International Journal of Food Microbiology 215 (2015) 40–48

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Metabolic footprinting of *Lactobacillus buchneri* strain LA1147 during anaerobic spoilage of fermented cucumbers☆

Suzanne D. Johanningsmeier *, Roger F. McFeeters

USDA-ARS, SAA Food Science Research Unit, 322 Schaub Hall, Box 7624, North Carolina State University, Raleigh, NC 27695, United States

ARTICLE INFO

Article history:
Received 20 December 2014
Received in revised form 15 May 2015
Accepted 8 August 2015
Available online 12 August 2015

Keywords:
GCxGC–ToFMS
Metabolomic platform
Lactic acid bacteria
Pickled vegetables
Spoilage organisms
Spoilage metabolites
L. buchneri metabolism

ABSTRACT

Lactobacillus buchneri has recently been associated with anaerobic spoilage of fermented cucumbers due to its ability to metabolize lactic acid into acetic acid and 1,2-propanediol. However, we have limited knowledge of other chemical components in fermented cucumber that may be related to spoilage and the unique metabolic capabilities of *L. buchneri*. Comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry metabolite profiling methods were applied for nontargeted detection of volatile and nonvolatile compounds to determine changes that occurred during anaerobic fermented cucumber spoilage by *L. buchneri* LA1147 and during reproduction of spoilage with natural microbiota. Univariate analysis of variance combined with hierarchial clustering analysis revealed 92 metabolites that changed during spoilage ($P < 0.01$). Decreases were observed in mono and disaccharides, amino acids, nucleosides, long chain fatty acids, aldehydes, and ketones, and increases were observed in several alcohols and butanoic and pentanoic acids. Most of the metabolite changes preceded lactic acid utilization, indicating that lactic acid is not a preferred substrate for anaerobic spoilage organisms in fermented cucumbers. The ability to detect biochemical changes that preceded lactate utilization revealed citrulline, trehalose, and cellobiose as compounds that may signify metabolic activity of *L. buchneri* spoilage strains prior to any significant product degradation.

Published by Elsevier B.V.

1. Introduction

Lactobacillus buchneri is a heterofermentative lactic acid bacterium that has been isolated from a wide variety of plant food environments. Its role in food and feed fermentations is usually secondary to other lactic acid bacteria (LAB), but it has a wider range of metabolic activities than some of its faster growing relatives. The unique metabolic activities of *L. buchneri* highlighted in recent literature include the ability to anaerobically convert lactic acid to acetic acid and 1,2-propanediol in microbiological media (Oude Elferink et al., 2001) and fermented cucumbers (Johanningsmeier et al., 2012). Although this metabolic activity is considered desirable in silage (Holzer et al., 2003), the metabolism of lactic acid in fermented cucumbers can lead to spoilage of the products due to the accompanying rise in pH (Johanningsmeier and McFeeters, 2013), which may allow other microorganisms to become metabolically active. To date, the complete set of metabolic activities of this organism in fermented cucumbers has yet to be characterized.

In fact, current knowledge of the biochemistry of fermented cucumber spoilage is largely limited to metabolites that have been measured using high performance liquid chromatography (HPLC) with ultraviolet (UV) and refractive index (RI) detection. Although this technology is excellent for targeted analysis of metabolites that change in millimolar concentrations, we have only limited knowledge of other components that may be related to spoilage processes. Given the potential diversity of chemical species in a food fermentation system, a discovery based, metabolomic approach for studying the chemistry of fermented cucumber spoilage is warranted.

The technologies that are primarily being used for metabolomic investigations include nuclear magnetic resonance, liquid chromatography–mass spectrometry, and gas chromatography–mass spectrometry (GC–MS). Each of these techniques has advantages and limitations, and a single analytical technique to comprehensively study the metabolome is not yet readily available (Wishart, 2008). Despite the limitation of requiring a volatile metabolite or a volatile metabolite derivative, two-dimensional gas chromatography–time-of-flight mass spectrometry (GCxGC–ToFMS) is a powerful tool for obtaining metabolite information. Two-dimensional gas chromatography (GCxGC) methods have been widely applied to the analysis of volatile compounds in foods. An increased number of compounds were detected, and significantly higher mass spectral similarity values were obtained for butter volatiles and grape monoterpenoids, providing evidence of the increased separation efficiency of GCxGC–ToFMS as compared to 1D GC–MS for complex

☆ Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture or North Carolina Agricultural Research Service, nor does it imply approval to the exclusion of other products that may be suitable.

* Corresponding author.
E-mail address: suzanne.johanningsmeier@ars.usda.gov (S.D. Johanningsmeier).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.08.004
0168-1605/Published by Elsevier B.V.

food matrices (Adahchour et al., 2005; Rocha et al., 2007). Analysis of lavender essential oil using a 2D separation resulted in a 25 fold increase in sensitivity and 3 fold increase in the number of resolved components as compared to traditional GC analysis (Shellie et al., 2001). This technology has been further developed for application to non-targeted profiling of volatile compounds in fermented cucumbers (Johanningsmeier and McFeeters, 2011). Similarly, GCxGC–ToFMS may be applied for the study of nonvolatile components that are first chemically derivatized with one or more trimethylsilyl (TMS) group(s) to make them volatile, allowing the analysis of a number of chemical classes including mono and disaccharides, sugar alcohols, sugar phosphates, organic acids, amino acids, and long chain fatty acids.

One-dimensional GC–MS based metabolomic platforms have been developed and applied for metabolite profiling in plants (Gullberg et al., 2004; Lisec et al., 2006; Roessner et al., 2000; Rudell et al., 2008; Weckworth et al., 2004; Zörb et al., 2006) and microorganisms (Barsch et al., 2004; Bölling and Fiehn, 2005; Koek et al., 2006; O'Hagan et al., 2005; Strelkov et al., 2004; van der Werf et al., 2008). Two-dimensional GC–ToFMS has been less extensively used for metabolomic investigations due to the challenges associated with the large volumes of data produced and the greater expense of the instrumentation itself. However, Welthagan et al. (2005) clearly demonstrated the advantages of GCxGC–ToFMS for biomarker discovery in spleen tissue extracts. GCxGC–ToFMS required approximately 10 fold less sample than GC–ToFMS, exhibited excellent peak resolution, and separated 7 fold more peaks with S/N > 50 and high quality mass spectra. Similarly, a GCxGC–ToFMS metabolomic platform was applied to rice extracts in a study of phenotypic variation. Peak responses were double that observed with the corresponding one-dimensional GC method, and approximately 620 peaks were observed in each sample (Kusano et al., 2007). Twenty-six metabolites that distinguished fermenting yeast cells from respiring yeast cells were identified from among more than 2500 peaks detected using non-targeted GCxGC–ToFMS metabolite profiling (Mohler et al., 2006). Similar GCxGC–ToFMS methods have been developed for metabolomic investigations in rye grass (Hope et al., 2005) and microbial metabolomics (Guo and Lidstrom, 2008).

The aforementioned studies have clearly demonstrated the usefulness of GC–MS for non-targeted metabolite profiling in a variety of matrices. Combining the information obtained from both volatile and nonvolatile analyses using a non-targeted GCxGC–ToFMS platform will allow a much more comprehensive view of the biochemical changes occurring during spoilage of fermented cucumbers than has been previously possible. The objective of this research was to apply a GCxGC–ToFMS metabolomic platform to study biochemical changes that occur during fermented cucumber spoilage by *L. buchneri* and during reproduction of spoilage with unidentified spoilage organisms obtained from reduced NaCl and commercial cucumber fermentation spoilage sources.

2. Materials and methods

2.1. Spoilage inocula sources

Brines from a reduced NaCl cucumber fermentation and a commercial cucumber fermentation that had undergone undesirable secondary fermentations were used as spoilage inocula. The reduced NaCl spoilage source has been previously described (Johanningsmeier and McFeeters, 2011; Johanningsmeier et al., 2012). The commercial spoilage source was obtained from a commercial brining facility after it was observed that a tank of fermented cucumbers was undergoing post-fermentation spoilage, described as Commercial 2 spoilage source by Johanningsmeier et al. (2012). *L. buchneri* (Culture Collection ID LA1147, USDA-ARS Food Science Research Unit, Raleigh, NC), a lactic acid degrading LAB isolated from the reduced NaCl spoilage source, was also used to reproduce spoilage in fermented cucumber slurry (FCS) media.

