Food Microbiology 77 (2019) 10–20

Contents lists available at ScienceDirect

Food Microbiology

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

Assessment of the non-lactic acid bacteria microbiota in fresh cucumbers and commercially fermented cucumber pickles brined with 6% NaCl☆

Ilenys M. Pérez-Díaz[a,*], Janet S. Hayes[a], Eduardo Medina[b,c], Ashlee M. Webber[a], Natasha Butz[d], Allison N. Dickey[e], Zhongjing Lu[f], Maria A. Azcarate-Peril[d]

[a] USDA-Agriculture Research Service, SEA, Food Science Research Unit, 322 Schaub Hall, North Carolina State University, Raleigh, NC, 27695, USA
[b] Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Raleigh, NC, 27695, USA
[c] Department of Food Biotechnology, Instituto de La Grasa, (CSIC), Universitary Campus Pablo de Olavide, Building 46, Crta Utrera Km 1, Seville, 41013, Spain
[d] Microbiome Core Facility, Department of Cell Biology and Physiology, School of Medicine, University of North Carolina, Chapel Hill, NC, 27599, USA
[e] Bioinformatics Research Center, North Carolina State University, Raleigh, NC, 27695, USA
[f] Department of Molecular and Cellular Biology, Kennesaw State University, Kennesaw, GA, 30144, USA

ARTICLE INFO

Keywords:
Aerobic bacteria
Gram-negative
Cucumber fermentation
Microbiome

ABSTRACT

Limited documentation of the cucumber fermentation microbiome has impeded the understanding of the role of microbes on the quality of finished products. We characterized the microbiome of fresh and fermented cucumber samples using culture dependent and independent techniques, with an emphasis on the non-lactic acid bacteria (non-LAB) population. Insubstantial microbiome variations were observed among fresh cucumber types with *Rhizobium* (31.04%), *Pseudomonas* (14.08%), *Pantoea* (9.25%), *Stenotrophomonas* (6.83%), and *Acinetobacter* (6.5%) prevailing. The relative abundance of LAB remained below 0.4% and 4.0% on fresh cucumbers and day 3 of the fermentations brined with 6% sodium chloride, respectively. Fermentation cover brine samples collected on day 1 harbored *Pseudomonas*, *Pantoea*, *Stenotrophomonas*, *Acinetobacter*, *Comamonas*, *Wautersiella*, *Microbacterium*, *Flavobacterium*, *Ochrobactrum* and the *Enterobacteriaceae*, *Citrobacter*, *Enterobacter* and *Kluyvera*. Plate counts for presumptive *Klebsiella* and *Pseudomonas* from fermentation cover brine samples reached 2.80 ± 0.36 and 2.78 ± 0.83 log of CFU/mL, respectively, in 30% and 60% of the nine tanks scrutinized with selective media. Both genera were found in cover brine samples with pH values at 4.04 ± 0.15. We aim at elucidating whether the low relative abundance of non-LAB in commercial cucumber fermentations, in particular *Pseudomonas* and *Enterobacteriaceae*, impacts the quality of fermented cucumbers.

1. Introduction

While lactic acid bacteria (LAB) prevail in commercial cucumber fermentations (Pérez-Díaz et al., 2016) and are credited with the inhibition of the acid sensitive microbiota, the microbiome of fresh and fermented cucumbers, in particular the population of Gram-negative aerobic bacteria, remains largely uncharacterized. The limited knowledge of the microbial diversity in fresh and fermented cucumbers has hindered the understanding of the impact of microbes on the quality of finished pickles and impedes the understanding of a number of fermented cucumber defects. *Enterobacter cloacae* is perhaps the most frequently isolated *Enterobacteriaceae* from spoiled cucumber fermentations. Twenty out of 29 cultures isolated from gas producing active cucumber fermentations were identified as *E. cloacae* which grew at an optimum pH value of 5.3 (ranging from 4.25 to 8.85) (Etchells, 1941; Veldhuis and Etchells, 1939). The bacterium was implicated in hydrogen production in cucumber fermentations brined with 5% sodium chloride (NaCl) or above (Etchells, 1941). This Gram-negative facultative anaerobe was also found in spoiled commercial cucumber fermentations brined with 5.8% NaCl at a pH of 3.4, which were characterized by the production of propionic and butyric acids and the reduction of lactic acid, conducive to an increase in pH (Franco et al., 2012). The putrefactive bacterium was additionally isolated from spoiled and laboratory scaled NaCl-free cucumber fermentations brined with 1.1% calcium chloride ($CaCl_2$) (Franco and Pérez-Díaz, 2013). *E. cloacae* is able to produce propionic acid at a pH of 4.6 in cucumber

☆ 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. USDA is an equal opportunity provider and employer.

* Corresponding author. USDA-Agriculture Research Service, Food Science Research Unit, 322 Schaub Hall, Box 7624, NCSU, Raleigh, NC, 27695, USA.
E-mail address: Ilenys.Perez-Diaz@ars.usda.gov (I.M. Pérez-Díaz).

https://doi.org/10.1016/j.fm.2018.08.003
Received 9 May 2018; Received in revised form 31 July 2018; Accepted 2 August 2018
Available online 07 August 2018
0740-0020/

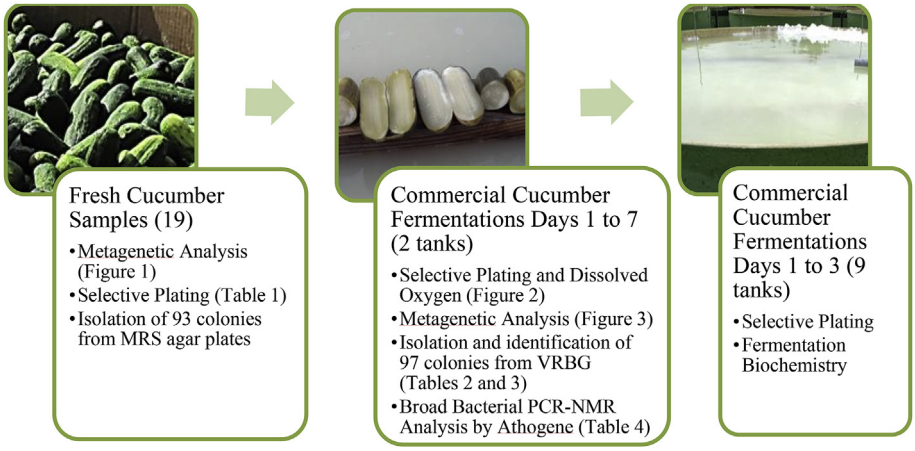

Fig. 1. Schematic representation of the experimental approach.

juice and the presence of yeasts enhances its survival at a pH of 3.2 in the same matrix (Franco and Pérez-Díaz, 2013). In the absence of LAB, the *Enterobacteriaceae* have been found to proliferate, causing internal cucumber bloating and an increase in pH conducive to putrefaction (Samish et al., 1963, 1957). Conversely, most *Enterobacteriaceae* are significantly suppressed by the acid produced by LAB during fermentations, if the vegetables are not blanched prior to brining (Garrido-Fernández et al., 1997; Samish et al., 1963).

Evidently, the presence of certain Gram-negative bacteria in cucumber fermentations seem to have more known negative than positive implications on the stability and quality of the brined fruits. However, a comprehensive compositional view of the indigenous microbiota in fresh and fermented cucumbers, particularly the population of non-LAB, may help to identify ways to manipulate the microbiome to produce desirable effects. Thus, it was the objective of this study to apply current microbial identification techniques to define the composition of the population of the non-LAB present in fresh cucumbers and modern commercial cucumber fermentations (Fig. 1). Nineteen fresh cucumber samples representing four commercial types and two commercial cucumber fermentations at the initial stage (day 1 through day 7) were studied using culture dependent and independent techniques, such as total and selective plating, biochemical characterization and 16S rDNA library PCR amplification and sequencing. The composition of the indigenous microbiota present in fresh cucumbers that is able to proliferate in Lactobacilli MRS (MRS) agar was also scrutinized to gain an understanding of the abundance of lactobacilli in such a fermentation substrate. The non-LAB population found in fermentation cover brine samples collected on days 1–3 from an additional nine commercial tanks was also studied using selective plating such as Violet Bile Salt agar, *Pseudomonas* Isolation agar, HiChrome *Klebsiella* agar, Leeds *Acinetobacter* Medium and McConkey agar to confirm the presence of selected microbes in commercial fermentations.

2. Materials and methods

2.1. Fresh cucumber samples processing and analysis

2.1.1. Fresh cucumber samples description and preparation

Nineteen fresh cucumber samples, including four different types, were collected from nearby grocery stores during the 2007 growing season and brought to the laboratory for immediate processing. Among the 19 cucumber samples there were six from Long English cucumbers, two from Mini cucumbers, six from Pickling cucumbers, and five from Super Select cucumbers. Fresh cucumbers were sliced using aseptic techniques and blended using a Waring Commercial Blender 700S (Torrington, CT, USA) equipped with a sterilized vessels for 90 s at medium speed. Cucumber slurries were homogenized using a Seward Stomacher 400 (Bohemia, NY, USA) in 6″ x 4.5” filter stomacher bags for 1 min at maximum speed.

