Food Microbiology 63 (2017) 217–227

Contents lists available at ScienceDirect

Food Microbiology

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

Reassessment of the succession of lactic acid bacteria in commercial cucumber fermentations and physiological and genomic features associated with their dominance☆

I.M. Pérez-Díaz [a, *], J. Hayes [a], E. Medina [b, c], K. Anekella [b], K. Daughtry [b], S. Dieck [b], M. Levi [b], R. Price [b], N. Butz [d], Z. Lu [e], M.A. Azcarate-Peril [d]

[a] USDA-Agriculture Research Service, SAA, Food Science Research Unit, 322 Schaub Hall-NCSU, 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, 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] Department of Molecular and Cellular Biology, Kennesaw State University, Kennesaw, GA, 30144, USA

ARTICLE INFO

Article history:
Received 26 August 2016
Received in revised form
4 November 2016
Accepted 30 November 2016
Available online 1 December 2016

Keywords:
Cucumber fermentation
Microbiota
Genome comparative analysis
Lactobacillus pentosus
Lactobacillus plantarum
Dominance

ABSTRACT

A compositional re-assessment of the microbiota present in commercial cucumber fermentation using culture independent and dependent methods was conducted, with emphasis on lactic acid bacteria (LAB). Two commercial cucumber fermentation tanks were monitored by measuring pH, dissolved oxygen and temperature, and used as sources of samples for microbial plating, genomic DNA extraction and measurement of organic acids and carbohydrates by HPLC. Six additional commercial tanks were included to identify the dominant microorganisms using molecular methods. A comparative analysis of the publically available genome sequences corresponding to the LAB found in cucumber fermentations was completed to gain an understanding of genomic features possibly enabling dominance. Analyses of the microbiota suggest Lactobacillales prevail in cucumber fermentations, including in order of prevalence *Lactobacillus pentosus*, *Lb. plantarum*, *Lb. brevis*, *Weissella* spp., *Pediococcus ethanolidurans*, *Leuconostoc* spp. and *Lactococcus* spp. It was observed that *Lb. pentosus* and *Lb. plantarum* have comparatively larger genomes, higher gene counts, uniquely distribute the ribosomal clusters across the genome as opposed to close to the origin of replication, and possess more predicted amino acids prototrophies and selected biosynthesis related genes. It is theorized that *Lb. pentosus* and *Lb. plantarum* dominance in cucumber fermentations is the result of their genetic make-up.

Published by Elsevier Ltd.

1. Introduction

Knowledge of the physiology of industrially relevant lactic acid bacteria (LAB) has increased exponentially as the result of technological advances associated with DNA sequencing, transcriptomics, proteomics, and diverse meta-analyses. Such advances generate innumerable opportunities to expand our understanding of the role of LAB in economically important fermentations. The study presented here focuses on advancing the understanding of the microbiota in modern commercial cucumber fermentations using culture independent techniques and discusses some of the physiological and genomic features possibly influencing the prevalence of selected LAB in such system.

The microbiology of cucumber fermentations has been studied since 1899, shortly after the discovery of yeasts, as active living cells responsible for transforming glucose to alcohol (Nanniga, 2010). Traditional knowledge of the cucumber fermentation microbiota provides evidence for the presence of various Gram-positive and Gram-negative bacteria, yeasts and molds at the outset. The main sources for such diverse microbiota on the cucumber exocarp are irrigation water and the growth supporting soil as well as preprocessing washing water and processing equipment. The

☆ 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.
E-mail address: Ilenys.Perez-Diaz@ars.usda.gov (I.M. Pérez-Díaz).

http://dx.doi.org/10.1016/j.fm.2016.11.025
0740-0020/Published by Elsevier Ltd.