2.2. Growth media preparation

Size 2B pickling cucumbers (32–38 mm in diameter) were obtained from a local processor, blended into slurry, and frozen at −10 °C until needed. Cucumber slurry was thawed, pressed through cheesecloth and centrifuged in 250-mL bottles at 23,400 ×*g* for 15 min to remove particulate matter. After centrifugation, NaCl, yeast extract, peptone and water were added to yield final concentrations of 67% cucumber, 4% NaCl, 1% yeast extract, and 1% peptone. The resulting modified cucumber slurry (mCS) growth media was sterile-filtered with a 0.2 μm bottle top filter (Nalgene FAST PES, 0.2 μm pore size, 90-mm-diameter membrane, Daigger, Vernon Hills, IL) and stored at 4 °C until use.

2.3. Fermented cucumber media preparation

Size 2B pickling cucumbers were washed, packed into 3.84-L glass jars, and covered with brine (55:45 cucumber:brine ratio) containing calcium chloride and NaCl so that the equilibrated concentrations were 0.25% and 4% (w/w), respectively. Brined cucumbers were inoculated with 10^6 CFU/g *Lactobacillus plantarum* MOP3 starter culture (Culture Collection ID LA0219, USDA-ARS, Food Science Research Unit, Raleigh, NC). Jars were closed with lids that were heated in boiling water to soften the plastisol liner, and a rubber septum was inserted into the lid of each jar to allow sampling of the brine with a sterile syringe. Fermentation was conducted at ambient temperature (21–25 °C) for 11 mo. Fermentation progressed normally as indicated by decreases in pH and production of organic acids as measured by HPLC with UV light detection (McFeeters and Barish, 2003). Sugars and alcohols were quantified in the same analysis using a refractive index detector in series. Fermented cucumbers were cut into pieces and blended into slurry to prepare FCS as a medium for inoculation with spoilage microorganisms. The FCS was pressed through cheesecloth and centrifuged in 250-mL bottles at 23,400 ×*g* for 15 min to remove particulate matter. The pH of the clarified slurry was raised from 3.1 to 3.8 by addition of 6 N NaOH to increase the likelihood that spoilage would occur (Fleming et al., 2002; Kim and Breidt, 2007), and NaCl was added to bring the equilibrated concentration to 6% w/w. The pH-adjusted, clarified FCS was sterile-filtered with a Nalgene FAST PES 0.2 μm pore size, 90-mm-diameter membrane, bottle-top filter apparatus (Daigger). Twelve mL of sterile-filtered FCS was then aseptically transferred into sterile 15-mL conical tubes. The loosely capped tubes were placed into an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) for 3 d prior to inoculation to remove dissolved oxygen from the media.

2.4. Reproduction of spoilage for metabolite profiling

Reduced NaCl and commercial spoiled fermentation brines (1 mL each) were inoculated into 9 mL mCS and incubated anaerobically at 30 °C for 2 wk. *L. buchneri* strain LA 1147 was streaked onto MRS and incubated anaerobically at 30 °C for 4 d. Three isolated colonies were transferred to 9 mL mCS and incubated anaerobically at 30 °C for 2 wk. One milliliter of each of the three resulting spoilage cultures was transferred to 25 mL mCS and incubated anaerobically at 30 °C for 6 d to prepare inocula for reproduction of spoilage in FCS. Spoilage inocula were centrifuged to pellet cells and the spent growth media was discarded. Cells were washed with 5 mL FCS, pelleted, and then resuspended in 15 mL FCS. Spoilage inocula were serially diluted and spiral plated onto MRS to count LAB. Conical centrifuge tubes containing 5 mL sterile-filtered FCS were inoculated in triplicate with 0.5 mL of each spoilage culture and incubated anaerobically at ambient temperature along with triplicate noninoculated FCS controls. Samples were aseptically withdrawn after 9, 22, 51, 77, 96, and 146 d of incubation and stored at −80 °C until analysis. Lactic acid utilization by spoilage inocula was quantified by HPLC. Non-targeted analysis of volatile and nonvolatile components was conducted using solid-phase microextraction–comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry

(SPME–GCxGC–ToFMS) and trimethylsilyl derivatization–comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (TMS–GCxGC–ToFMS), respectively.

2.5. Volatile compound analysis by SPME–GCxGC–ToFMS

Non-targeted volatile compound analysis was performed with slight modification of the analytical method described by Johanningsmeier and McFeeters (2011). Briefly, spoilage samples (100 μL) were diluted 1:10 with deionized water (896 μL) and acidified with 4 μL of 3 N sulfuric acid (H_2SO_4) in 10 mL screw-cap headspace vials (Microliter Analytical Supplies, Inc., Suwanee, GA). NaCl (0.40 g) was added to "salt out" volatile components from the samples. Samples were blocked by replicate, randomized for analysis order (PROC PLAN, version 9.1.3 SAS® software, SAS Institute, Cary, NC) and placed into a refrigerated sample tray (2 °C). Automated sampling was performed using a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies, Carrboro, NC). Headspace vials containing the diluted samples were agitated at 500 rpm (5 s on and 2 s off) for 15 min at 40 °C prior to extraction. Volatile compounds were collected by insertion of a 1-cm, 50/30 μm DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco, Bellefonte, PA) into the headspace above the sample for 30 min at 40 °C with 100 rpm agitation (5 s on and 2 s off). Extracted volatile compounds were desorbed from the SPME fiber into the GC inlet at 250 °C for 15 min. A blank sample (1.0 mL deionized water containing 6 mM sulfuric acid and 0.4 g NaCl) was included in order to determine which detected components were system artifacts.

A LECO® Pegasus III® GCxGC–ToFMS instrument (Model# 614-100-700, Leco Corporation, St. Joseph, MI) included an Agilent GC (Model# 6890 N, Agilent Technologies, Santa Clara, CA) fitted with a secondary oven and cryogenic modulator. The two-dimensional separation was achieved using a SolGel-Wax™, 30 m × 0.25 mm ID × 0.25 μm film thickness (SGE, Austin, TX), polyethylene glycol 1st dimension column in the primary oven and an RTX 17–01, 1.0 m × 0.1 mm ID × 0.1 μm film thickness (Restek, Bellefonte, PA), 14% cyanopropylphenyl–86% dimethyl polysiloxane 2nd dimension column in the secondary oven. A 0.75-mm-ID Siltek deactivated SPME liner (Restek) was used in the inlet, set at 250 °C and operated in pulsed splitless mode with a pulse pressure of 37 psi for 1 min. The split vent was opened 2 min following injection, and the GC was operated in constant flow mode with 1.3 mL/min helium carrier gas. The primary oven temperature was maintained at 40 °C for 2 min and then increased at 5 °C/min to 140 °C. The temperature ramp was then increased to 10 °C/min to 250 °C and the temperature was held at 250 °C for 3 min. The secondary oven followed the same temperature program except the temperature was maintained at 10 °C higher than the main oven until the temperature reached a maximum of 250 °C in the secondary oven. The transfer line temperature was maintained at 250 °C. The modulator offset was + 30 °C with a 2.75 s 2nd dimension separation time and 0.55 s hot pulse. Compressed air (35 psi) was used for the hot pulses, and liquid nitrogen-cooled nitrogen gas (18 psi) was used for the cold pulses.

The mass spectrometer was operated with − 70 eV and an ion source temperature of 200 °C. The detector voltage was set at 1500 V and masses 25–500 were collected at 200 spectra per second. No solvent delay was employed.