2.1.2. Microbial analysis of fresh cucumber samples

The filtered homogenized juice was aseptically collected and serially diluted in a 0.85% NaCl solution prior to spiral plating using an Autoplate 4000 automated plater (Spiral Biotech, Norwood, MA, USA). Full strength cover brine samples and samples diluted 100 and 10,000-fold were used for spiral plating. Enumeration of total aerobic microbes, yeasts and molds and fecal coliforms was conducted from three types of plates: Plate Count agar (PCA; Difco 247940, Becton Dickinson and Co., Franklin Lakes, NJ, USA), Yeast and Mold agar (YMA; Difco 271120, Becton Dickinson and Co.) supplemented with 0.01% chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) and 0.01% chlortetracycline (Sigma-Aldrich) to inhibit bacterial growth, and Violet Red Bile (VRBG, Difco 211695, Becton Dickinson and Co.) agar supplemented with 1% glucose, respectively. Agar plates were incubated aerobically at 30 °C for 48 h, except for VRBG plates, which were incubated at 35 °C ± 2. LAB were enumerated using Lactobacilli MRS agar (Difco 288130, Becton Dickinson and Co.), supplemented with 1% of a 0.1% cycloheximide solution (Oxoid, Basingstoke, UK) to prevent the growth of yeasts. MRS agar plates were incubated anaerobically using a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, MI, USA) at 30 °C for 48 h. Actual plate counts were obtained using a Flash and Go automated plate colony counter (Neutec Group, Inc., Barcelona, Spain).

2.1.3. Isolation and identification of presumptive lactobacilli colonies from MRS agar plates inoculated with fresh cucumber samples homogenate

A total of 150 isolated colonies were picked from MRS agar plates inoculated with fresh cucumber homogenate and streaked on MRS agar plates for purification. Of the colonies streaked, 14% did not subsequently grow on MRS agar plates and several others could not be identified. Only 93 cultures were identified out of 150 streaked for purity. The 93 colonies purified included 27 ± 2 collected from MRS plates inoculated with each fresh cucumber type, except for mini-cucumbers with nine representative colonies. Each purified colony was transferred to MRS broth supplemented with 15% glycerol (v/v) (Cat No. G5516, Sigma Aldrich) for the preparation of frozen stocks.

Purified cultures were transferred from frozen stocks to 1 mL of MRS broth, individually, and incubated at 30 °C ± 2 for 48–72 h under anaerobic conditions using a Coy Chamber (Coy Laboratory Products, Inc.) to obtain bacterial pellets for DNA extraction. Total genomic bacterial DNA was obtained using an InstaGene Matrix DNA extraction kit (Bio-Rad Laboratories, Hercules, CA, USA) used following the manufacturer instructions. Extracted DNA was used for the partial amplification of the 16S rDNA gene for the purpose of sequencing and

identification. The Polymerase Chain Reaction (PCR) amplification mixture contained 2X master mix (Bio-Rad), 10 μL of the resulting total genomic DNA extracted from each of the 93 bacterial isolates, and 0.25 μM of primers 8f (5-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Wilson et al., 1990). The PCR amplification steps consisted of 1 cycle of 4 min at 94 °C followed by 25 cycles of 1 min at 94 °C, 2 min at 50–60 °C, and 2 min at 72 °C, with a final extension step of 7 min at 72 °C. Variations in the annealing temperature between 50 and 60 °C were imperative in order to get an amplicon with all the different extracted DNA samples. These PCR amplifications were an iterative process. The amplicons were stored at 4 °C until sequenced by Eton Bioscience Inc. (Durham, NC, USA). Sequence data were formatted and analyzed using the BioEdit software (www.mbio.ncsu.edu/bioedit). Only bases that had quality scores greater than or equal to 20 were used for the alignment. The sequences obtained were subjected to the basic local alignment search tool (BLAST) (Altschul et al., 1990; Benson et al., 1997) using the 16S ribosomal RNA sequence database to determine the identity of the isolates. Sequences can be accessed via NCBI through accession numbers: MH062673 to MH062740.

2.1.4. Preparation and sequencing of the fresh cucumbers 16S rDNA library

The fresh cucumber juices derived from the homogenization step described above (in the fresh cucumber samples description and preparation section) were used for DNA extraction. One mL aliquots were centrifuged (Sorvall, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 10,621 rcf (9.5 rad) for 10 min at 21 °C. The pellets were re-suspended in 490 μL sterile NaCl solution (0.85%) prior to DNA extraction using a Power Soil DNA Isolation Kit (12888, MoBio Laboratories, Inc., Carlsbad, CA, USA) with proteinase K, according to the manufacturer instructions. Extracted DNAs were stored at −20 °C until use. DNA concentrations were quantified using PicoGreen dsDNA reagent (Invitrogen, Life Technologies, Carlsbad, CA) on a 96-well plate reader and mixed at equimolar concentrations. The 16S rDNA gene regions V3 to V4 were amplified by PCR from the total genomic DNA samples using the S-D-Bact-0341-b-S-17/S-*d*-Bact-0785-a-A-21 primer pair with an amplicon size of 464 bp (Klindworth et al., 2012; also known as Bakt_341F/Bakt_805R primer pair designed by Sinclair et al. (2015)). Primers were barcoded as described by the manufacturer for the Illumina MiSeq sequencing technology. Sequencing services were obtained from the Carver Biotech Laboratory at the WM Keck Center for Comparative and Functional Genomics (Chicago, IL, USA). The SRA accession number for the raw fastq files for the 19 samples processed is SRP132735 and were associated with the Bio-project titled “Assessment of the Microbiota in Fresh Cucumber Samples” with code number PRJNA433862.

2.1.5. Processing of the 16S rDNA amplicon sequences data corresponding to fresh cucumber samples

The reads from the 19 fresh cucumber samples were quality-trimmed using Trimmomatic (version 0.36), and any reads with lengths less than 36 bp were removed (Bolger et al., 2014). Using MacQIIME 1.9.1–20150604 (Caporaso et al., 2010a), primer sequences were removed, the reads were merged using fastq-join with the default parameters (Aronesty, 2011), and merged reads with Phred quality scores less than 20 were removed. Of the starting sequences for the 19 samples, 77.0% ± 2.4 remained with a median length of 405 bp after the QC and merging steps. VSearch (Rognes et al., 2016) was used to de-replicate the sequences and remove singletons, sort on sequence abundance and cluster the sequences at 97%. The most abundant read from each cluster was selected as the cluster centroid and with VSearch, chimeras were removed from the list of centroids. The remaining sequences served as a set of representative sequences. To construct a phylogenetic tree for downstream alpha diversity analyses, PYNAST was used to align the sequences using the SILVA (version 128) core alignment sequences (Caporaso et al., 2010b). The alignments were filtered on the entropy_threshold (0.10) and the allowed_gap_frac (0.80) parameters. The read sequences were mapped to the representative sequences to generate the Orthologue Taxonomic Units (OTUs) table. Taxonomy was assigned to the OTUs using the SILVA (version 128) database (Quast et al., 2013; Yilmaz et al., 2014). The OTU table was filtered to remove any OTUs that represented less than 0.005% of the total sequences. In addition, OTUs that corresponded to chloroplasts or mitochondria were removed. The average number of reads remaining for the 19 samples was then 5933 reads (15.6% of the reads that remained after the QC and merging steps). MacQIIME 1.9.1–20150604 was used to calculate the alpha diversity metrics including Phylogenetic Diversity (PD), Chao1, and the Observed OTUs count to assess the sampling depth. The phylogenetic tree used in the alpha diversity analyses was produced with FastTree (Price et al., 2009). Based on the resulting alpha diversity plots, a rarefaction level of 1000 sequences per sample was selected. Samples with fewer than 1000 sequences were not considered for statistical analysis, which raised the average number of reads per sample to 8474 and reduced the total number of samples to 13. The relative abundances for the 13 samples are shown in Fig. 1 and the number of reads in the samples along with the alpha diversity values for PD, Chao1, and the Observed OTUs are shown in Table S1.

2.2. Commercial cucumber fermentations and fermentation cover brine samples collection, processing and analysis

2.2.1. Commercial scale fermentations carried out by processors

Commercial scale fermentations were carried out in 28,400 to 37,854 L open-top fiberglass tanks containing 50–60% whole cucumbers (size 3A: 39–51 mm diameter and about 13.7 cm long), and 50 to 40% cover brine solutions. All of the tanks studied were brined with ~1.04 M NaCl (~6%). Tanks A and C to K were located in North Carolina, USA, while Tank B was located in Minnesota, USA. A mixture of size 3A and 2B whole cucumbers (2.7–3.8 cm diameter and about 12.7 cm long) were used for the fermentations in Tank A and Tank B. Recycled cover brine was used in Tank A while freshly prepared cover brine containing 15 ± 5 mM acetic acid (added as a 20% vinegar solution) was used in Tank B. Relish stock, whole cucumbers size 2B and nubs of size 2B cucumbers were used for the fermentations in tanks C to K (Table 5) and covered with recycled cover brines. Cushion cover brine was added into the tanks, prior to the addition of the cucumbers. In-tank cucumbers were immediately covered with wooden boards and subjected to air-purging circulation at a rate of 25–65 SCFH to mix-in rain water and release the CO_2 formed during fermentation, so that bloater defect is prevented. Air purging was applied for the first 21 d of the fermentation, with a routine of 20 h on and 4 h off at variable flow rates (estimated between 25 and 65 SCFH). The air purging system included an L-shaped PVC pipe placed upside down in the tanks with the horizontal portion laying at the interface of the cover brine and the atmosphere and the vertical leg tighten to the tank walls. The vertical

Table 1
Plate counts obtained in various culture media inoculated with fresh cucumber juice samples. The columns include plate counts for the total aerobic microbes (PCA), yeast and molds (YM/A), presumptive *Enterobacteriaceae* (VRBG) and lactobacilli (MRS).