microbiota originally present in the cucumbers initially compete for dominance, remaining active for several days or weeks depending on factors such as temperature, dissolved oxygen and the salt concentration used in the cover brines (Pérez-Díaz et al., 2014). In the majority of cases LAB dominate the fermentations for a long time, given their ability to generate and tolerate acidic conditions. LAB are known to reach maximum cell densities by the third day of cucumber fermentations brined with 5–10% NaCl (Jones et al., 1940). *Lactobacillus plantarum* was the first LAB associated with cucumber fermentations (Etchells and Jones, 1946; Rosen and Fabian, 1953). This was the result of the characterization of isolates from cover brine samples using carbohydrate fermentation patterns, carbon dioxide production ability and the description of *Lb. plantarum* by Orla-Jensen released in 1938 (Etchells and Jones, 1946; Rosen and Fabian, 1953). It is currently understood that one family and 8 species of LAB may be naturally and actively present in many vegetable fermentations, which in order of dominance are *Enterobacteriacae*, *Enterococcus* spp., *Leuconostoc mesenteroides*, *Weissella* spp., *Pediococcus pentosaceous* (previously known as *P. cerevisiae*), *P. acidilactici*, *Lb. brevis*, *Lb. plantarum* and *Lb. pentosus* (Chen et al., 2012; Costilow et al., 1956; Etchells and Goresline, 1940; Jung et al., 2014; Lee et al., 2015; Paramithiotis et al., 2014; Pederson and Albury, 1950, 1956; Plengvidhya et al., 2007; Vahlteich et al., 1935; Wouters et al., 2013). Generally, *Lc. mesenteroides* survives best in cucumber fermentations at temperatures below 18 °C (Pederson and Albury, 1950), while *P. pentosaceous* ceases to proliferate at the same temperature (Pederson and Albury, 1950). *Lb. plantarum* is more resistant to the acidic pH as compared to *Lc. mesenteroides* (McDonald et al., 1990), which precedes most other LAB in cucumber fermentations (Etchells and Goresline, 1940; Singh and Ramesh, 2008; Vahlteich et al., 1935). Ninety percent of the *P. pentosaceous* isolated from cucumber fermentation cover brines in the 1940s were obtained from samples collected early in the process, while the *Lb. plantarum* and *Lb. brevis* were isolated after the numbers of pediococci started to decline (Fig. 1). In a study conducted in 2008 by Singh and Ramesh, pediococci were detected after 30 h of a cucumber fermentation brined with 2% NaCl (m/v; after equilibration). Such event coincided with a reduction in the number of leuconostoc (Singh and Ramesh, 2008). Generally, cucumber fermentation is defined as an anaerobic process conducted by 2×10^8 CFU/mL of microorganisms, primarily LAB, found in cover brines and able to produce 0.6–1.2% lactic acid (Hamilton and Johnston, 1960).

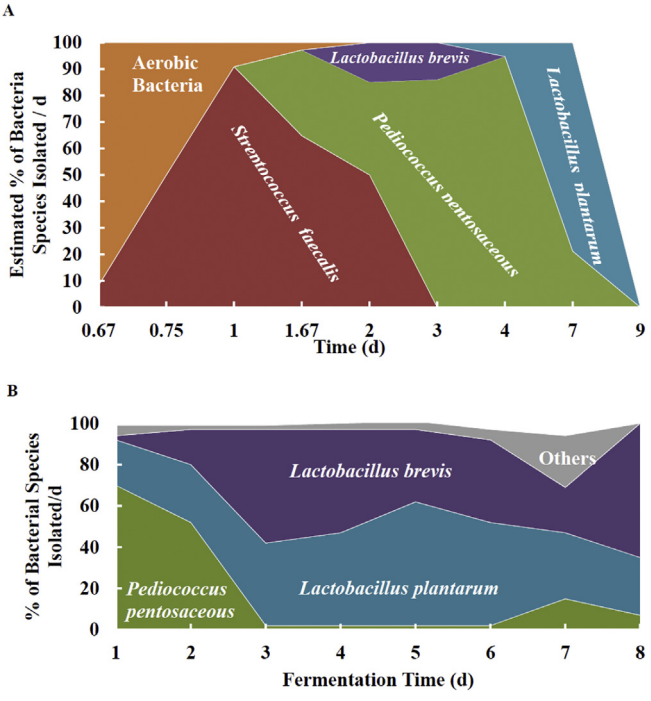

Fig. 1. Microbial succession in cucumber fermentations brined with 5% NaCl (m/v) at 30 °C (86 °F) as described by Pederson and Albury in 1950 (a) or with 5–10% NaCl (m/v) at 15–28 °C as described by Costilow et al., in 1956 for a sample size of 84 commercial fermentations (b).

Current commercial cucumber fermentation practices differ significantly from those in the 1940s, when most of the studies described above were completed. Modern commercial cucumber fermentations are carried out in 40,000 L open top tanks containing 50–70% whole cucumbers or pre-cut pieces of the vegetable, and 50-30% cover brine solution containing acetic acid, added as concentrated vinegar, and NaCl to achieve average equilibrated concentrations of 25 mM and 1.03 M (6%), respectively. Cucumbers are packed in fiberglass tanks and immediately covered with wooden boards to prevent them from floating until an equilibrium between the vegetables and cover brine solution components is achieved. Air purging is applied in combination with vinegar supplementation to reduce the incidence of bloating; a defect involving the entrapment of carbon dioxide produced during the fermentation in the whole cucumber tissue, forming hollow cavities, similar to those desired in Swiss cheese (Costilow et al., 1977; Fleming and Pharr, 1980). The decrease in the concentration of the sodium chloride used for modern cucumber fermentation as compared to the traditional process containing up to 17% NaCl (m/v), translates into a reduction of the amount of chlorides discharged to the environment. The current practice to reuse fermentation cover brines provides for the reclamation of salt and reduces the amount of water needed to process cucumbers (McFeeters et al., 1977). The wooden fermenting vessels used in the 1940s have been replaced with fiberglass tanks to reduce the cost of maintenance and loss of salty cover brines that would end up leaking into the ground.