2.6. Nonvolatile compound analysis by TMS–GCxGC–ToFMS

Spoilage samples were thawed, briefly vortexed, and centrifuged at 16,000 ×*g* for 7.5 min (Marathon 16KM microcentrifuge, Fisher Scientific, Pittsburgh, PA). A 40 μL aliquot of supernatant was transferred to a microcentrifuge tube, and 10 μL internal standard stock solution containing 2 nmol ribitol (Sigma-Aldrich, St. Louis, MO), 4 nmol deuterated valine (Icon Isotopes, Summit, NJ), and 4 nmol C^{13}-labeled salicylic acid (Icon Isotopes) was added. Sample pH was adjusted to neutral by addition of 0.05 M sodium carbonate (Na_2CO_3) to each sample as indicated by titration with 1% bromothymol blue indicator. Samples were dried by centrifugal evaporation under vacuum (Savant SpeedVac model SVC100D, Instruments, Inc., Farmingdale, NY). Metabolites with aldehyde and keto groups were oximated by addition of 98 μL of 20 mg/mL *O*-methoxylamine HCl in pyridine to the dried samples, which were heated for 90 min at 70 °C. Formation of the TMS derivatives was done by heating the oximated samples for 30 min at 70 °C after addition of 98 μL of N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA). After the 30 min incubation, sample tubes were placed on ice for 5 min, centrifuged to pellet reaction precipitate, and transferred to an autosampler vial. Samples were blocked by replicate for derivatization, randomized for run order and placed into a refrigerated sample tray (2 °C). A 1 μL hot needle injection was performed using a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies). Three pre and post washes of the syringe with ethyl acetate and hexane and 3 sample prewashes were employed to avoid carryover. A 4-mm-ID deactivated tapered splitless injection liner (Agilent Technologies) was used in the inlet set at 260 °C and operated in pulsed splitless mode with a pulse pressure of 24 psi for 1 min. The split vent was opened 1 min following injection, and the GC was operated in constant flow mode with 1.0 mL/min helium carrier gas. The two-dimensional separation was achieved using a BPX50, 30 m × 0.25 mm ID × 0.25 μm film thickness (SGE), 50% phenyl polysilphenylene-siloxane 1st dimension column in the primary oven and a BPX5, 1.0 m × 0.15 mm ID × 0.15 μm film thickness (SGE), 5% phenyl polysilphenylene-siloxane 2nd dimension column in the secondary oven. Although most of the previously published 2D GC metabolomic studies used a nonpolar/polar column combination, Koek et al. (2008) found that a reverse column combination resulted in better resolution of components and greater use of the separation space with similar separation efficiency. The primary oven temperature was maintained at 70 °C for 4 min and then increased at 5 °C/min to 230 °C. The temperature ramp was then increased to 15 °C/min to 290 °C and the temperature was held at 290 °C for 15 min. The secondary oven followed the same temperature program except the temperature was maintained at 15 °C higher than the main oven. The transfer line temperature was maintained at 280 °C. The modulator offset was + 30 °C with a 2.75 s 2nd dimension separation time and 0.55 s hot pulse. The mass spectrometer was operated at − 70 eV with an ion source temperature of 250 °C. The detector voltage was set at 1550 V and masses 35–800 were collected at 200 spectra per second. A solvent delay of 7 min was employed to reduce wear on the detector.

2.7. GCxGC–ToFMS data processing

ChromaTOF® software version 4.33 data processing methods were used to detect and quantify peaks based on unique masses as determined by the deconvolution algorithm. A library search of the NIST/EPA/NIH Mass Spectral Library (National Institute of Standards and Technology (NIST), Gaithersburg, MD, 2005) or LECO-Fiehn Rtx5 Mass Spectral Library (Leco Corporation) was utilized for tentative identification of deconvoluted chromatographic peaks for volatile and nonvolatile compounds, respectively. Masses 85–800 were used for library matching of nonvolatile compound peaks in order to remove the influence of the abundant mass 73 that is encountered with TMS-derivatives. Chemical names were assigned to peaks that had a minimum mass spectral similarity ≥ 800 (1000 is an exact match). The unique mass (U) for each peak, as assigned by the ChromaTOF® deconvolution algorithm, was used for peak area calculations. Alignment of chromatograms was accomplished using the StatCompare® feature in ChromaTOF®, and aligned peak information was exported for statistical analysis.

2.8. Statistical analysis

Analysis of variance (ANOVA) and hierarchial cluster analysis (HCA) were performed in JMP Genomics version 4.1 (SAS Institute). Aligned

peak information exported from ChromaTOF® was reformatted using Excel (Microsoft Corporation, Redmond, WA). A column was added in order to give each peak a unique numerical identifier for statistical analysis. Missing value replacement was performed using the "randbetween" function in Excel to provide substitution data that reflected possible responses below the method's detection limit for undetected components, and peak areas were subjected to log2 transformation to normalize peak area variances prior to statistical analysis. An ANOVA of log2 peak areas was conducted to detect differences in volatile and nonvolatile compounds among treatments. Significance was established at $P < 0.01$ after adjustment of *P*-values to control the false discovery rate using the method of Benjamini and Hochberg (1995). Metabolite peaks that were found to be significantly different among treatments were subjected to HCA that used the Fast Ward method as the default clustering process. The resulting heat maps were visually examined for biologically relevant trends. Clusters of metabolites that represented increases or decreases during spoilage for all spoilage inocula were selected for further examination. Components that changed during anaerobic incubation of noninoculated FCS were presumed to have been formed as a result of chemical changes that occurred during the extended incubation period and were manually excluded when necessary.

3. Results and discussion

Non-targeted profiling of the volatile and nonvolatile chemical composition during reproduction of anaerobic fermented cucumber spoilage revealed a number of biochemical changes that were previously unknown. Generally, decreases were observed in sugars, amino acids, nucleosides, long chain fatty acids, aldehydes, and ketones, and increases were observed in alcohols and short chain fatty acids. Hundreds of metabolite peaks were found to change as a result of anaerobic metabolic activity of spoilage organisms, and several key metabolites were identified. The majority of these changes preceded lactic acid utilization, indicating that lactic acid is not a preferred substrate for spoilage organisms in fermented cucumbers.

Although lactic acid was utilized at a similar rate by *L. buchneri* and the reduced NaCl and commercial spoilage cultures (Fig. 1), the three spoilages differed somewhat in their overall metabolite profiles. Approximately 692 volatile compounds were detected at S/N ≥ 250 in FCS media inoculated with *L. buchneri* and spoilage cultures. Of these, 195 were significantly different among treatments after 146 d anaerobic incubation ($P < 0.01$). These differential metabolites were subjected to HCA resulting in 11 distinct clusters (Fig. 2). The clusters contained groups of metabolites that represented several biologically significant trends. From top to bottom, clusters labeled 1, 2, 3, and 8 represent volatile metabolites present in FCS (6% NaCl, pH 3.8) that were used differentially by the three spoilage inocula, indicating that there were significant metabolic activities of other organisms present in the two spontaneous spoilage sources. This was not surprising because a variety of microbiota have been identified in spoiling fermented cucumbers from different sources at varying stages of spoilage (Breidt et al., 2013; Johanningsmeier et al., 2012; Franco and Pérez-Díaz 2013; Franco et al., 2012; Fleming et al., 1989). Although a wide variety of LAB and other organisms have been isolated or identified by non-culture based methods, only *L. buchneri* and *Lactobacillus parafarraginis* have been shown to initiate anaerobic lactic acid degradation at the low pH and high NaCl conditions of a fermented cucumber (Franco and Pérez-Díaz, 2012; Johanningsmeier et al., 2012; Johanningsmeier and McFeeters, 2013). The other organisms may be present without the specific ability to metabolize lactic acid, such as *Pediococcus ethanolidurans* (Franco and Pérez-Díaz, 2012; Johanningsmeier et al., 2012); may work in concert with *L. buchneri* to convert 1,2-propanediol to propionic acid and 1-propanol, such as *Lactobacillus rapi* (Johanningsmeier and McFeeters, 2013); or may participate in the latter stages of spoilage after the pH has risen, such as *Clostridium tertium* (Fleming et al., 1989), *Clostridium bifermentans* and *Enterobacter cloacae* (Franco and Pérez-Díaz, 2012, 2013), *Propionibacteria acidilactici* (Breidt et al., 2013), and *Pectinatus sottaceto* (Breidt et al., 2013; Caldwell et al., 2013). These organisms may have other metabolic activities that typically go undetected without the comprehensive approach applied in this study. For example, several LAB isolated from commercial and reduced salt fermented cucumber spoilage brines were unable to metabolize lactic acid when inoculated in pure culture (Johanningsmeier et al., 2012). Although these LAB were not contributing to the prominent metabolic activity that characterizes spoilage of

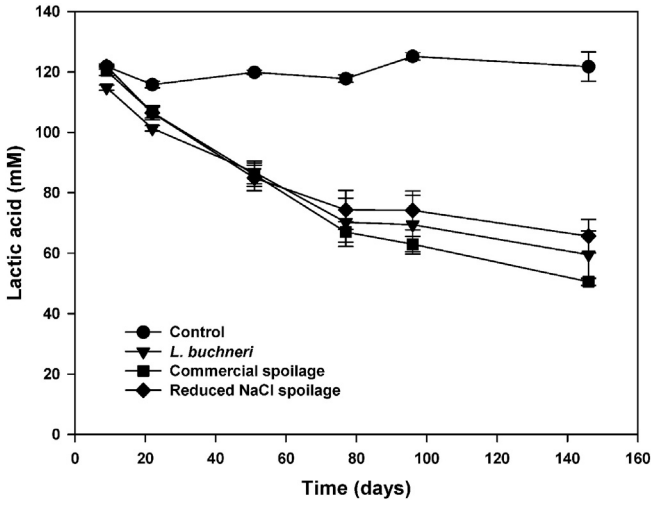

Fig. 1. Anaerobic lactic acid utilization in fermented cucumber medium (pH 3.8, 6% NaCl) by *L. buchneri* and spoilage organisms from reduced NaCl and commercial fermentation sources.