Samples	No.	PCA	YM/A	VRBG	MRS
	Samples	log of CFU/g			
Pickling Cucumbers	6	5.2 ± 0.8	2.8 ± 1.0	4.6 ± 1.0	3.8 ± 1.2
Super Select Cucumbers	5	5.5 ± 0.6	2.8 ± 0.5	4.6 ± 0.5	3.2 ± 0.3
Mini Cucumbers	2	5.6 ± 0.1	3.6 ± 0.2	5.3 ± 0.5	4.7 ± 1.1
Long English Cucumbers	6	6.2 ± 1.1	3.5 ± 0.8	5.3 ± 1.0	3.2 ± 0.3
Total Number of Samples	19	5.7 ± 0.9	3.2 ± 0.8	5.0 ± 0.9	3.5 ± 0.8

Table 2
The number of isolates from VRBG plates inoculated with fermentation cover brine samples from Tanks A and B and the corresponding genera and species identification using the 16S rDNA (1st column) and *dnaJ* (2nd column) sequences homology. The number of colonies per day and tank are shown on the last two columns to the right.

Identification using 16S *rRNA* sequencing	Identification using *dnaJ* sequencing	Tank A			Tank B		
		Fermentation Age (d)					
		1	3	7	1	3	7
Ochrobactrum pseudogrignonense	no amplicon	4	1		2	1	
Citrobacter freundii	*Citrobacter freundii*	2	3				
Citrobacter freundii or *brakii*	*Citrobacter brakii*					1	
Enterobacter spp.	*Enterobacter kobei, E. nimipressuralis, E. cloacae* subsp. *cloacae, E. asburiae*	5	9	1	2		1
Enterobacter cancerogenus	*Enterobacter cancerogenus*						1
Enterobacter xiangfangensis	*Enterobacter cancerogenus* or *cloacae complex*					2	1
Enterobacter tabaci	*Enterobacter hormaechei/cloacae/asburiae*					1	3
Enterobacter aerogenes	*Lelliottia amnigena*					2	
multiple questionable hits	*Enterobacter cloacae*	3					
Leclercia adecarboxylata	*Enterobacter cloacae* complex or *Leclercia adecarboxylata*						3
Enterobacteriaceae	*Leclercia adecarboxylata* or *Enterobacter* spp., *Escherichia vulneris*		3				2
Erwinia rhapontici	*Erwinia amylovora*				1		
Serratia marcescens	*Serratia marcescens*				2		
Providencia rettgeri	no amplicon	2					
Pseudomonas sp.(*parafulva, putida, plecoglossicida* and *taiwanensis*),	no amplicon	1				2	2
Stenotrophomonas maltophilia	no amplicon						3
Pantoea eucalypti, P. agglomerans, P. anthophila	*Pantoea agglomerans*				9		3
Pantoea ananatis	*Pantoea ananatis*				8		1
Pantoea eucrina	*Pantoea* sp.				1		
Obesumbacterium proteus or *Hafnia alvei*	no amplicon					2	
Kluyvera sp.	*Kluyvera cryocrescens*	1					
Kluyvera intermedia	*Citrobacter amalonaticus* or *Kluyvera intermedia*						3
Kluyvera cryoscrescens	*Enterobacter cloacae* complex						1
Micrococcus yunnanensis	no amplicon						1

pipe was perforated and hosted a purging stone attached to a plastic tubing for the delivery of air to about 4 feet below the cover brine surface. An air blower was connected to the plastic tubing to deliver the gas. The gas flow displaced the cover brine in its vicinity forcing it to circulate in an upward direction along the pipe walls initiating cover brine circulation, while the gas bubbles were forced to move towards the bottom of the tanks. Tanks were replenished with cover brine prepared at the equilibrated concentrations to compensate for volume losses due to evaporation.

2.2.2. Microbial analysis of fermentation cover brine samples

For the first experiment, fermentation cover brine samples (100 mL)

Table 3
Identification of certain VRBG isolates using the miniaturized biochemical test strips, API 20E and the respective % identity and quality scores. The results of the identification by the sequencing of the 16S *rRNA* are shown in the 3rd column for reference. The first, second and third digit of the identification code refers to the fermentation day in which the source cover brine sample was collected, the height in the tank from which the cover brine sample was collected from top to bottom and a unique sequential number assigned to a particular isolate, respectively.

Source	Identification Code	Identification by 16S *rRNA* amplicon sequencing	Primary Identification by API 20E	% identity	Identification Quality
Tank A	1.2.1E	*Ochrobactrum pseudogrignonense*	*Ochrobactrum anthropi*	47.8	Low Discrimination
Tank A	1.2.3E	*Citrobacter freundii*	*Citrobacter freundii*	84.2	Good Identification
Tank A	1.2.4E	*Enterobacter kobei*	*Enterobacter cloacae*	97.7	Good Identification
Tank A	1.2.7E	*Enterobacter nimipressuralis*	*Leclercia adecarboxylata*	85.6	Low Discrimination
Tank A	1.2.10E	*Enterobacter cloacae*	*Pantoea* spp	91.1	Good Identification
Tank B	1.2.4E	*Pantoea agglomerans*	*Pantoea* spp	99.8	Very Good Identification
Tank B	1.2.16E	*Pantoea ananatis*	*Pantoea* spp	99.1	Very Good Identification
Tank B	1.2.20E	*Erwinia toletana*	*Pantoea, Klebsiella,*	–	Unacceptable Profile
Tank B	1.2.21E	*Ochrobactrum pseudogrignonense*	*Ochrobactrum anthropi*	47.8	Low Discrimination
Tank A	1.8.2E	*Providencia rettgeri*	*Providencia rettgeri*	99.9	Very Good Identification
Tank A	1.8.4E	*Pseudomonas*	*Pseudomonas fluorescens*	75	Good Genus Identification
Tank A	1.8.5E	*Kluyvera cryocrescens*	*Kluyvera* spp	98.7	Good Identification
Tank B	1.8.3E	*Serratia marcescens*	*Serratia marcescens*	99.8	Doubtful Profile
Tank A	3.8.2E	*Enterobacter cloacae*	*Enterobacter cloacae*	97.7	Good Identification
Tank A	3.8.3E	*Enterobacter nimipressuralis*	*Leclercia adecarboxylata*	85.6	Low Discrimination
Tank A	3.2.3E	*Citrobacter freundii*	*Citrobacter freundii*	99.9	Excellent Identification
Tank A	3.2.4E	*Escherichia vulneris*	*Escherichia vulneris*	99.1	Very Good Identification
Tank A	3.2.8E	*Enterobacter cloacae*	*Enterobacter cloacae*	97.7	Good Identification
Tank A	3.2.9E	*Enterobacter kobei*	*Enterobacter cloacae*	97.7	Good Identification
Tank A	3.2.10E	*Leclercia adecarboxylata*	*Leclercia adecarboxylata*	85.6	Low Discrimination
Tank B	3.2.13E	*Enterobacter cancerogenus*	*Enterobacter cancerogenus*	99.9	Excellent Identification
Tank B	3.2.17E	*Kluyvera intermedia*	*Acinetobacter baumannii*	67.7	Low Discrimination
Tank A	7.2.2E	*Enterobacter* sp.	*Enterobacter cloacae*	99.3	Very Good Identification
Tank B	7.2.7E	*Stenotrophomonas maltophila*	*Stenotrophomonas maltophilia*	99.3	Very Good Identification
Tank B	7.8.20E	*Leclercia adecarboxylata*	*Leclercia adecarboxylata*	73	Doubtful Profile

Table 4
Results of the broad bacterial test using PCR amplification coupled with NMR analysis (an Athogene proprietary analysis) for fermentation cover brine samples collected on days 1 and 3 from Tanks A and B.

Tank ID	Fermentation Age (d)	Bacterial Identification	Genomes/Well	Quality Score
A	1	*Citrobacter freundii/Enterobacter cloacae*	209	0.82
A	1	*Acinetobacter lwoffii, Acinetobacter haemolyticus, Acinetobacter johnsonii*	44	0.96
A	1	*Kluyvera intermedia/Enterobacter cancerogenus*	25	0.89
A	3	*Citrobacter freundii/Enterobacter cloacae*	186	0.85
B	1	*Pantoea ananatis/Pantoea vagans*	38	0.96
B	1	*Aureobasidium pullulans*	122	0.95
B	1	*Pseudomonas fluorescens*	29	0.95
B	3	LAB only	NA[a]	NA

[a] NA: not applicable.

were aseptically collected in two 50 mL Corning conical tubes (430849 Corning Inc., Corning NY, USA) at the purging system outlet on days 1, 3, 7, 14, 30 and 90 from Tanks A and B. However, the last plating on VRBG was done on day 14 due to lack of microbial growth. Cover brine samples were immediately transported to our laboratory for same day processing. Samples were serially diluted using a 0.85% NaCl solution and spiral plated on PCA and VRBG for the enumeration of total aerobic microbes and presumptive lactose positive coliforms, respectively, using an automated plater, Autoplate 4000 (Spiral Biotech). Full strength cover brine samples and samples diluted 100 and 10,000-fold were used for spiral plating. PCA and VRBG plates were incubated at 30 and 35 °C, respectively, for 24–48 h. Plate counts were obtained using a Flash and Go automated plate counter (Neutec Group, Inc.).