It is an objective of this study to apply current microbial identification techniques to review and define the microbiota of modern cucumber fermentations at the commercial scale. Two commercial cucumber fermentation tanks located in the eastern and northern parts of the USA and exposed to slightly different climates were monitored to characterize the microbial ecology of cover brine samples using culture and non-culture based methods. The fermentation biochemistry was evaluated using HPLC. Other physical parameters such as pH, dissolved oxygen and temperature were also monitored. An additional 6 commercial tanks located across the USA from Texas to Michigan were included in the study to characterize the dominant microorganisms present in cover brine samples during the peak of the fermentation, using molecular biology methods.

The second objective of this study was to review the current knowledge of the genomics of LAB to gain an insight into the genetic basis for the dominance of particular LAB in cucumber fermentations. Significant progress has been made in the comparative genomics of LAB and the evolutionary patterns associated with this group of industrially relevant bacteria. A comparison of the publically available genome sequences corresponding to the LAB prevailing in cucumber fermentations was completed.

Comprehensively, this study provides an update of the microbiota in commercial cucumber fermentations and describes some of the known physiological and genomic features that allow *Lb. pentosus*, *Lb. plantarum* and *Lb. brevis* to prevail in such system.

2. Materials and methods

2.1. Commercial scale fermentations

Commercial scale fermentations were carried out in 28,400–37,854 L open-top fiberglass tanks containing 50–60% whole cucumbers (size 3A: 39–51 mm diameter and 13.7 cm long), and 50 to 40% cover brine solutions. All of the tanks studied were located in different geographic regions within the USA, brined with ~1.04 M NaCl and subjected to air purging for the first 21 days of the fermentations, with a routine of 20 h on and 4 h off at variable flow rates. Recycled brine and a mixture of size 3A and 2B whole cucumbers (27–38 mm diameter and 12.7 cm long) were used for the fermentation in Tank 1, while acetic acid was added to the cover brine formulation in Tank 2. Acetic acid was added to the cover brine in Tank 2 as a 20% vinegar solution to 15 mM. A *P. pentosaceous* starter culture was added in the cover brine of Tank 5. Cushion cover brine was added into the tanks, prior to the addition of the vegetables. In-tank vegetables were immediately covered with wooden boards and air-purging circulation used to mix-in rain water. Tanks were replenished with cover brine prepared at the equilibrated concentrations to compensate for volume losses due to evaporation.

2.2. Sample collection

On each sampling day (1, 3, 7 and 14) approximately 50 mL of cover brine samples were taken from an average of 1.067 and 2.286 m from the cover brine surface via a perforated pipe placed next to the air purging system in the tanks. Four cover brine samples were taken from two independent locations of two fermentation tanks over a 14 d period after purging for 1 h, for a total of 16 samples. Cover brine samples were collected after ~100 mL of the cover brines had moved through the sampling tubing. The sampling apparatus consisted of a buffer siphon PVC pump (BSP-1000; CBS Scientific Inc., San Diego, CA) connected to 1/2″ diameter thin walled, Tygon® tubing. The samples were placed in 50 mL sterile conical tubes and immediately transported to our laboratory for same day processing. Additional cover brine samples were collected by processors, delivered to our laboratory via expedited mail and processed upon arrival.

2.3. Measurement of pH, temperature and dissolved oxygen

Measurements of pH were done from cover brine samples in the laboratory using a Fisher Accumet pH meter (model AR25, Fisher Scientific, Pittsburgh, PA) combined with a Gel-Filled Pencil-Thin pH Combination Electrode (Acumet Fisher Scientific). Temperature and dissolved oxygen were measured on-site, upon sample collection, at variable depths through the perforated pipes placed in the tanks for this study. 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's instructions. Temperature data were collected using the dissolved oxygen probe meter.