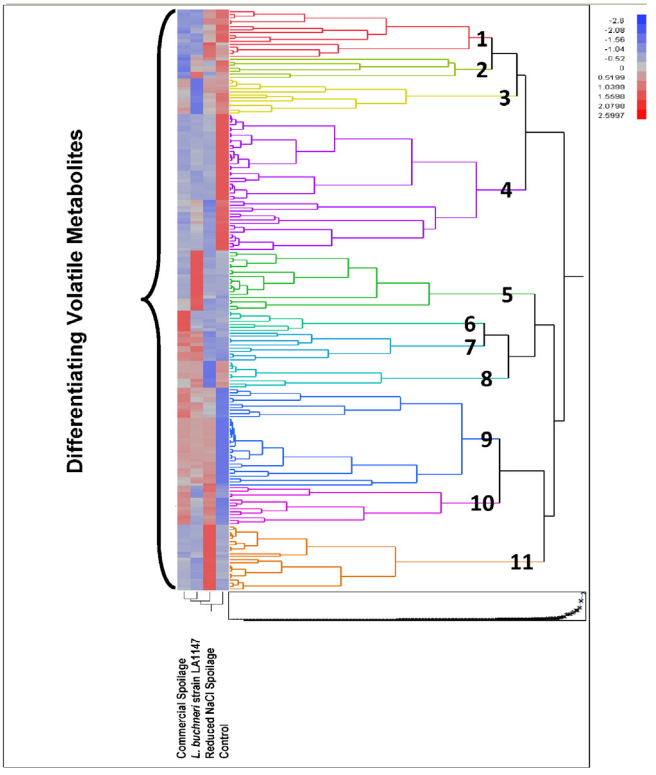

Fig. 2. Hierarchial clustering of 195 volatile metabolites that changed significantly ($P < 0.01$) in fermented cucumber medium after 146 d anaerobic incubation with spoilage organisms.

fermented cucumbers, lactic acid degradation with a concomitant rise in pH, their repeated isolation and identification indicates that they were metabolically active.

The volatile metabolites that grouped into the largest cluster, denoted as cluster 4, were present in FCS and decreased substantially by *L. buchneri*, as well as by undefined organisms in commercial and

Table 1
Volatile metabolites that changed significantly ($P < 0.01$) during anaerobic spoilage of fermented cucumber medium by *L. buchneri* and spoilage cultures from reduced NaCl and commercial fermentation sources.

Metabolite ID	CAS[a] registry #	Method of identification[b]	Mass spectral match quality[c]			RI[d]	Retention time[e]		Mass[f]
			Similarity	Reverse	Probability		t_{r1} (sec)	t_{r2} (sec)	
Volatile metabolites that decreased									
Cis-1,2-dimethyl-cyclopropane	930-18-7	MS	909	910	1773	508	165.00	0.645	55
Acetaldehyde	75-07-0	MS, RI, ST	944	945	9193	702	181.50	0.615	29
Acetone	67-64-1	MS, RI, ST	902	903	6644	815	220.00	0.695	43
Butanal	123-72-8	MS, RI	900	900	8303	874	255.75	0.790	72
Methanol	67-56-1	MS, RI	921	936	9174	897	269.50	0.635	31
3-Methylbutanal	590-86-3	MS, RI, ST	840	840	8454	915	286.00	0.890	41
Ethyloxirane	106-88-7	MS	842	862	7098	934	305.25	0.785	72
2,4,5-Trimethyl-1,3-dioxolane	3299-32-9	MS	836	885	9052	940	310.75	0.970	101
3,3-Dimethyl-2-butanone	75-97-8	MS, RI, ST	858	870	5026	945	316.25	1.000	57
Acetonitrile	75-05-8	MS, RI, ST	834	915	5965	1003	374.00	0.690	41
Methyl isobutyl ketone	108-10-1	MS, RI, ST	900	900	5639	1007	379.50	1.035	43
Propanenitrile	107-12-0	MS, RI	884	918	9115	1025	404.25	0.775	54
Hexanal	66-25-1	MS, RI, ST	905	906	8095	1078	475.75	1.085	41
5-Hexen-2-one	109-49-9	MS, RI	820	864	5169	1084	484.00	0.970	43
Trans-2-methyl-2-butenal	497-03-0	MS, RI	888	919	6232	1095	497.75	0.940	84
3-Methyl-2-hexanone	2550-21-2	MS, RI	839	874	5141	1103	508.75	1.215	72
1-Cyclopropylethanone	765-43-5	MS	871	881	3464	1126	544.50	0.910	69
Trans-2-pentenal	1576-87-0	MS, RI, ST	887	887	4466	1129	550.00	0.950	57
Trans-2-ethyl-2-butenal	63883-69-2	MS, RI	903	906	5826	1156	591.25	1.070	41
4-Methyl-4-penten-2-ol	2004-67-3	MS	873	883	6556	1178	624.25	1.155	56
5-Methylisoxazole	5765-44-6	MS	854	904	9107	1199	657.25	0.820	83
3-Methyl-2-butenal	107-86-8	MS, RI	922	935	5715	1199	657.25	0.940	59
1-Chloro-2-propanone	78-95-5	MS	915	916	9447	1209	673.75	0.780	49
Carbonic acid, ethyl isobutyl ester	0-00-0	MS	838	863	7776	1213	679.25	1.045	56
Furfural	98-01-1	MS, RI, ST	851	879	7237	1460	1064.25	0.780	95
1,1-Dimethyl-3-chloropropanol	1985-88-2	MS	874	933	8508	1471	1080.75	0.835	59
2-Acetylfuran	1192-62-7	MS, RI	635	858	6023	1501	1124.75	0.825	95
Benzaldehyde	100-52-7	MS, RI	884	886	6061	1520	1152.25	0.870	77
4-Methyl-benzaldehyde	104-87-0	MS, RI	935	935	3796	1645	1328.25	0.940	91
Neodecanoic acid	26896-20-8	MS	804	811	4468	2038	1691.25	0.695	88
Volatile metabolites that increased									
2-Butanol	78-92-2	MS, RI	920	921	4783	1021	398.75	0.800	45
3-Methyl-2-butanol	598-75-4	MS, RI	917	919	6239	1092	495.00	0.825	45
3-Pentanol	584-02-1	MS, RI, ST	906	916	6628	1106	514.25	0.830	59
2-Propen-1-ol	107-18-6	MS, RI	855	875	7402	1111	522.50	0.690	57
2-Pentanol	6032-29-7	MS, RI	918	921	5523	1118	533.50	0.825	45
3,3-Dimethylbutane-2-ol	464-07-3	MS, RI	831	845	5275	1122	539.00	0.875	57
2-Methyl-3-pentanol	565-67-3	MS, RI	931	932	8508	1152	585.75	0.905	59
Cyclopentanone	120-92-3	MS, RI	883	907	8047	1179	627.00	0.980	55
4-Penten-1-yl acetate	1576-85-8	MS, RI	816	817	6838	1190	643.50	1.065	43
3-Hexanol	623-37-0	MS, RI	797	856	5901	1192	646.25	0.900	55
4-Methyl-2-pentanol	108-11-2	MS, RI, ST	905	905	3802	1216	684.75	0.885	45
3,3-Dimethyl-1-butanol	624-95-3	MS, RI	814	839	3967	1252	742.50	0.850	69
2-Heptanol	543-49-7	MS, RI, ST	941	942	6955	1315	841.50	0.950	45
3-Hexen-1-ol	544-12-7	MS, RI	902	921	6325	1358	910.25	0.830	67
3-Methyl-1-hexanol	13231-81-7	MS, RI	881	895	3781	1414	995.50	0.890	70
Butylcyclopropane	930-57-4	MS	869	870	1741	1454	1056.00	0.895	56
3-Ethyl-1-pentyn-3-ol	6/9/6285	MS	821	831	4002	1478	1091.75	0.815	57
1-Octanol	111-87-5	MS, RI	800	902	2082	1557	1207.25	0.930	56
Butanoic acid	107-92-6	MS, RI, ST	869	870	8859	1622	1298.00	0.680	60
Pentanoic acid	109-52-4	MS, RI, ST	910	910	8007	1730	1430.00	0.665	60
à-Methylbenzyl alcohol	98-85-1	MS, RI	857	860	3018	1804	1507.00	0.720	79
Benzyl alcohol	100-51-6	MS, RI	910	911	7213	1869	1562.00	0.675	79
4-Methylbenzyl alcohol	589-18-4	MS, RI, ST	903	904	4285	1957	1630.75	0.695	79
2-Methylbenzyl alcohol	89-95-2	MS, RI	850	879	7041	1986	1652.75	0.685	104