In the second experiment, an additional group of nine commercial tanks (Tanks C, D, E, F, G, H I, J, and K) were evaluated for plate counts of fecal coliforms, *Klebsiella* spp., *Acinetobacter* spp. and *Pseudomonas* spp. using VRBG and MacConkey (MCA) with Crystal Violet, NaCl and 0.15% Bile Salts (Sigma M8302, Sigma-Aldrich) agar plates, HiCrome *Klebsiella* Selective agar (HCK; 90925 Sigma-Aldrich) supplemented with *Klebsiella* Selective Supplement containing 50 mg of carbenicillin per liter (15821 Sigma-Aldrich), Leeds *Acinetobacter* Medium (LAM; 1839 HiMedia Laboratories Pvt., Mumbai, India) supplemented with

Table 5
Plate counts for lactose fermenting bacteria, *Klebsiella* spp., *Acinetobacter* and *Pseudomonas* from Violet Bile agar supplemented with glucose and McConkey agar, HiCrome *Klebsiella* agar, Leeds *Acinetobacter* medium and *Pseudomonas* Isolation agar in log of CFU/mL. The limit of detection for colony forming units was 0.4 log of CFU/mL of cover brine. Also shown are the fermentation cover brine pH and lactic and acetic acids concentrations (mM).

Tank ID	Fermented Stock	Time (h)	VRBG	McConkey Agar	HiChrome *Klebsiella* Agar	Leeds *Acinetobacter* Medium	*Pseudomonas* Isolation Agar	pH	Lacic Acid (mM)	Acetic Acid (mM)
C	relish	36	BDL[a]	1.56	BDL					
		48	BDL	BDL	BDL					
		66	BDL	BDL	BDL					
		72	BDL	BDL	BDL		BDL	3.7	67.9	16.0
D	relish	36	3.03	1.65	3.06					
		48	2.73	1.82	2.90					
		66	0.72	1.32	2.73					
		72	BDL	BDL	BDL		3.35	4.0	34.2	3.5
E		36	3.02	1.73	2.68					
	relish	48	2.73	1.56	2.60					
		66	0.72	1.40	BDL					
		72	BDL	BDL	BDL		3.10	4.3	24.4	3.1
F	whole	18	BDL	BDL	BDL					
	cucumbers	24	BDL	BDL	BDL	BDL	BDL	4.0	22.0	2.4
G	whole	18	BDL	BDL	BDL					
	cucumbers	24	BDL	BDL	BDL	BDL	BDL	4.1	22.5	2.6
H	whole	18	BDL	BDL	BDL					
	cucumbers	24	BDL	BDL	BDL	BDL	BDL	4.1	20.9	2.1
I		18	2.96	3.02	3.37					
	whole	24	BDL	2.36	2.24	2.08	3.97	4.1	20.4	2.6
	cucumbers	48	BDL	BDL	BDL	BDL	3.96	4.3	22.8	3.3
	and nubs	72					2.51	3.9	36.3	4.3
J		18	BDL	BDL	BDL					
	whole	24	BDL	BDL	BDL	BDL	BDL	3.9	26.0	2.9
	cucumbers	48	BDL	BDL	BDL	BDL	1.76	4.0	23.3	2.8
	and nubs	72					1.87	4.1	22.7	2.7
K		18	BDL	BDL	BDL					
	whole	24	BDL	BDL	BDL	BDL	2.66	3.9	25.8	3.1
	cucumbers	48	BDL	BDL	BDL	BDL	2.79	4.1	23.7	3.0
	and nubs	72					1.74	4.1	21.6	2.9

[a] BDL: Below the Limit of Detection.

Leeds *Acinetobacter* Selective Supplement containing 50 mg cephradine, 15 mg cefsulodin, and 10 mg vancomycin per liter of media (FD335, HiMedia Laboratories Pvt. Ltd., Mumbai, India) and *Pseudomonas* Isolation agar (PI; Difco 292710), respectively. These agar plates were inoculated with fermentation cover brine samples collected from commercial tanks packed with size 2B (3.2–3.8 cm in length) whole cucumbers, size 2B cucumber ends or relish stocks containing whole cucumbers, nubs and pieces of cucumbers, as indicated on the text. Fermentation cover brine samples were collected at various time points between days 0 and 3 as described in Table 5. From 10 to 100 mL of selected cover brine samples were spun using a Sorvall Centrifuge (Thermo Fisher Scientific Inc.) at 15,294 rcf for 10 min at 21 °C and the pellets re-suspended in 1 mL of the supernatant to concentrate the cells and lower the limit of detection to 0.4 log of CFU/mL. Concentrated and full strength cover brine samples as well as samples serially diluted with a 0.85% NaCl solution were subjected to spiral plating using an automated plater, Autoplate 4000 (Spiral Biotech). Agar plates were incubated at 35 °C under aerobic conditions for 24–48 h. Plate counts were obtained using a Flash and Go automated plate counter (Neutec Group, Inc.).

2.2.3. Isolation and identification of gram negative bacteria from VRBG plates inoculated with fermentation cover brine samples generated in the first experiment

A maximum of 20 isolated colonies were picked from each VRBG plate inoculated with fermentation cover brine samples collected on days 1, 3 and 7 from Tanks A and B. A total of 150 colonies were streaked on VRBG for purification prior to the preparation of frozen stocks in Tryptic Soy broth (TSB, Bacto 286220, Becton Dickinson and Co.) supplemented with 15% glycerol (v/v) (Cat No. G5516, Sigma Aldrich). Not all VRBG plates had 20 colonies that could be isolated and no colonies were recovered from VRBG plates inoculated with cover brine samples collected on day 14 from Tanks A and B. A total of 150 frozen stocks were prepared.

Purified cultures were transferred from frozen stocks to 1 mL of TSB, individually, and incubated at 35 °C ± 2 for 24–48 h under static conditions to obtain bacterial pellets for DNA extraction using an InstaGene Matrix DNA extraction kit (Bio-Rad) following the manufacturer instructions. 14% (21) of the isolates was lost after freezing due to loss of viability. Extracted DNA was used for the partial amplification of the 16S rDNA and the *dnaJ* genes for the purpose of identification. The PCR amplification mixture contained 2X master mix (Bio-Rad), 10 μL of the resulting total genomic DNA extracted from each one of the 100 bacterial isolates, and 0.25–0.4 μM of primers 8f (5-AGAGTTTGA TCCTGGCTCAG-3′) and 1492r (5′-GTTACCTTGTTACGACTT-3′) (Wilson et al., 1990) or the degenerated primers DN1-1F (5′-GATYTR-CGHTAYA ACATGGA-3′) and DN1-2R (5′-TTCACRCCRTYDAAGA-ARC-3′) (Pham et al., 2007) for the amplification of the 16S rDNA or *dnaJ* genes, respectively. The PCR amplification steps for 16S rDNA consisted of one cycle of 4 min at 94 °C followed by 25 cycles of 1 min at 94 °C, 2 min at 50–60 °C, and 2 min at 72 °C, with a final extension step of 7 min at 72 °C. The PCR amplification conditions for *dnaJ* were 3 min at 94 °C followed by 35 cycles of 30 s at 50 °C, and 1 min at 72 °C, with a final extension step of 7 min at 72 °C. Amplicons were stored at 4 °C. No amplicons could be obtained using the primer pair described above for 16% (21) of the extracted DNA samples. The PCR amplification products were sequenced by Eton Bioscience Inc. Sequence data were formatted and analyzed using the BioEdit software (www.mbio.ncsu.edu/bioedit). Only bases that had quality scores greater than or equal to 20 were used for the alignment. The sequences obtained were subjected to the basic local alignment search tool (BLAST) (Altschul et al., 1990; Benson et al., 1997) using the 16S ribosomal RNA sequence database to determine the identity of the isolates. 14% of the sequences obtained did not align to the sequences in the database. The sequences obtained are available in GenBank with accession numbers MG678916 to MG678938 (Tank A dnaJ); MG706072 – MG706116 (Tank B dnaJ); MH045680 - MH045712 (Tank A 16S rRNA) and MG681197-MG681257 (Tank B 16S rDNA).

Selected isolates, representing each of the species detected by gene amplification, were subjected to the miniaturized biochemical testing using the API 20E system by BioMérieux (Marcy l'Etoile, France) following the manufacturer instructions. Each isolate identified by API20E (a total of 24 isolates) was deposited in the USDA-ARS Food Science Unit (Raleigh, NC, USA) culture collection with sequential identification numbers from B0520 to B0544.

2.2.4. 16S rDNA gene amplicon sequencing using Ion Torrent PGM platform for fermentation cover brine samples collected from tanks A and B

Genomic DNA was extracted from fermentation cover brine samples collected on day 1 from Tanks A and B, for the amplification of the 16S rDNA pool as described by Medina et al. (2016). Amplicons were barcoded and sequenced using the Ion Torrent PGM sequencing platform as described by Medina et al. (2016). The forward and reverse amplification primers contained Ion Torrent specific adapters on the 5′ end and a primer linker on the 3′ end with the following generic sequences, respectively, where the string of N represents the barcode sequences: 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNN.

NNNNNNAGAGTTTGATCCTGGCTCAG-3′, and 5′-CCTCTCTATGGG CAGTCGGTGATGC.

TGCCTCCCGTAGGAG T-3'. The ribosomal amplicon sequencing analysis using the Ion Torrent PGM sequencing platform targeted the V1-V2 fragment of the 16S rDNA gene. Amplicon high-throughput sequencing data analysis was conducted from raw Ion Torrent fastq files with accession no. KF998365 - KF998547 by the Microbiome Core Facility at the University of North Carolina-Chapel Hill, NC, USA. QIIME (Caporaso et al., 2010a) was used to calculate alpha diversity on rarefied OTUs to assess sampling depth coverage using the observed species, Phylogenetic Diversity and Chao1 diversity metrics (Table S1).