2.4. Analysis of fermentation metabolites

Fermentation cover brine samples were spun at 12,000 rpm for 10 min in an Eppendorf benchtop refrigerated centrifuge 5810R (Hamburg, Germany) to remove residual particulate matter, and the supernatant diluted 10X with a 0.03 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) (McFeeters and Barish, 2003). The column was heated to 37 °C and eluted with 0.03N sulfuric acid at a flow rate of 0.6 mL/min. A Thermo Separations UV6000 diode array detector (Spectra System Thermo Scientific, Waltham, MA) set to collect data at 210 nm was used to detect malic, lactic, acetic, propionic, and butyric acids. A refractive index detector (model 410, Waters Corp., Millipore Corp., Billerica, MA) connected in series with the diode array detector was used to measure glucose, fructose, and ethanol. External standardization of the detectors was done using 4 concentrations of the standard compounds for the acids and carbohydrates measured.

2.5. Analysis of microbial counts

Aseptically collected brine samples were serially diluted in saline solution (0.85% NaCl (m/v)), and spiral plated using an Autoplate 4000 (Spiral Biotech, Norwood, MA). LAB were enumerated on Lactobacilli MRS agar (cat. 288130, Difco™, Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 0.001 cycloheximide (v/v) (0.1% stock solution, Oxoid, Basingstoke, England) to prevent yeasts growth. The MRS agar plates were incubated anaerobically in a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, MI) at 30 °C for 48 h. Colony forming units on MRS plates were quantified with a Q-Count plate counter (Spiral Biotech, Norwood, MA). One hundred isolated colonies per time point were picked and streaked on Lactobacilli MRS agar for purification prior to the preparation of frozen stocks in Lactobacilli MRS broth containing 15% glycerol (v/v) (Cat No. G5516, Sigma Aldrich, St. Louis, MO). A total of 800 frozen stocks were transferred to 1 mL of MRS broth, individually, and incubated at 30 °C for 48 h under static conditions to obtain bacterial pellets for DNA extraction.

2.6. Isolates identification by the partial sequencing of the 16S rRNA gene

DNA extraction from each pure culture in MRS broth was conducted using an InstaGene Matrix DNA extraction kit (Bio-Rad) following the manufacturer's instructions. Extracted DNA was used for the partial amplification of the 16S *rRNA* gene sequence for identification. The PCR mixture contained 2X master mix (Bio-Rad), 10 μL of the resulting total genomic DNA extracted from each of all the 800 bacterial isolate, and 0.6 μM of primers 8f (5-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Wilson et al., 1990). The PCR steps consisted of 4 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 2 min at 57 °C, and 2 min at 72 °C, with a final extension step of 7 min at 72 °C. Amplicons were stored at 4 °C until sequenced by Eton Bioscience Inc. (Durham, NC). Sequence data were formatted and analyzed using 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 (accession no. JX826519 to JX826575, KX648716 to KX649067, KX649068 to KX649146, KX649150, and KX649153 to KX649202).

2.7. recA amplification screening for the discrimination of Lb. plantarum and Lb. pentosus

The *recA* gene was partially amplified as described by Torriani et al. (2001) using genomic DNA isolated from a subset of 161 isolates ambiguously identified as *Lb. plantarum*/*pentosus* using the partial sequence of the 16S *rRNA* gene. Genomic DNA was extracted as described above for the partial sequencing of the 16S *rRNA* gene.

Torriani's multiplex PCR protocol yields amplicons of 318, 218 and 107 bp for *Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum* genomic DNA, respectively. The subset utilized for the *recA* screening was composed of all *Lb. plantarum*/*pentosus* isolates found to be resistant to bacteriophage infection (Lu et al., 2012).

2.8. 16S rRNA gene amplicon sequencing using Ion Torrent PGM platform for fermentation cover brine samples

Genomic DNA was extracted for the amplification of the 16S *rRNA* gene pool as described by Medina et al. (2016). Amplicons were barcoded (Table S1) 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 described in Table S1: 5′CATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNAGAGTTT-GATCCTGGCTCAG3′, and 5′CCTCTCTATGGGCAGTCGGTGATGCTGC CTCCCGTAGGAGT3′. The ribosomal amplicon sequencing analysis using the Ion Torrent PGM sequencing platform targeted the V1-V2 fragment of the 16S *rRNA* gene. Amplicon high-throughput sequencing data analysis was conducted from raw Ion Torrent fastq files with accession no. KF998365 - KF998547. QIIME was used to calculate alpha diversity on rarefied Operational Taxonomic Units (OTUs) to assess sampling depth coverage using the observed species, Shannon and phylogenetic diversity (*PD*) metrics shown in Table S1.

2.9. AthoGen analysis of bacterial populations

Broad bacterial culture independent testing was conducted by AthoGen (Carlsbad, CA) using the total DNA extracted from cover brine samples as described for the Ion Torrent analysis. A total of 8 cover brine samples from 6 independent sources were analyzed. The 16S *rRNA* gene 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 is ~10^4 CFU/mL.

