U.S. pork both domestically and internationally by demonstrating that Trichinella is not found in U.S. commercial pork. Recent guidelines from the Codex Alimentarius provide an opportunity for countries to establish a "compartment of negligible risk" for Trichinella by auditing good production practices and demonstrating, by initial testing, that infection is < 1/1,000,000 pigs.

To document the absence or very low prevalence of Trichinella spiralis in market hogs. ARS will conduct surveillance testing for a period of 24 months. Testing will include a statistical sampling of a population of 60-80 million pigs each year and will be designed to demonstrate a prevalence of no more than 0.000001 at a 95% confidence level. The proposal seeks to test approximately 3,000,000 samples each year for a 2-year period.

This project is still underway, and thus there are no data at this time