2.2.5. AthoGen analysis of the bacterial populations in the samples generated in the first experiment

Broad bacterial culture independent testing was conducted by AthoGen (Carlsbad, CA, USA) using the total DNA extracted from cover brine samples collected from Tanks A and B, as described for the Ion Torrent analysis. A total of 12 cover brine samples were analyzed corresponding to duplicates of six independent heights in Tanks A and B. The 16S rDNA was amplified by PCR and analyzed by NMR to determine the abundance of the predominant bacteria present in the samples using AthoGen's proprietary technology and database. The detection limit of the assay was $\sim 10^4$ CFU/mL.

2.2.6. Measurement of pH and dissolved oxygen in commercial fermentation tanks

The pH of cover brine samples was measured in the laboratory using a Fisher Accumet pH meter (model AR25, Fisher Scientific, Pittsburgh, PA, USA) combined with a Gel-Filled Pencil-Thin pH Combination Electrode (Acumet, Fisher Scientific). Dissolved oxygen was measured on-site and upon sample collection, at variable depths in the tanks, through the air purging system pipe typically installed in cucumber fermentation vessels. A CellOx 325 probe attached to an Oxi 330i meter (WTW, Welheim, Germany) via a 3.66 m cable was used to measure dissolved oxygen after adjusting for 6% salinity. The probe was calibrated and maintained following the manufacturer instructions.

2.2.7. Analysis of fermentation metabolites by HPLC

Fermentation cover brine samples (1.5 mL) were spun at 15,294 rcf for 10 min in an Eppendorf benchtop refrigerated centrifuge 5810R (Hamburg, Germany) to remove residual particulate matter. The supernatants were diluted 10X with a 0.01 N sulfuric acid solution in HPLC vials. Organic acids and carbohydrate concentrations were measured using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and the HPLC method described by McFeeters and Barish

(2003) with some modifications. The operating conditions of the system included a column temperature of 65 °C and a 0.01 N H_2SO_4 eluent at 0.9 mL/min. A SPD- 20A UV–vis detector (Shimadzu Corporation, Canby, OR, USA) was set at 210 nm at a rate of 1 Hz to quantify malic, lactic, succinic, propionic and butyric acids. An RID-10A refractive index detector (Shimadzu Corporation) connected in series with the diode array detector was used to measure acetic acid, lactic acid, glucose, fructose and ethanol. External standardization of the detectors was done using at least five concentrations of the standard compounds.

3. Results

3.1. Microbial plate counts from fresh cucumber samples

Minimal differences were observed in plate counts for the various groups of microbes tested among cucumber type (Table 1). Evidently, total aerobic counts (PCA) were higher than those corresponding to yeasts and molds (YMA), presumptive *Enterobacteriaceae* (VRBG) and lactobacilli (MRS) (Table 1). Yeasts and mold plate counts were not higher than 3.6 ± 0.2 log of CFU/g (Table 1). Plate counts for presumptive *Enterobacteriaceae* were on average at least 1.5 log of CFU/g higher than those corresponding to lactobacilli from MRS agar plates (Table 1).

3.2. Isolation and characterization of bacterial colonies from MRS agar plates inoculated with fresh cucumber juice

Enterococcus (25%), *Exiguobacterium* (15%), *Lactococcus* (15%), *Staphylococcus* (13%), *Lactobacillus* (11%) and *Leuconostoc* (10%) were frequently isolated (> 5%) from MRS plates inoculated with fresh cucumber homogenate. Three genera were infrequently encountered including *Bacillus* (6%), *Aerococcus* (4%) and *Clostridium* (1%). Of the colonies isolated from MRS agar plates, 10% could not be identified using the 16S rDNA sequence. Additionally, PCR amplicons could not be generated for 14% of the extracted DNAs and 10% of the isolates lost viability from frozen stocks. A number of the cultures identified as *Enterococcus* spp. grew on MRS agar plates incubated under anaerobic conditions, but not in the presence of air. Eight isolates out of the 93 had 16S rDNA sequences similar to that of the *Lb. plantarum* cluster.

3.3. Metagenetic analysis of fresh cucumber samples

Rhizobium (31.04%), *Pseudomonas* (14.08%), *Pantoea* (9.25%), *Stenotrophomonas* (6.83%) and *Acinetobacter* (6.50%) were the genera more frequently present in fresh cucumber samples in relatively high abundance (Fig. 2). *Acinetobacter* was abundantly present in Pickling (14.42%), Super Select (3.50%) and Long English (5.62%) cucumbers. *Sphingomonas* (4.6%), *Klebsiella* (3.1%), *Oxalobacteriaceae* (3.0%), *Clavibacter* (2.9%), *Aureimonas* (1.9%), *Sphingobacterium* (1.2%), *Methylobacterium* (1.2%) and *Citrobacter* (1.1%) were occasionally present in certain cucumber types (Fig. 2). The average relative abundance of a lactic acid bacterium in fresh cucumbers was less than 0.37% including *Lactococcus* (0.36%), *Lactobacillus* (0.20%), *Leuconostoc* (0.18%) *Weissella* (0.09%) and *Enterococcus* (0.01%).

3.4. Total aerobic and presumptive Enterobacteriaceae plate counts from commercial cucumber fermentation cover brine samples collected from tanks A and B

The plate counts for total aerobic microbes follows the trend of a typical cucumber fermentation (Pérez-Díaz et al., 2014) (Fig. 3). As expected, the detectable number of *Enterobacteriaceae* cultivated in VRBG from cover brine samples decreased below detectable levels by day 14, after most of the lactic and acetic acids was produced (55.2 ± 13.1 and 10.4 ± 6.5, respectively), the cover brine pH reached 3.44 ± 0.02 and the dissolved oxygen levels decreased to less than 0.3 mg/L (Fig. 3).

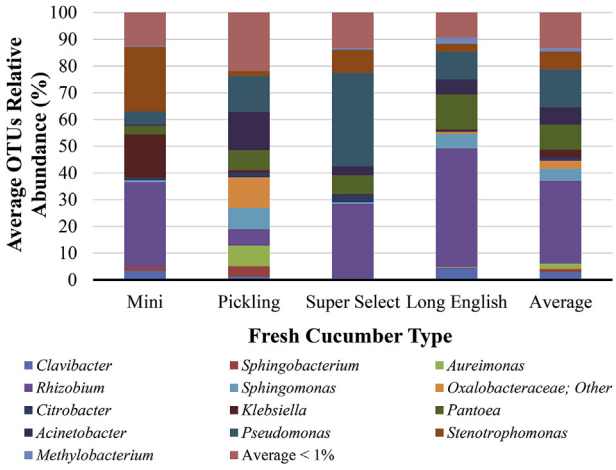

Fig. 2. Relative abundance (%) corresponding to the orthologous taxonomic units (OTU) found in fresh cucumber samples from 4 commercial types. The number of mini cucumber, pickling cucumber, super select cucumber and long English cucumber samples considered in the analysis were 2, 3, 2 and 6, respectively. Average for all samples (n = 13) are also shown for each OTU.

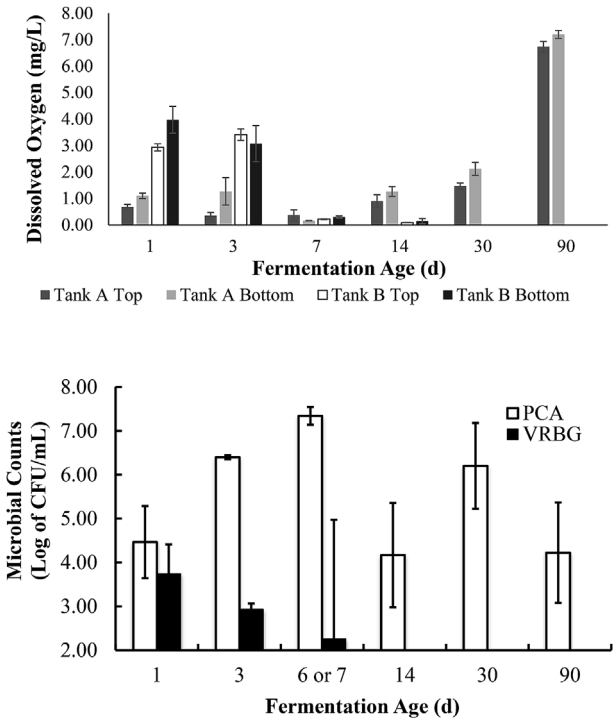

Fig. 3. Plate counts for total aerobes (PCA) and presumptive *Enterobacteriaceae* (VRBG) and dissolved oxygen from commercial cucumber fermentations in Tanks A and B as a function of time (d). Results shown for plate counts are average and standard deviation for duplicates of two independent replicates (n = 4). Samples were collected on days 7 and 6 from Tanks A and B, respectively, and plated on the same day. Minimal detection limit for plate counts was 2.4 log of CFU/mL. Dissolved oxygen measurements represent technical duplicates and were not taken from Tank B on days 30 and 90.