2.10. Comparative genome sequences analyses

The genome sequences corresponding to the species detected in the fermentation cover brine samples studied were analyzed using the Joint Genome Institute-Integrated Microbial Genomics and Metagenome (IMG/M) online tool (Markowitz et al., 2014a, 2014b). Specific tools used are those for genome statistic comparative analysis, abundance profile for COG functions, and individual genome amino acid biosynthetic pathway annotations and reconstructions.

3. Results and discussion

3.1. Reassessment of the microbiota present in modern cucumber fermentations

Relevant differences between the two commercial scale cucumber fermentations studied included the utilization of recycled cover brine in Tank 1, containing 12.8 ± 2.3 mM lactic acid, and the supplementation of the fresh cover brine in Tank 2 with 22.2 ± 1.7 mM acetic acid, to facilitate texture retention (Fleming et al., 1978). Such differences resulted in an initial cover brine pH of 4.39 ± 0.01 and 3.70 ± 0.02, respectively, prior to the equilibration of the cover brines and vegetables (Fig. 2A). Upon equilibration of the fermentation vessel contents by day 3, the pH differential

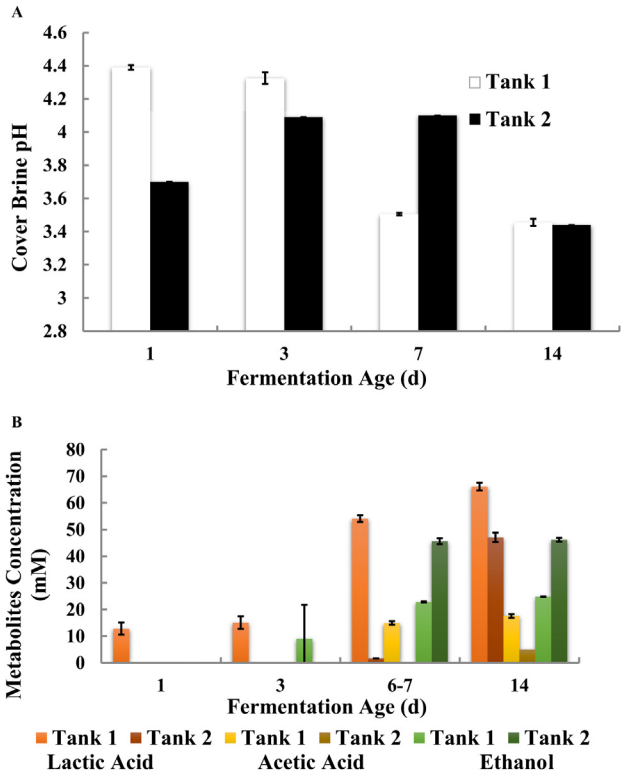

Fig. 2. Commercial cucumber fermentation biochemistry including pH measurements (A) and concentration of acetic acid, lactic acid and ethanol (B) from cover brine samples collected at various time points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was 0.21 ± 0.02 units (Fig. 2A). A final pH of 3.44 ± 0.5 was measured in cover brine samples after 14 days, suggesting that complete conversion of carbohydrates occurred in both tanks (Fig. 2A).

The analysis of fermentation metabolites suggest there were significant differences between the two vessels studied based on the conversion of an estimated 40.8 ± 2.3 mM glucose and 44.5 ± 3.1 mM fructose naturally present in the fresh vegetables. A total of 85.3 ± 2.7 mM of combined carbohydrates in the fresh vegetables were estimated to represent 51.2 ± 2.7 mM in the fermentation vessel, after equilibration of the vegetables with the cover brine (60:40 pack out ratio w/v). Those carbohydrates were converted to 61.9 ± 9.3 mM lactic acid, 17.4 ± 1.9 mM acetic acid and 21.4 ± 0.9 mM ethanol in Tank 1, and 48.5 ± 2.9 mM lactic acid, 5.2 ± 1.8 mM acetic acid and 46.2 ± 0.2 mM ethanol in Tank 2 after adjusting for the original composition of the cover brines used (Fig. 2B). Evidently, more lactic acid production and less acetic acid and ethanol formation occurred in Tank 1, which was packed with recycled cover brine, compared to Tank 2, in which acetic acid was added to the cover brine (Fig. 2B). Lactic acid production occurred faster in Tank 1 compared to Tank 2 (Fig. 2B). Additionally, malic acid, which was naturally present in cucumbers at 12.3 ± 1.9 mM, was not detected in Tank 1 by day 7 of the fermentation after reaching 3.5 ± 0.4 mM on day 3; while remaining at 4.3 ± 0.1 mM in Tank 2 by day 6 of the fermentation and disappearing by day 14.