[a] Chemical Abstracts Service registry number.
[b] MS: identification based on mass spectral match to the NIST 05 library with >800 similarity, RI: comparison with published retention indices on polyethylene glycol column phase, ST: mass spectral and retention index match to authentic standard.
[c] Mass spectral match quality based on comparison to the NIST 05 library; Similarity values based on spectral match with a perfect match = 1000; Reverse values represents the mass spectral match with masses not present in the reference spectrum removed; Probability refers to the uniqueness of the spectrum in comparison to that of similar compounds: higher values represent a more unique mass spectrum and greater likelihood of correct identification.
[d] Retention indices based on first dimension retention of components on a SOL–GEL-WAX (polyethylene glycol) column using SPME GCxGC–ToFMS.
[e] GCxGC peak retention information for each metabolite; t_{r1} (sec) represents the time in seconds for the compound to elute from the first dimension column and t_{r2} (sec) represents the time in seconds for the compound to elute from the second dimension column.
[f] Mass selected by ChromaTOF software during automated data processing to represent an interference free mass for each analyte; The unique mass for each component was used for calculation of peak areas.

reduced NaCl spoilage inocula. Since lactic acid was utilized in FCS to a similar degree by all three spoilage inocula (Fig. 1) and the primary goal was to identify metabolites related to anaerobic degradation of lactic acid in fermented cucumbers, this group of 46 metabolites was selected for manual inspection. The resulting 32 metabolites that decreased significantly as a result of spoilage by all three spoilage inocula, *L. buchneri*, commercial and reduced NaCl sources, are presented in Table 1. These compounds were mostly aldehydes and ketones and a few alcohols, hydrocarbons, and unidentified components. Clusters labeled 5, 6, 7, 10, and 11 represent groups of metabolites not present or in low abundance in FCS that were produced differentially by *L. buchneri*, commercial and reduced NaCl spoilage inocula. Cluster 9 was comprised of 33 metabolite peaks that were increased similarly in FCS by all three spoilage inocula. Three artifact peaks were removed by manual inspection, and the 30 volatile metabolites that increased during fermented cucumber spoilage are presented in Table 1. There were increases in a number of alcohols and butanoic and pentanoic acids. The decreases in aldehydes and ketones combined with increases in alcohols suggests alcohol dehydrogenase activity by these spoilage microorganisms. NAD dependent alcohol dehydrogenases (E.C. 1.1.1.1), aryl alcohol dehydrogenases (E.C. 1.1.1.90) and NADP dependent alcohol dehydrogenases (E.C. 1.1.1.2) are widely distributed in nature, and have been annotated in *L. buchneri* by gene sequence homology (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/). The conversion of an aldehyde or ketone to an alcohol by these types of enzymes would generate $NAD(P)^+$ and therefore, these reactions may act collectively as electron acceptors necessary for energy production from metabolism of other molecules.

The volatile metabolites that were increased or decreased by all three spoilage inocula were further analyzed over the time course of lactic acid utilization to determine which components may be related to this activity by spoilage bacteria in fermented cucumbers. Several of the changes in volatile compounds occurred within the first 9 d of anaerobic incubation ($P < 0.001$), which was prior to the onset of lactic acid degradation. These changes included decreases in acetone, butanal, 3-methylbutanal, 3,3-dimethyl-2-butanone, 2-methyl-2-butenal, 2-ethyl-2-butenal, 3-methyl-2-butenal, 2-pentenal, furfural, benzaldehyde, and 4-methylbenzaldehyde. Increases were observed in 2-butanol, 3-methyl-2-butanol, 3-pentanol, 2-propen-1-ol, 2-pentanol, 3,3-dimethylbutan-2-ol, 2-methyl-3-pentanol, 4-penten-1-yl acetate, 4-methyl-2-pentanol, 3-ethyl-1-pentyn-3-ol, butanoic and pentanoic acids, alpha-methylbenzyl alcohol, and 4-methylbenzyl alcohol. Only a few of the changes occurred during the time that lactic acid was being degraded ($P < 0.001$). These included decreases in acetaldehyde, methanol, acetonitrile, propanenitrile, and hexanal and increases in cyclopentanone, 3,3-dimethyl-1-butanol, and 2-methylbenzyl alcohol.

Non-targeted detection of nonvolatile spoilage metabolites was accomplished by chemical derivatization of samples to form volatile TMS-metabolites. TMS-metabolites were separated and detected using comprehensive GCxGC–ToFMS that employed a 50% phenyl (BPX-50) first dimension separation followed by a 5% phenyl (BPX-5) second dimension separation. The complexity of the TMS-derivatized samples is illustrated in Fig. 3 panel A. Despite the complexity, the derivatization reagent artifacts were clearly separated from metabolites in the second dimension, and separation of metabolite peaks was achieved with a relatively short modulation time of 2.75 s. The yellow box highlighted in panel A is expanded in view B to demonstrate the effectiveness of the BPX-50/BPX-5 column combination in resolving TMS-metabolites in fermented cucumbers. Panel C is a one dimensional representation of the TMS-glycerol peak that was captured in 13 slices. Although the peak shape of the TMS-glycerol in one dimension suggests a well-separated single component, panel D illustrates the separation of at least 8 components by the second dimension column at a single first dimension elution time. Further separation of peaks based on individual masses was achieved by the ChromaTOF® software deconvolution algorithm applied during data processing. Of the 4888 aligned peaks per sample, only 1033 peaks were tentatively identified by mass spectral match with >800 similarity to the Fiehn metabolomics library, indicating the need for continued development of mass spectral libraries for TMS-metabolites. Tentatively identified metabolites were from a wide variety of chemical classes, including amino acids, organic acids, sugars, sugar alcohols, nucleotides, amines, disaccharides and fatty acids.

More than 600 metabolite peaks (approximately 14% of total metabolite peaks) changed significantly with spoilage ($P < 0.01$) after 146 d of anaerobic incubation. Similar trends were observed in the clustering of these differentiating, nonvolatile metabolites as previously described for the volatile spoilage metabolites (Fig. 4). However, very few nonvolatile metabolites increased during spoilage. The identified metabolites that decreased consistently in FCS as a result of spoilage by both *L. buchneri* and mixed spoilage cultures are presented in Table 2. Of particular interest was the disappearance of citrulline from the fermented cucumber media.