3.5. Isolation and identification of isolates from VRBG plates inoculated with commercial cucumber fermentation cover brines collected from tanks A and B

No colonies were observed on VRBG plates inoculated with fermentation cover brine samples collected on day 14 (Fig. 3). The number

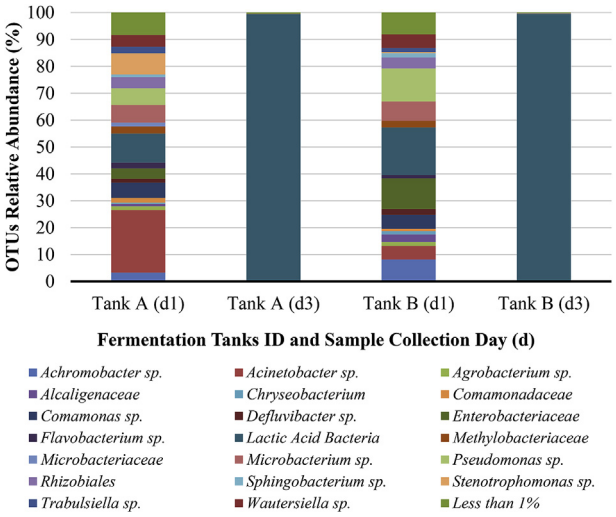

Fig. 4. Relative abundance (%) corresponding to the orthologous taxonomic units (OTU) found in cucumber fermentation cover brine samples collected 1 and 3 days after brining. Data for day 3 is adapted from Pérez-Díaz et al. (2016) for reference.

of isolates from fermentation cover brine samples as a function of fermentation time and their identification using the partial sequencing of the 16S rDNA and *dnaJ* genes are shown in Table 2. In order of prevalence, *Enterobacter, Ochrobactrum, Citrobacter, Pantoea, Pseudomonas, Kluyvera* and *Stenotrophomonas* were found in cover brine samples. Identification of selected isolates using the miniaturized biochemical testing strips, API 20E, are shown in Table 3 with members of the *Enterobacteriaceae* family dominating the list.

3.6. Metagenetic analysis of commercial cucumber fermentation samples collected from tanks A and B

Two independent fermentation cover brine samples collected from Tanks A and B on day 1 were analyzed with average post-data processing reads of 16,432 ± 39 and a phylogenetic diversity metric of 9.22 ± 0.07 (Table S1). Fig. 3 shows a common qualitative distribution of taxonomical families in both of the fermentation cover brine samples collected on day 1 which are compared to the OTUs found on day 3 in Tanks A and B by Pérez-Díaz et al. (2016). The relative abundance of LAB matching OTUs in Tanks A and B on day 1 fluctuated between 3.25 and 3.98% and co-dominated with *Acinetobacter* (14%) (Fig. 4). As expected *Pseudomonas* (9%) and *Enterobacteriaceae* (8%) followed LAB and *Acinetobacter* in relative abundance (Fig. 4). All other OTUs identified in Fig. 4 were detected at or below 7% relative abundance.

3.7. Broad bacterial testing using PCR-NMR of the fermentation samples generated in the first experiment

Table 4 shows the bacterial genera and species detected using this technique showing a predominance of *Enterobacteriaceae*, *Acinetobacter* spp., *Pseudomonas* sp. and *Aureobasidium* sp. but not of LAB.

3.8. Plate counts for lactose fermenting bacteria, Klebsiella spp., Acinetobacter spp. and Pseudomonas spp. in cover brine samples collected on days 1, 2 and 3 from 9 commercial fermentation tanks

Plate counts from Tanks C to K on the 5 selective media used for the screening of Gram-negative bacteria were consistently 1 to 3 log of CFU/mL higher in fermentation cover brines collected from tanks packed with relish stocks as compared to whole cucumber or nub stocks (Table 5). Plate counts for lactose fermenting bacteria and presumptive *Klebsiella* spp. from VRBG and MC, and HCK, respectively, were detected in samples collected from relish tanks up to 2 days after tanking. One tank out of the six tanks packed with whole cucumbers or nubs also harbored viable cells of lactose fermenting bacteria and presumptive *Klebsiella* spp. on the second day of fermentation. Presumptive *Acinetobacter* spp. were only detected on day 1 of fermentation from one out of the nine tanks studied, which was packed with whole cucumbers. Presumptive *Pseudomonas* spp., however, were detected in five cover brine samples out of the nine studied, which were collected on day 3 of the fermentation. Initial levels of acetic and lactic acids were measured from cover brine samples at 2.8 ± 0.4 and 23.2 ± 1.9 mM, respectively, as expected for the use of recycled brines (Table 5). The average pH value of 4.1 ± 0.4 was measured after 3 days of fermentation (Table 5). Only Tank C presented substantially different acetic and lactic acids concentrations (16.0 mM and 67.9 mM, respectively) and a pH of 3.71 (Table 5).

4. Discussion

Although commercial cucumber fermentations are typically managed following the principles and guidelines established for the production of safe foods and employ cover brines containing preservatives such as acid and salt, a distinct microbiota, mostly defined by microbial load in fresh cucumbers and the surroundings, seems to be present in each tank determining the long term stability of the preserved stock. Opportunities for the introduction of microbes exist in every step of the process. This includes the use of sanitized and non-sterile tanks that in theory may be hosting microbial biofilms, the use of recycled cover brines that may be serving as an unintended source of inocula, the tanking of fresh fruits grown in a diversity of geographical regions and soils, and the packing of cucumber pieces or nubs with exposed nutrients-containing-flesh, potentially harboring a higher load of microbes, to mention a few. However, the observations made here suggest that microbial inoculation in a fermentation vessel from the various potential sources in the tank yard does not substantially impact the relative abundance of the diverse members of the microbiome or course of the fermentation, given that LAB are equipped to dominate in such habitat. Thus, the ability of certain microbes to thrive in a cucumber fermentation seems to define the microbiome more so than the presence of a microbe in the fresh cucumbers or the fermentation habitat.

This study shows the dominance of Gram-negative bacteria in fresh cucumbers and on day 1 of cucumber fermentations (Figs. 1 and 3). Bacteria present in fresh cucumbers such as *Rhizobium*, *Pseudomonas*, *Acinetobacter*, *Sphingomonas*, *Sphingobacterium*, *Methylobacterium* and *Citrobacter* were also detected in samples of fermentation cover brine samples collected on day 1, and in lower relative abundance in samples collected on day 3 (Figs. 1 and 3). While *Pantoea* spp. were not specifically detected in fermentation cover brine samples by metagenetics (but perhaps as *Enterobacteriacea*), it was found in fresh cucumber samples using the same technique and in fermentation cover brine samples by the broad bacterial analysis and by plating on VRBG. Although, *Klebsiella* spp. were detected in fresh cucumber samples using metagenetics but not in fermentation cover brine samples, selective plating for the members of this genera revealed their presence in three out of nine commercial cucumber fermentation tanks scrutinized (Figs. 1 and 3 and Table 4). *Stenotrophomonas* was detected in fresh cucumber samples and in commercial cucumber fermentation brines after 1 day of tanking but not after 3 days, indicating their die-off (Figs. 1 and 3). The fact that a decreasing number of the microbes indicated above were detected in commercial fermentations by day 3 post-tanking confirms that the community of aerobic bacteria, mostly Gram-negative, is excluded early in commercial cucumber fermentations. Together these data also suggest that a substantial number of the Gram-negative bacteria able to colonize fresh cucumbers perish in a fermentation process, as expected in a preserved food.

Isolation and identification of *Pseudomonas*, *Bacillus*, *Sphingomonas*,

Microbacterium, *Stenotrophomonas*, *Acinetobacter*, *Chryseomonas* and *Enterobacetriaceae* such as *Enterobacter*, *Hafnia*, *Erwinia*, *Klebsiella*, *Serratia* and *Pantoea* from fresh and fermented cucumbers is in line with the findings made by others for fresh and fermented vegetables such as tomatoes, corn lettuce and cabbage (Lee et al., 2016 Manani et al., 2006; Ottesen et al., 2016; Rastogi et al., 2012; Samish et al., 1963; Shi et al., 2009; Weiss et al., 2007). Should a core vegetables microbiome be defined, it would likely include such 13 genera but surely contain the endophytes belonging to *Pseudomonadaceae* and *Enterobacteriaceae*.

Based on our observations, it is presumed that a combination of factors such as a decreasing dissolved oxygen concentration (Fig. 2), production of organic acids by LAB (Pérez-Díaz et al., 2016) and the presence of up to 18% NaCl in unequilibrated cover brines, induces the die-off of Gram-negative bacteria in cucumber fermentations. Plate counts for lactose-fermenting bacteria, mostly Gram-negative *Enterobacteriaceae*, from VRBG, decreased from 5.0 ± 0.9 log of CFU/mL in fresh cucumbers and 3.74 ± 1.2 log of CFU/mL in brined cucumbers on day 1 of the fermentation to undetectable levels by day 3 (Table 1 and Fig. 2). It is presumed that upon initiation of the fermentation, the majority of the microbial population on fresh cucumbers, which localizes on the cucumbers exocarp (Mattos et al., 2005), is exposed to the full strength cover brine containing between 12 and 18% NaCl, a known preservative. Concomitant with a decrease in the number of Gram-negative bacteria is the retention or increase in total aerobic counts (Fig. 3) and the LAB population. Total aerobes plate counts increased from 5.7 ± 0.9 log of CFU/mL in fresh cucumbers and 4.46 ± 0.24 log of CFU/mL in brined cucumbers 1 d post-tanking to 7.34 ± 0.2 log of CFU/mL on day 7 of the fermentation (Table 1 and Fig. 2). Such an increment in plate counts for total aerobes, is mostly due to the increase in organic acid producing LAB, anaerobes able to proliferate in the presence of oxygen on PCA, as evidenced by plate counts at 8.5 ± 0.3 log of CFU/mL on day 7 of the fermentation from MRS agar plates (Pérez-Díaz et al., 2016). Thus, it is confirmed that the decrease in the numbers of Gram-negative bacteria is fully or partially due to an increasing concentration in organic acids, mostly lactic and acetic acids, which are able to suppress microbial growth as a function of fermentation age by LAB, as presumed.