The characterization of the microbial populations using various techniques confirms observations made by others in that the dominant microbial community in cucumber fermentations are lactic acid bacteria such as *Lb. plantarum*/*pentosus*, *Lb. brevis*, and *Pediococcus* spp. (Table 1 and Figs. 4 and 5), which reached maximum cell densities by days 6 and 7 at 7.84 ± 0.56 Log CFU/mL,

Table 1
Identification of colonies randomly selected from MRS plates, streaked for purification and classified using the partial sequence of the 16S *rRNA* gene. The first value provided for each row and column corresponds to Tank 1. The value provided after the backslash represents the number of colonies isolated from Tank 2 for the respective identity.

Bacterial identification	No. of isolates					
	Fermentation age (days)					
	1	3	7	14	30	90
Predominant						
Lactobacillus plantarum/pentosus	39/0	50/3	46/24	58/31	88/4	16/0
Lactobacillus brevis		8/0	51/0	32/33	0/6	1/0
Pediococcus ethanolidurans	13/0	2/0	0/1	6/18	9/80	27/0
Excluded						
Enterococcus sp.	1/0	0/1				
Leuconostoc lactis	0/69					
Leuconostoc mesenteroides	5/7	0/17				
Leuconostoc fallax	5/8	0/1				
Leuconostoc citreum	2/3	0/3				
Weisella hellenica	2/0	1/0				
Lactobacillus graminis		0/3				
Leuconostoc holzapfelii		0/3				
Weisella paramesenteroides		26/3	0/4			
Pediococcus pentosaceus		0/1	0/11			
Lactobacillus alimentarius			2/0			
Weisella cibaria	1/2	7/26	0/17			
Lactococcus lactis	6/0	0/32	0/1			
Persistant						
Lactobacillus namurensis	23/0	1/0				2/0
Bacillus sp.						5/0
Lactobacillus buchneri						4/0
Lactobacillus paracollinoides						1/0
Lactobacillus zymae						1/0
Streptococcus parasanguinis					0/1	

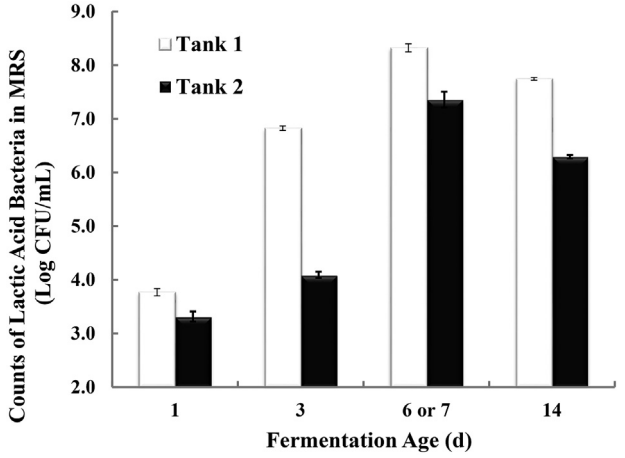

Fig. 3. Counts of lactic acid bacteria from MRS plates inoculated with cover brine samples collected at various time points. Results shown are averages and standard deviations for duplicates of two independent replicates. Minimal detection limit was 2.4 log CFU/mL.

with the highest variability on day 3 (Fig. 3). Table 1 and Fig. 4 also confirm the presence of *Leuconostoc* spp. at the beginning of cucumber fermentation (days 1 and 3), including not only the previously reported species, *mesenteroides*, but also *fallax*, *citreum*, and *holzapfelii*. *Lactococcus* spp. were also detected, in particular *Lc. lactis* (Table 1 and Fig. 4). *Weisella mesenteroides* and *W. cibaria* were found to overlap with *Leuconostoc* spp. during the third day of the fermentation and persisted in detectable numbers until day 7 on which the lactobacilli prevailed (Table 1 and Fig. 4). While in one fermentation *Lb. plantarum/pentosus* were detected in higher