Citrulline, a non-protein amino acid naturally present in cucumber (Fish and Bruton, 2010), had remained after primary fermentation and was metabolized by *L. buchneri* during anaerobic incubation. Citrulline is a known intermediate in the arginine deiminase pathway, which has been shown to function as an acid tolerance mechanism in some LAB (Budin-Verneuil et al., 2004; Casiano-Colón and Marquis, 1988; Rollan et al., 2003). The conditions for synthesis and activity of the arginine deiminase pathway enzymes in *L. buchneri* NCDO110 showed that this system is not glucose repressed (Manca de Nadra et al., 1986a), and two of the key enzymes, ornithine transcarbamylase and carbamate kinase were purified and characterized from this strain (Manca de Nadra et al., 1986b, 1987). Together, ornithine transcarbamylase and carbamate kinase facilitate the conversion of citrulline to ornithine with concomitant production of 1 molecule of ATP and ammonia, resulting in energy production and deacidification of the cell cytoplasm. Liu et al. (1995) found relatively high activities of these enzymes in *L. buchneri* strain CUC-3 compared to other LAB of wine origin. In this study, the metabolism of citrulline in fermented cucumber media occurred prior to the onset of lactic acid degradation, indicating that it is a preferred substrate for anaerobic spoilage organisms in fermented cucumber. This metabolic pathway in *L. buchneri* may be serving a vital role in its unique ability to survive and grow during the post-fermentation bulk storage of fermented cucumbers. In fact, addition of citrulline to simulated wine media increased growth yields of *L. buchneri* CUC-3 at acidic pHs of 3.3–3.7 (Terrade and Mira de Orduña, 2009), which are similar to the terminal pH of cucumber fermentations, 3.2–3.6 (Pérez-Díaz et al., 2014). Since citrulline is present in cucurbits and does not appear to be metabolized by most homofermentative LAB (Liu et al., 1995) during the primary fermentation of cucumbers, we propose that depletion of citrulline may be a biomarker for the onset of spoilage-associated secondary fermentation of cucumbers by *L. buchneri* and closely related species.

Similarly to what was observed in the volatile composition changes in FCS, the majority of the nonvolatile metabolite changes preceded lactic acid utilization. HCA of TMS-metabolite peaks during the timecourse of anaerobic lactic acid utilization revealed that sample treatments were clustered by their metabolite profiles according to the inoculum source that was used rather than the time course of lactic acid utilization (data not shown). The exception to this was the numerous nonvolatile metabolite changes that occurred in the first 9 d of anaerobic incubation, prior to lactic acid utilization. Several of these metabolites were carbohydrates (Table 2). Collectively, these mono- and disaccharides present in levels undetectable by traditional HPLC methods (<2 mM) may be serving as energy sources for the growth of spoilage organisms after primary fermentation is complete, which would account for the small increase in cell density that has been observed during this early period of spoilage (Johanningsmeier et al., 2012). The unique carbohydrate utilization patterns of selected *L. buchneri* strains have been studied for the conversion of lignocellulosic biomass

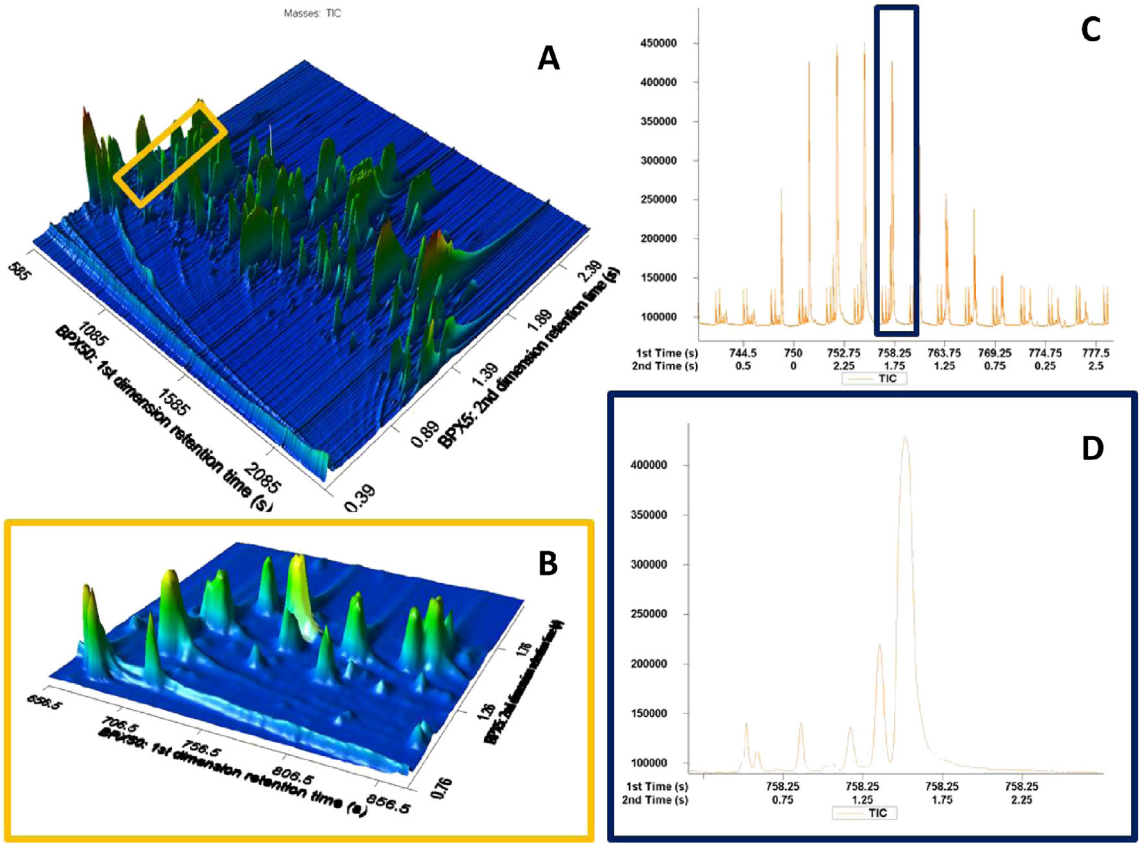

Fig. 3. Two-dimensional separation of TMS-metabolites of fermented cucumber medium by GCxGC–ToFMS.

to ethanol (Liu et al., 2008). Among the unique capabilities of strain NRRL B-0929, was the ability to ferment D-trehalose and D-cellobiose. It was also noted by Heinl et al. (2012) that *L. buchneri* CD034 isolated from silage had acquired additional genes encoding enzymes that facilitate the utilization of plant cell walls as substrates for metabolism. Interestingly, it seems that this capability by certain strains of *L. buchneri* may be involved to some degree in the spoilage of fermented cucumbers. In this study, both D-trehalose and D-cellobiose were utilized in the fermented cucumber media prior to the onset of lactic acid degradation. The metabolism of these carbohydrates may explain the small increases in cell density that have been observed prior to lactic acid utilization (Johanningsmeier et al., 2012) and the long lag time between inoculation of spoilage organisms and the initiation of lactic acid metabolism (Fig. 1). Catabolite repression is a common biological phenomenon; for example, the ability of bacteria from human feces to metabolize lactate to butyrate was completely inhibited in the presence of glucose (Duncan et al., 2004). More specifically, the ability of *L. buchneri* to anaerobically metabolize lactic acid to acetic acid and 1,2-propanediol was demonstrated using cell cultures that were grown in glucose limited conditions (Oude Elferink et al., 2001). Therefore, we hypothesize that the ability of *L. buchneri* strains to degrade lactic acid in fermented cucumbers will depend on the ability to metabolize specific carbohydrates that remain after primary fermentation is complete and/or the presence of other microbiota that can metabolize the residual carbohydrate energy sources. This new knowledge and the ability to detect biochemical changes that precede lactate utilization provides an opportunity to identify indicator compounds for the initiation of spoilage prior to any significant product degradation and to develop improved technologies to prevent lactic acid degradation during bulk storage of fermented vegetables. The regulatory mechanisms that influence lactic acid utilization by *L. buchneri* are currently unknown. Future research in this area

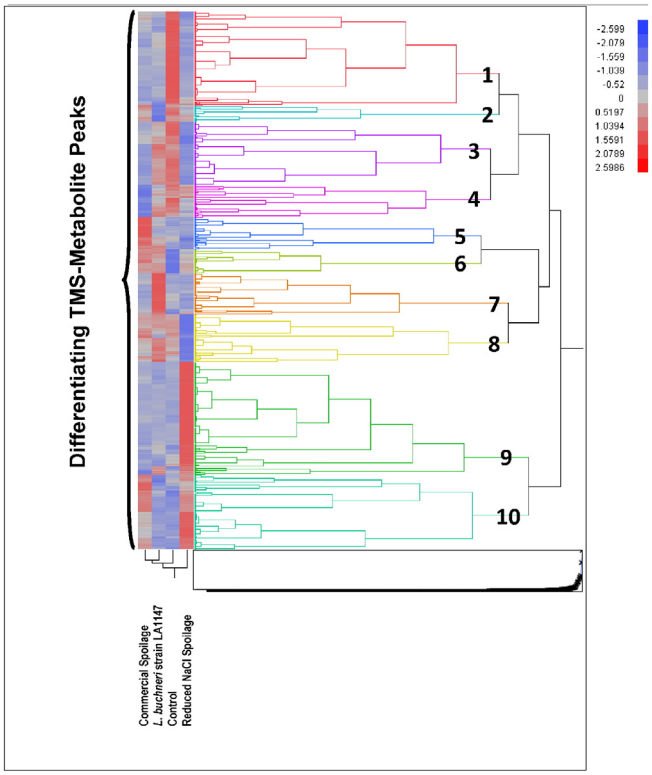

Fig. 4. Hierarchial clustering of 684 TMS-metabolite peaks that changed significantly ($P < 0.01$) in fermented cucumber medium after 146 d anaerobic incubation with spoilage organisms.