This study also confirms the presence of LAB to less than 1% in fresh cucumber samples and fermentation cover brines 1 d after tanking (Figs. 1 and 3 and Table 1). Plate counts from MRS agar were recorded at 3.5 ± 0.8 log of CFU/mL on fresh cucumbers (Table 1). Although, MRS agar was developed for the cultivation of lactobacilli (De Man et al., 1960), the data presented here suggest that organisms other than LAB are also able to proliferate in such non-selective medium containing a significant amount of sodium acetate at pH 6.5. The proliferation of several Gram-positive bacteria such as *Exiguobacterium*, *Staphylococcus*, *Enterococcus*, *Lactococcus* and *Leuconostoc* on MRS agar plates was also observed. Ten percent (9 colonies) of the isolates from MRS agar plates inoculated with fresh cucumber juice were classified as lactobacilli and only four were identified as *Lb. plantarum/pentosus/paraplantarum*. The metagenetic analysis also detected a low relative abundance for LAB in fresh cucumbers totaling 0.87% of the OTUs population, with lactobacilli representing 0.20%. *Lb. plantarum* and *Lb. brevis* have been detected in young cucumber fermentations packed with recycled cover brines in significant numbers, but not in the counterparts brined with freshly prepared cover brines (Pérez-Díaz et al., 2016). Together these data suggest that plate counts from MRS agar plates are likely to overestimate the population of LAB, particularly lactobacilli, in the early stage of cucumber fermentations.

Although, a combination of culture dependent and independent techniques enabled the identification of more than 25 bacterial genera in fresh and freshly-brined cucumbers, including members of the *Enterobacteriaceae* family not previously associated with cucumbers, no pathogens of public health significance (*Salmonella*, *Listeria* and *Escherichia coli*) were found in fresh or brined cucumbers. An *Escherichia* sp. was detected in fermentation cover brine samples to 1.3 and 0.7% after 1 d of packing, which was undetectable by day 3 of the fermentation. *Escherichia vulneris* was isolated from VRBG plates inoculated with cover brine samples collected on day 3 of the fermentation (Table 3). *Salmonella* and *Listeria* were also not detected in fresh cucumbers or cover brine samples using culture dependent and independent techniques. These findings are in agreement with those reported by Fiedler et al. (2017) and support the safety record of fermented cucumber products, evidenced by the lack of outbreaks involving such food products.

The broad bacterial PCR-NMR test and the sequencing of the 16S rDNA and *dnaJ* from the VRBG isolates revealed the presence of *Citrobacter*, in particular *C. freundii* (an opportunistic pathogen), *Trabulsiella*, which is often confused with *Salmonella*, *Kluyvera*, *Leclercia*, *Providencia, Serratia*, *Erwinia* and *Pantoea* (Tables 2–4). Given the fermentative abilities of selected *Enterobacteriaceae*, it would be of interest to establish the role of the genera listed above in the development of cucumber bloating defect during the early stages of commercial fermentations and defined survival parameters for NaCl content and acidic pH in conditions similar to those present in cucumber fermentations.

Species belonging to the *Enterobacter cloacae* complex (Hoffmann and Roggenkamp, 2003), previously associated with hydrogen production in cucumber fermentations (Etchells and Goresline, 1940), were detected in cover brine samples collected on days 1 and 3 (Tables 2–4). A number of isolates from VRBG plates inoculated with cover brine samples collected on days 1 and 3 of the fermentation were identified as *Enterobacter* by the sequencing of the 16S rDNA and *dnaJ* (Table 2) and the API 20E miniaturized biochemical testing (Table 3), including *En. kobei, En. nimipressuralis* and *En. cancerogenous. Enterobacter* spp. were also found among isolates from lactobacilli MRS agar plates inoculated with fresh cucumber juice. Thus, these observations confirm the presence of the *Enterobacter* genus in commercial cucumber fermentations likely derived from the fresh fruit.

This is the first report of the presence of members of the *Pseudmonadaceae* family in cucumber fermentations, confirming their persistence despite the relatively high salt concentration and developing acidic pH in commercial cucumber fermentations. The *Pseudomonadaceae* family was found in fresh cucumbers by Samish et al. (1963). The average relative abundance of *Pseudomonas* in the 13 fresh cucumber samples analyzed in this study was 14.08% with a maximum detected abundance of 33.12 and 36.90% in Pickling and Super Select cucumbers, respectively (Fig. 1). Multiple *Pseudomonas* species, which are Gram-negative aerobic rods, were found in cover brine samples collected on days 1, 3 and 7 (Table 2). The *Pseudomonadaceae* family was represented to 9% relative abundance on day 1 of fermentations (Fig. 1). It was also found in cover brine samples collected on day 3 of the fermentation to a relative abundance of 0.60% in Tank B (data not shown). The psychrophilic pathogen *P. fragi* and the plant pathogen *P. viridiflavans* were found in fermentation cover brine samples collected on day 1 to relative abundances of 3.1 and 2.93%, respectively (data not shown). *P. fluorescens* was identified by the broad bacterial PCR-NMR test to 29 genomes/well with a quality score of 0.95 out of 1 (Table 4), and was isolated from VRBG agar plates on days 1, 3 and 7 of commercial cucumber fermentations (Tables 2 and 3). Selective plating for *Pseudomonas* spp. on PI agar suggested the presence of this genus in five cover brine samples out of nine collected on day 3 of commercial fermentations to 2.46 ± 0.67 log of CFU/mL, when the pH was at 4.08 ± 0.14 corresponding to lactic and acetic acids concentrations of 27.84 ± 6.88 and 3.31 ± 0.63 mM, respectively (Table 5). However, further studies are granted to conclusively define the processing parameters that enable the presence of *Pseudomonas* spp. in commercial cucumber fermentations.

Together the data described above suggest that *Pseudomonas* spp. may be present in substantial numbers in the early stage of cucumber fermentations and thus may be impacting the quality of fermented cucumbers. The three *Pseudomonas* species found in cucumber fermentations (*P. fragi*, *P. fluorescens* and *P. viridiflava*) are flagellated

(Palleroni, 1981), may derive energy for growth from a variety of organic compounds (Zago and Chugani, 2009), and although strictly aerobic, some species may grow in anaerobic conditions in the presence of nitrate, a substrate naturally present in cucumbers (Blekkenhorst et al., 2017). *P. fluorescens* is known to produce heat stable lipases activated by calcium ions (Fox and Stepaniak, 1983), an ingredient in cucumber fermentation cover brines, and a heat stable protease (Mayerhofer et al., 1973) that causes a number of defects in refrigerated milk such as bitterness and casein breakdown (Scatamburlo et al., 2015). Thus, it is speculated that *Pseudomonas*, naturally present in fresh cucumbers, could reach microenvironments within the fermenting cucumber meso- and endocarps, colonizing it and impacting tissue firmness. This could also result in the formation of hollow cavities, better known as bloater defect, in particular in fermentations proceeding at low environmental temperatures (i.e. 4–10 °C). The impact of this microbe on the texture of fermented cucumbers by proteolysis or lipolysis remains to be investigated.

Bacillus cereus was the only spore former and one of the few Gram-positive bacteria detected on cover brine samples collected from day 1 of the fermentations. This spore former is a ubiquitous aerobic rod previously found as a survivor in acidified cucumbers in jars by McFeeters and Pérez-Díaz (2008) upon inoculation of cover brine samples in aerobic media. Although, some Bacilli strains are harmful to humans, some strains are used as probiotic cultures in animal feed given their ability to compete with *Salmonella* (Vila et al., 2009).

Contrary to *Methylobacteriaceae*, which were present in the fresh cucumbers (1.2%) and fermentation cover brine samples collected on day 1 (3%), and persist in fermentations for months (Medina et al., 2016), *Acetobacteriaceae* were not detected in the fresh cucumber samples or fermentation cover brine samples collected on day 1 (Figs. 1 and 3). However, *Acetobacteriaceae* were detected in fermentation cover brine samples collected on day 3 to less than 1% relative abundance (Pérez-Díaz et al., 2016) and have been associated with fermented cucumber spoilage (Medina et al., 2016). *Acetobacter peroxydans*, *Acetobacter aceti* and *Acetobacter pasteurianus* were found in commercial cucumber fermentation cover brine samples at pH below 3.4. *Acetobacter* colonies were isolated from Mannitol Yeast Peptone agar inoculated with cover brine samples collected from tanks holding 110 day-old stock or older, that had been subjected to air purging regularly (Medina et al., 2016); and is known to be the microbe of choice for acetic acid production for vinegar. Thus, it is speculated that the main source of *Acetobacteriaceae* inocula in cucumber fermentations is the vinegar used as an ingredient in cover brines. Alternatively, the bacterium may be present in fresh cucumbers at below 0.01% relative abundance.

5. Conclusion

The utilization of a combination of microbiological and molecular genetic techniques enabled the identification of a variety of non-LAB in fresh and fermented cucumbers, including those previously not thought to be associated with the fermentation of such fruit, such as *C. freundii*, *Pseudomonas*, *Ochrobactrum, Pantoea, Kluyvera, Stenotrophomonas, Comamonas*, *Acinetobacter*, *Wautersiella*, *Microbacterium* and *Flavobacterium* and the taxonomical family *Methylobacteriaceae*. Data presented suggest that the presence of non-LAB in fermentations is limited by reduced levels of dissolved oxygen and the decrease in pH, which remained at 4.08 ± 0.14 on day 3 of commercial cucumber fermentations. Observations made confirm the commonly accepted notion that non-LAB are inhibited by acidity early in the fermentation of cucumbers. Thirteen out of the 25 genera found in this study to be associated with fresh and fermented cucumbers have also been identified as members of other vegetable microbiota. Further studies are needed to understand the parameters that impact the persistence and relative abundance of non-LAB in commercial fermentations. We aim at elucidating the role of *Pseudomonas* spp. and *Enterobacteriaceae* on cucumber texture degradation, via heat stable proteases and lipases, and cucumber bloating, via the production of CO_2, respectively.