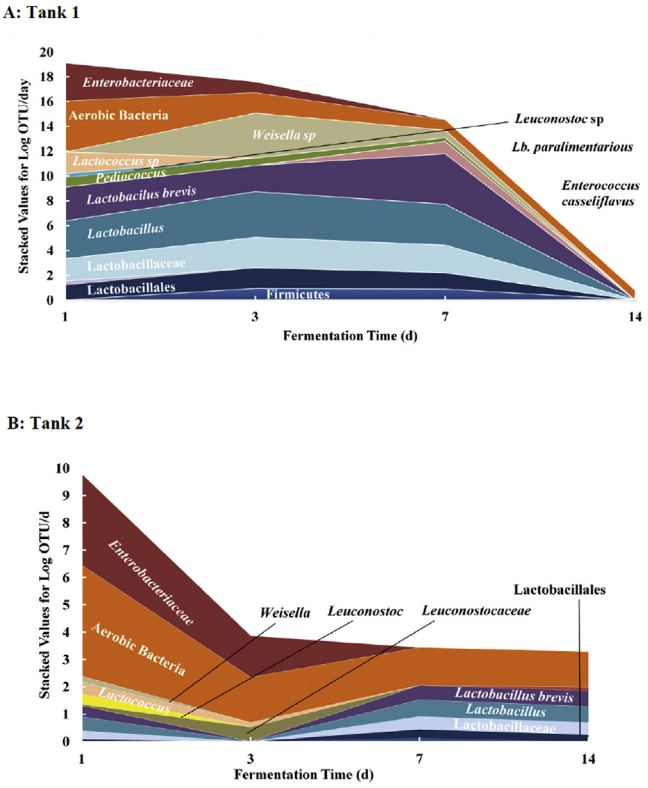

Fig. 4. Results of the 16S *rRNA* gene amplicon sequencing data analysis for cover brine samples collected at variable time points from Tanks 1 (A) and Tank 2 (B). *Lb.*: *Lactobacillus*; *E.*: *Enterobacter*.

abundance from day 1 (Table 1), in the second slower fermentation the abundance of the lactobacilli was obviously increased on day 7 and preceded by *Leuconostoc* spp. and *Weisella* spp. which dominated the fermentation on day 3 (Table 1 and Fig. 4). *Lb. brevis* persisted in both fermentations during the monitoring period, although it did not outcompete other LAB. *Lb. plantarum/pentosus* and *Lb. brevis* were also found to prevail in 7 out of 8 other commercial cucumber fermentations studied (Fig. 5). According to 16S *rRNA* gene amplicon sequencing data, *Pediococcus* spp. were more abundant in the fermentation weakly colonized by *Leuconostoc* spp. (Fig. 4). *P. ethanolidurans* persisted up to 90 days, while *P. pentosaceous*, previously associated with cucumber fermentation, was only detected until day 7 (Table 1). *Lb. plantarum/pentosus* and *P.*

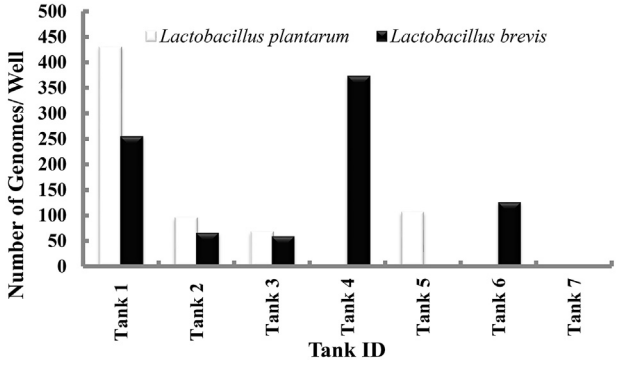

Fig. 5. Results for the AthoGen Broad Bacterial Testing from cover brine samples collected on day 7 of fermentations conducted at various locations across the United States of America.

ethanolidurans were detected well after the primary fermentation was completed along with *Lb. namurensis* (also detected on day 1), *Bacillus* sp., *Lb. buchneri*, *Lb. paracollinoides*, *Lb. zymae* and *Streptococcus parasanguinis* (Table 1).

Physiological features associated with the succession of LAB in modern cucumber fermentations. Lactobacilli and pediococci belong to the family of Lactobacillaceae. These microorganisms have complex nutritional requirements for amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters, and fermentable carbohydrates (deVos et al., 2009). Although, their optimum growth temperature range fluctuates between 30 and 40 °C, some strains may also grow at temperatures as low as 2 °C and as high as 53 °C (deVos et al., 2009), which confirms that cucumber fermentations in outdoor open top tanks may proceed during multiple seasons depending on the geographical location. Having the ability to produce lactic acid, these microorganisms have an optimal pH between 5.5 and 6.2, with growth generally occurring at pH 5.0 or below (deVos et al., 2009). *Lb. plantarum* in particular is known to grow in cucumber fermentations to a pH of 3.3 with the ability to produce acid to a pH of 3.1 (McDonald et al., 1993; Passos et al., 1994). The aciduric nature of these microorganisms is understood to represent an advantage for dominance in cucumber fermentations, aiding in the exclusion of undesired microorganisms such as *Enterobacteriaceae*, whose numbers declined from 1623 ± 747 Operational Taxonomic Unit (OTU) on day 1 of the fermentations to undetectable levels by day 7 (Fig. 4).