Table 2
Nonvolatile metabolites that decreased significantly ($P < 0.01$) during anaerobic spoilage of fermented cucumber medium by *L. buchneri* and spoilage cultures from reduced NaCl and commercial fermentation sources.

Metabolite ID	CAS[a] registry #	Method of identification[b]	Mass spectral match quality[c]			RI[d]	Retention time[e]		Mass[f]
			Similarity	Reverse	Probability		t_{r1} (sec)	t_{r2} (sec)	
D-Alanyl-D-alanine	923-16-0	MS	844	844	7299	1065	483.25	0.820	116
Lactic acid	50-21-5	MS	921	939	9796	1180	653.57	0.990	43
Isoleucine	73-32-5	MS, ST	876	880	7564	1296	833.71	1.420	45
Uracil	66-22-8	MS	923	938	9832	1461	1088.25	1.110	99
Lyxose	1114-34-7	MS, ST	963	963	4741	1617	1312.86	1.590	103
Xylose	6763-34-4	MS, ST	871	871	3946	1625	1324.66	1.570	103
Fructose	57-48-7	MS, ST	968	970	4528	1784	1534.88	1.750	103
Citrulline (TMS derivative 1)	372-75-8	MS, ST	934	950	9768	1879	1656.09	1.350	157
Citrulline (TMS derivative 2)	372-75-8	MS, ST	841	865	9451	1890	1672.04	1.170	70
Glucose	50-99-7	MS, ST	918	918	2419	1892	1576.25	1.790	206
Palmitic acid	57-10-3	MS, ST	955	973	9035	2104	1919.09	1.420	87
Cellobiose	528-50-7	MS, ST	893	893	2150	2518	2295.73	1.390	204
Trehalose	99-20-7	MS, ST	895	898	4800	2558	2317.74	1.350	361
Gentiobiose	554-91-6	MS, ST	929	929	3903	2662	2369.06	1.250	204
Adenosine	58-61-7	MS	770	775	9566	2776	2417.77	0.990	236
5′-Methylthioadenosine	2457-80-9	MS	864	870	9815	3067	2559.81	1.190	236

[a] Chemical Abstracts Service registry number.
[b] MS: identification based on mass spectral match to the LECO-Fiehn Rtx05 library with >800 similarity, ST: mass spectral and retention index match to authentic standard.
[c] Mass spectral match quality based on comparison to the LECO-Fiehn Rtx05 library; Similarity values based on spectral match with a perfect match = 1000; Reverse values represents the mass spectral match with masses not present in the reference spectrum removed; Probability refers to the uniqueness of the spectrum in comparison to that of similar compounds: higher values represent a more unique mass spectrum and greater likelihood of correct identification.
[d] Retention indices of the trimethylsilylated metabolite based on first dimension retention of components on a BPX50 (50% phenyl–50% polysilphenylene-siloxane) column using GCxGC–ToFMS.
[e] GCxGC peak retention information for each metabolite; t_{r1} (sec) represents the time in seconds for the compound to elute from the first dimension column and t_{r2} (sec) represents the time in seconds for the compound to elute from the second dimension column.
[f] Mass selected by ChromaTOF software during automated data processing to represent an interference free mass for each analyte; The unique mass for each component was used for calculation of peak areas.

would allow better control of spoilage in fermented foods and the enhancement of this activity in silage where it yields a desirable outcome.

Acknowledgments

The authors wish to express their appreciation to Dr. Jason Osborne of the NC State Statistics Department for statistical consulting. We also gratefully acknowledge that this investigation was partially supported by Pickle Packers International Inc., Washington, DC.

References

Adahchour, M., Wiewel, J., Verdel, R., Vreuls, R.J.J., Brinkman, U.A.Th., 2005. Improved determination of flavour compounds in butter by solid-phase (micro)extraction and comprehensive two-dimensional gas chromatography. J. Chromatogr. A 1086, 99–106.
Barsch, A., Patschkowski, T., Niehaus, K., 2004. Comprehensive metabolite profiling of *Sinorhizobium meliloti* using gas chromatography–mass spectrometry. Funct. Integr. Genomics 4, 219–230.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Methodol. 57, 289–300.
Bölling, C., Fiehn, O., 2005. Metabolite profiling of *Chlamydomonas reinhardtii* under nutrient deprivation. Plant Physiol. 139, 1995–2005.
Breidt, F., Medina-Pradas, E., Wafa, D., Pérez-Díaz, I.M., Franco, W., Huang, H., Johanningsmeier, S.D., Kim, J., 2013. Characterization of cucumber fermentation spoilage bacteria by enrichment culture and 16S rDNA cloning. J. Food Sci. 78 (3), M470–M476.
Budin-Verneuil, A., Maguin, E., Auffray, Y., Ehrlich, S.D., Pichereau, V., 2004. An essential role for arginine catabolism in the acid tolerance of *Lactococcus lactis* MG1363. Dairy Sci. Technol. 84 (1–2) (61–38).
Caldwell, J.M., Brown, J., Breidt, F., 2013. *Pectinatus sottaceto* sp. nov., isolated from commercial pickle spoilage tank. Int. J. Syst. Evol. Microbiol. 63 (10), 3609–3616.
Casiano-Colón, A., Marquis, R.E., 1988. Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Appl. Environ. Microbiol. 1318–1324.
Duncan, S.H., Louis, P., Flint, H.J., 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl. Environ. Microbiol. 70 (10), 5810–5817.
Fish, W.W., Bruton, B.D., 2010. Quantification of L-citrulline and other physiologic amino acids in watermelon and selected cucurbits. Cucurbitaceae 2010 Proceedings, Charleston, South Carolina, pp. 152–154.
Fleming, H.P., Daeschel, M.A., McFeeters, R.F., Pierson, M.D., 1989. Butyric acid spoilage of fermented cucumbers. J. Food Sci. 54 (3), 636–639.
Fleming, H.P., Humphries, E.G., Thompson, R.L., McFeeters, R.F., 2002. Acidification of commercially fermented cucumbers in bulk tanks to increase microbial stability. Pickle Pak. Sci. 8 (1), 38–43.
Franco, W., Pérez-Díaz, I.M., 2012. Role of selected oxidative yeasts and bacteria in cucumber secondary fermentation associated with spoilage of the fermented fruit. Food Microbiol. 32, 338–344.
Franco, W., Pérez-Díaz, I.M., 2013. Microbial interactions associated with secondary cucumber fermentation. J. Appl. Microbiol. 114, 161–172.
Franco, W., Pérez-Díaz, I.M., Johanningsmeier, S.D., McFeeters, R.F., 2012. Characteristics of spoilage-associated secondary cucumber fermentation. Appl. Environ. Microbiol. 78 (4), 1273–1284.
Gullberg, J., Jonsson, P., Nordström, A., Sjöström, M., Moritz, T., 2004. Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/mass spectrometry. Anal. Biochem. 331, 283–295.
Guo, X., Lidstrom, M.E., 2008. Metabolite profiling analysis of *Methylobacterium extorquens* AM1 by comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry. Biotechnol. Bioeng. 99 (4), 929–940.
Heinl, S., Wibberg, D., Eikmeyer, F., Szczepanowski, R., Blom, J., Linke, B., Goesmann, A., Grabherr, R., Schwab, H., Pühler, A., Schlüter, A., 2012. Insights into the completely annotated genome of *Lactobacillus buchneri* CD034, a strain isolated from stable grass silage. J. Biotechnol. 161, 153–166.
Holzer, M., Mayrhuber, E., Danner, H., Braun, R., 2003. The role of *Lactobacillus buchneri* in forage preservation. Trends Biotechnol. 21 (6), 282–287.
Hope, J.L., Prazen, B.J., Nilsson, E.J., Lidstrom, M.E., Synovec, R.E., 2005. Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry detection: analysis of amino acid and organic acid trimethylsilyl derivatives, with application to the analysis of metabolited in rye grass samples. Talanta 65, 380–388.
Johanningsmeier, S.D., McFeeters, R.F., 2011. Detection of volatile spoilage metabolites in fermented cucumbers using nontargeted comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GCxGC–ToFMS). J. Food Sci. 76 (1), C168–C177.
Johanningsmeier, S.D., McFeeters, R.F., 2013. Metabolism of lactic acid in fermented cucumbers by *Lactobacillus buchneri* and related species, potential spoilage organisms in reduced salt fermentations. Food Microbiol. 35 (2), 129–135.
Johanningsmeier, S.D., Franco, W., Pérez-Díaz, I.M., McFeeters, R.F., 2012. Influence of sodium chloride, pH, and lactic acid bacteria on anaerobic lactic acid utilization during fermented cucumber spoilage. J. Food Sci. 77 (7), M397–M404.
Kim, J.-H., Breidt, F., 2007. Development of preservation prediction chart for long term storage of fermented cucumber. Kor. J. Life Sci. 17 (12), 1616–1621.
Koek, M.M., Muilwijk, B., van der Werf, M.J., Hankemeier, T., 2006. Microbial metabolomics with gas chromatography/mass spectrometry. Anal. Chem. 78, 1272–1281.
Koek, M.M., Muilwijk, B., van Stee, L.L.P., Hankemeier, T., 2008. Higher mass loadability in comprehensive two-dimensional gas chromatography–mass spectrometry for improved analytical performance in metabolomics analysis. J. Chromatogr. A 1186, 420–429.
Kusano, M., Fukushima, A., Kobayashi, M., Hayashi, N., Jonsson, P., Moritz, T., Ebana, K., Saito, K., 2007. Application of a metabolomic method combining one-dimensional