Acknowledgements

The authors thank Ms. Sandra Parker with the USDA-ARS Food Science Research Unit located in Raleigh, NC for administrative support. The authors also thank the Spanish Government (MECD) for the postdoctoral fellowship support for Dr. E. Medina. The authors thank AthoGen located in Carlsbad, CA, USA for the Rapid Research Acceleration Program which partially sponsored the PCR-NMR analysis. The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.08.003.

References

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Aronesty, E., 2011. Ea-utils: “Command-line tools for processing biological sequencing data”. https://github.com/ExpressionAnalysis/ea-utils.
Benson, D.A., Boguski, M.S., Lipman, D.J., Ostell, J., 1997. GenBank. Nucleic Acids Res. 25, 1–6.
Blekkenhorst, L.C., Prince, R.L., Ward, N.C., Croft, K.D., Lewis, J.R., Devine, A., Shinde, S., Woodman, R.J., Hodgson, J.M., Bondonno, C.P., 2017. Development of a reference database for assessing dietary nitrate in vegetables. Mol. Nutr. Food Res. 61 (8), 1600982. https://doi.org/10.1002/mnfr.201600982.
Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for illumina sequence data. Bioinformatics btu170.
Caporaso, G.J., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Gonzalez-Pena, A., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010a. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7 (5), 335–336. https://doi.org/10.1038/nmeth.f.303.
Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R., 2010b. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26 (2), 266–267. https://doi.org/10.1093/bioinformatics/btp636.
De Man, J.C., Rogosa, M., Sharpe, M.Elisabeth, 1960. A medium for the cultivation of Lactobacilli. J. Appl. Bacteriol. 23, 130–135.
Etchells, J.L., 1941. A New Type of Gaseous Fermentation Occurring during the Salting of Cucumbers, vol. III. Univ Microfilms Abstr, Ann Arbor, MI), pp. 7–8 (2), (Abstract).
Etchells, J.L., Goresline, H.E., 1940. Methods of examination of fresh cucumber pickle. Fruit Prods. J. 19 (11), 331–335.
Fiedler, G., Kabisch, J., Böhnlein, C., Huch, M., Becker, B., Cho, G.S., Franz, C.M.P.A., 2017. Presence of human pathogens in produce from retail markets in northern Germany. Foodb. Pathog. Dis. 14 (9), 502–509. https://doi.org/10.1089/fpd.2016.2258.
Fox, P.F., Stepaniak, L., 1983. Isolation and some properties of extracellular heat-stable lipases from *Pseudomonas fluorescens* strain AFT 36. J. Dairy Res. 50 (1), 77–89.
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.
Garrido-Fernández, A., Fernández-Díez, M.J., Adams, R.M., 1997. Table Olives: Production and Processing. Chapman & Hall, London.
Hoffmann, H., Roggenkamp, A., 2003. Population genetics of the nomenspecies *Enterobacter cloacae*. Appl. Environ. Microbiol. 69 (9), 5306–5318.
Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2012. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41 (1), e1. https://doi.org/10.1093/nar/gks808.
Lee, S.A., Park, J., Chu, B., Kim, J.M., Joa, J.H., Sang, M.K., Song, J., Weon, H.Y., 2016. Comparative analysis of bacterial diversity in the rhizosphere of tomato by culture-dependent and -independent approaches. J. Microbiol. 54 (12), 823–831.
Manani, T.A., Collison, E.K., Mpuchane, S., 2006. Microflora of minimally processed frozen vegetables sold in Gaborone, Botswana. J. Food Protect. 69 (11), 2581–2586.
Mattos, F.R., Fasina, O.O., Reina, L.D., Fleming, H.P., Breidt Jr., F., Damasceno, G.S., Passos, F.V., 2005. Heat transfer and microbial kinetics modeling to determine the location of microorganisms within cucumber fruit. J. Food Sci. 70 (5), E324–E330.
Mayerhofer, H.J., Marshall, R.T., White, C.H., Lu, M., 1973. Characterization of a heat-stable protease of *Pseudomonas fluorescens* P26. Appl. Microbiol. 25 (1), 44–48.
McFeeters, R.F., Barish, A.O., 2003. Sulfite analysis of vegetables and vegetables by high-performance liquid chromatography (HPLC) with ultraviolet spectrophotometric detection. J. Agric. Food Chem. 51 (6), 1513–1517.

McFeeters, R.F., Pérez-Díaz, I.M., 2008. Microbiological preservation of cucumbers for bulk storage by the use of acetic acid and food preservatives. J. Food Sci. 73 (6), M287–M291.
Medina, E., Pérez-Díaz, I.M., Breidt, F., Hayes, J.S., 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. 80 (1), M121–M129. https://doi.org/10.1111/1750-3841.13158.
Ottesen, A.R., Gorham, S., Reed, E., Newell, M.J., Ramachandran, P., Canida, T., Allard, M., Evans, P., Brown, E., White, J.R., 2016. Using a control to better understand phyllosphere microbiota. PLoS One 11 (9), e0163482. https://doi.org/10.1371/journal.pone.0163482.
Palleroni, N.J., 1981. Introduction to the family *Pseudomonadacea*. In: Starr, M.P., Stolp, H., Trüper, H.G., Balows, A., Schlegel, H.G. (Eds.), The Prokaryotes. vol. 1. Springer-Verlag, Berlin and New York, pp. 655–665.
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, fifth ed. American Public Health Association (Chapter 51).
Pérez-Díaz, I.M., Hayes, J.S., Medina-Pradas, E., Anekella, K., Daughtry, K.V., Dieck, S., Levi, M., Price, R., Butz, N., Lu, Z., Azcarte-Peril, M., 2016. Reassessment of the succession of lactic acid bacteria in commercial cucumber fermentations and physiological and genomic features associated with their dominance. Food Microbiol. 63, 217–227. https://doi.org/10.1016/j.fm.2016.11.025.
Pham, H.N., Ohkusu, K., Mishima, N., Noda, M., Monir Shah, M., Sun, X., Hayashi, M., Ezaki, T., 2007. Phylogeny and species identification of the family Enterobacteriaceae based on *dnaJ* sequences. Diagn. Microbiol. Infect. Dis. 58 (2), 153–161.
Price, M.N., Dehal, P.S., Arkin, A.P., 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol. Biol. Evol. 26 (7), 1641–1650.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41 (D1), D590–D596.
Rastogi, G., Sbodio, A., Tech, J.J., Suslow, T.V., Coaker, G.L., Leveau, J.H., 2012. Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. ISME J. 6 (10), 1812–1822. https://doi.org/10.1038/ismej.2012.32.
Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ Preprints 4https://doi.org/10.7287/peerj.preprints.2409v1. e2409v1.
Samish, Z., Etinger-Tulczynska, R., Bick, M., 1957. The presence of bacteria within cucumber tissue. Records of the Agricultural Research Station 8, 101.
Samish, Z., Etinger-Tulczynska, R., Bick, M., 1963. The microflora within the tissue of fruits and vegetables. J. Food Sci. 28 (3), 259–266.
Scatamburlo, T.M., Yamazi, A.K., Cavicchioli, V.Q., Pieri, F.A., Nero, L.A., 2015. Spoilage potential of *Pseudomonas* species isolated from goat milk. J. Dairy Sci. 98 (2), 759–764. https://doi.org/10.3168/jds.2014-8747. Epub 2014 Dec 12.
Shi, X., Wu, Z., Namvar, A., Kostrzynska, M., Dunfield, K., Warriner, K., 2009. Microbial population profiles of the microflora associated with pre- and postharvest tomatoes contaminated with Salmonella typhimurium or Salmonella montevideo. J. Appl. Microbiol. 107 (1), 329–338. https://doi.org/10.1111/j.1365-2672.2009.04211.x. Epub 2009 Mar 9.
Sinclair, L., Osman, O.A., Bertilsson, S., Eiler, A., 2015. Microbial community composition and diversity via 16S rRNA gene amplicons: evaluating the illumina platform. PLoS One 10 (2), e0116955. https://doi.org/10.1371/journal.pone.0116955.
Veldhuis, M.K., Etchells, J.L., 1939. Gaseous products of cucumber pickle fermentations. Food Res. 4 (6), 621–630.
Vila, B., Fontgibell, A., Badiola, I., Esteve-Garcia, E., Jiménez, G., Castillo, M., Brufau, J., 2009. Reduction of *Salmonella enterica* var. Enteritidis colonization and invasion by *Bacillus cereus* var. *toyoi* inclusion in poultry feeds. Poultry Sci. 88 (5), 975–979. https://doi.org/10.3382/ps.2008-00483.
Weiss, A., Hertel, C., Grothe, S., Ha, D., Hammes, W.P., 2007. Characterization of the cultivable microbiota of sprouts and their potential for application as protective cultures. Syst. Appl. Microbiol. 30 (6), 483–493.
Wilson, K.H., Blitchington, R.B., Greene, R.C., 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. 28, 1942–1946.
Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glöckner, F.O., 2014. The SILVA and "All-species living tree project (LTP)" taxonomic frameworks. Nucleic Acids Res. 42, D643–D648.
Zago, A., Chugani, S., 2009. Pseudomonas. In Reference Module in Biomedical Sciences, Encyclopedia of Microbiology, third ed. Academic Press, pp. 245–260. https://doi.org/10.1016/B978-012373944-5.00203-0.