and two-dimensional gas chromatography–time-of-flight/mass spectrometry to metabolic phenotyping of natural variants in rice. J. Chromatogr. B 855, 71–79.
Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., Fernie, A.R., 2006. Gas chromatography mass spectrometry-based metabolite profiling in plants. Nat. Protoc. 1 (1), 387–396.
Liu, S.-Q., Pritchard, G.G., Hardman, M.J., Pilone, G.J., 1995. Occurrence of arginine deiminase pathway enzymes in arginine catabolism by wine lactic acid bacteria. Appl. Environ. Microbiol. 61 (1), 310–316.
Liu, S., Skinner-Nemec, K.A., Leathers, T.D., 2008. *Lactobacillus buchneri* strain NRRL B-30929 converts a concentrated mixture of xylose and glucose into ethanol and other products. J. Ind. Microbiol. Biotechnol. 35, 75–81.
Manca de Nadra, M.C., Nadra Chaud, C.A., de Ruiz, Pesce, Holgado, A., Oliver, G., 1986a. Synthesis of the arginine dihydrolase pathway enzymes in *Lactobacillus buchneri*. Curr. Microbiol. 13, 261–264.
Manca de Nadra, M.C., Nadra Chaud, C.A., de Ruiz, Pesce, Holgado, A., Oliver, G., 1986b. Carbamate kinase of *Lactobacillus buchneri* NCDO110. I. Purification and properties. Biotechnol. Appl. Biochem. 8 (1), 46–52.
Manca de Nadra, M.C., de Ruiz, Pesce, Holgado, A.A., Oliver, G., 1987. Carbamate kinase of *Lactobacillus buchneri* NCDO110. II. Kinetic studies and reaction mechanism. Biotechnol. Appl. Biochem. 9 (2), 141–145.
McFeeters, R.F., Barish, A.O., 2003. Sulfite analysis of fruits and vegetables by high-performance liquid chromatography (HPLC) with ultraviolet spectrophotometric detection. J. Agric. Food Chem. 51, 1513–1517.
Mohler, R.E., Dombek, K.M., Hoggard, J.C., Young, E.T., Synovec, R.E., 2006. Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry analysis of metabolites in fermenting and respiring yeast cells. Anal. Chem. 78 (8), 2700–2709.
O'Hagan, S., Dunn, W.B., Brown, M., Knowles, J.D., Kell, D.B., 2005. Closed-loop, multiobjective optimization of analytical instrumentation: gas chromatography/time-of-flight mass spectrometry of the metabolomes of human serum and of yeast fermentations. Anal. Chem. 77, 290–303.
Oude Elferink, S.J.W.H., Krooneman, J., Gottschal, J.C., Spoelstra, S.F., Faber, F., Driehuis, F., 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. Appl. Environ. Microbiol. 67 (1), 125–132.
Pérez-Díaz, I.M., Breidt, F., Buescher, R.W., Arroyo-Lopez, F.N., Jimenez-Diaz, R., Bautista-Gallego, J., Garrido-Fernandez, A., Yoon, S., Johanningsmeier, S.D., 2014. Fermented and acidified vegetables. In: Pouch Downes, F., Ito, K.A. (Eds.), Compendium of Methods for the Microbiological Examination of Foods, 5th ed. American Public Health Association (Chapter 51).
Rocha, S.M., Coelho, E., Zrostlíková, J., Delgadillo, I., Coimbra, M.A., 2007. Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry of monoterpenoids as a powerful tool for grape origin traceability. J. Chromatogr. A 1161, 292–299.
Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N., Willmitzer, L., 2000. Simultaneous analysis of metabolites in potato tuber by gas chromatography–mass spectrometry. Plant J. 23 (1), 131–142.
Rollan, G., Lorca, G.L., Font de Valdez, G., 2003. Arginine catabolism and acid tolerance response in *Lactobacillus reuteri* isolated from sourdough. Food Microbiol. 20, 313–319.
Rudell, D.R., Mattheis, J.P., Curry, E.A., 2008. Prestorage ultraviolet-white light irradiation alters apple peel metabolome. J. Agric. Food Chem. 56, 1138–1147.
Shellie, R., Marriott, P., Morrison, P., 2001. Concepts and preliminary observations on the triple-dimensional analysis of complex volatile samples by using GCxGC–TOFMS. Anal. Chem. 73, 1336–1344.
Strelkov, S., von Elstermann, M., Schomburg, D., 2004. Comprehensive analysis of metabolites in *Corynebacterium glutamicum* by gas chromatography/mass spectrometry. Biol. Chem. 385, 853–861.
Terrade, N., Mira de Orduña, R., 2009. Arginine and citrulline do not stimulate growth of two *Oenococcus oeni* strains in wine. FEMS Microbiol. Lett. 290, 98–104.
van der Werf, M.J., Overcamp, K.M., Muilwijk, B., Koek, M.M., van der Werff-van der Vat, B.J.C., Jellema, R.H., Coulier, L., Hankemeier, T., 2008. Comprehensive analysis of the metabolome of *Pseudomonas putida* S12 grown on different carbon sources. Mol. BioSyst. 4, 315–327.
Weckworth, W., Loureiro, M.E., Wenzel, K., Fiehn, O., 2004. Differential metabolic networks unravel the effects of silent plant phenotypes. PNAS 101 (20), 7809–7814.
Welthagan, W., Shellie, R.A., Spranger, J., Ristow, M., Zimmermann, R., Fiehn, O., 2005. Comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GCxGC–TOF) for high resolution metabolomics: biomarker discovery on spleen tissue extracts of obese NZO compared to lean C57BL/6 mice. Metabolomics 1 (1), 65–73.
Wishart, D.S., 2008. Metabolomics: applications to food science and nutrition research. Trends Food Sci. Technol. 19, 482–493.
Zörb, C., Langenkämper, G., Betsche, T., Niehaus, K., Barsch, A., 2006. Metabolite profiling of wheat grains (*Triticum aestivum* L.) from organic and conventional agriculture. J. Agric. Food Chem. 54, 8301–8306.