While the dominance of *Lb. plantarum* in cucumber fermentation was well documented in the 1940s, modern techniques for the taxonomical classification of LAB have revealed that *Lb. plantarum* is closely related to, but distinguishable from *Lb. pentosus* (Dellaglio et al., 1975; Huang et al., 2010; Kandler and Weiss, 1986; Torriani et al., 2001). The average sequence similarity for five type strains belonging to the *Lb. plantarum* cluster, which includes *Lb. pentosus*, *Lb. paraplantarum* and *Lb. plantarum*, has been defined as 99.4% by Huang et al. (2010). The inability to discriminate species of this group using the 16S *rRNA* gene amplicon sequencing was likely influencing the ambiguous identification of a significant number of lactobacilli in both fermentations (Fig. 4). Attempts to identify 359 colonies isolated from MRS agar plates inoculated with cucumber fermentation cover brine samples using sequencing of the 16S *rRNA* gene amplicon resulted in ambiguities between *Lb. plantarum* and *Lb. pentosus* (Table 1). Unlike most *Lb. plantarum*, *Lb. pentosus* is able to degrade certain pentose carbohydrates, including arabinose, melezitose and xylose (deVos et al., 2009; Zanoni et al., 1987). Kandler and Weiss (1986) demonstrated that the two species are genomically distinct. This observation was confirmed by Zanoni et al. (1987) using DNA-DNA molecular hybridization. Alignments of the *Lb. plantarum* and *Lb. pentosus* genome sequences available to date suggest the later encodes for CRISPR system, a putative LytSR two-component regulatory system associated with biofilm formation and capsular polysaccharides not found in *Lb. plantarum* (Anukam et al., 2013). Methods for the differentiation of the two species were proposed and developed after most of the microbial ecology studies for commercial cucumber fermentations were completed in the 1940s. Thus, it was imperative to revisit the results obtained from the monitoring of the two commercial fermentations studied here. The screening of the *recA* gene size, proposed by Torriani et al. (2001) as a primary tool for the discrimination of *Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum*, suggested that 89% of the isolates scrutinized are *Lb. pentosus* (Table 2). This observation indicates that *Lb. pentosus* has the ability to outcompete *Lb. plantarum* in modern commercial cucumber fermentations.

Lb. pentosus and *Lb. plantarum* are facultative heterofermentative LAB commonly found in plant material and sewage, and considered spoilage organisms in certain fermented foods. As reflected in Fig. 2A the main fermentation catabolite is lactic acid followed by ethanol and acetic acid. As facultative heterofermentative LAB, *Lb. plantarum* and *Lb. pentosus* are expected to produce mainly lactic acid from the metabolism of glucose and fructose via the Embden-Meyerhof pathway, although they may also produce acetic acid and ethanol in the presence of oxygen and under glucose limitation, via the phosphoketolase pathway. Both species are able to produce exopolysaccharides, which may aid in their survival and dominance in the extremely acidic conditions, characteristic of cucumber fermentations (Cerning et al., 1994; Donot et al., 2012; Harutoshi, 2013).

Proliferation of obligate heterofermentative LAB such as *Lb. brevis* in vegetable fermentations is presumed to evolve from the energetic advantages associated with the production of acetic acid in lieu of lactic acid. Obligate heterofermentative lactobacilli metabolize hexoses, such as the glucose and fructose naturally present in fresh cucumbers, to lactic acid, acetic acid, and carbon dioxide via the phosphogluconate pathway (deVos et al., 2009). Ethanol can also be produced by obligate heterofermentative lactobacilli in the presence of oxygen (deVos et al., 2009). Pentoses are fermented to lactic acid and acetic acid by the pentose phosphate pathway. Some acetic acid (Tank 1: 17.4 ± 1.9 mM and Tank 2: 5.2 ± 1.8 mM) and more than 20 mM ethanol were detected in the fermentations on day 7. Dissolved oxygen levels were measured at 0.85 ± 0.3 and 3.35 ± 0.3 mg/L during the first 3 d of the fermentations in Tank 1 and Tank 2, respectively, declining to 0.39 ± 0.09 mg/L on day 7. Additionally, the growth of *Lb. brevis* occurred between days 3 and 7 (Fig. 4). This circumstantial evidence suggests that the obligately heterofermentative LAB significantly contributed to the production of acetic acid, ethanol and, consequently, carbon dioxide in the fermentations studied.

The fact that *Pediococcus* spp. can thrive in cucumber fermentations represents an advantage in the reduction of the incidence of fermented cucumber bloating. *Pediococcus* spp. are homofermentative LAB producing L- and D-lactic acid and able to grow at pH values as low as 4.5 (deVos et al., 2009). Conversion of glucose and fructose to lactic acid exclusively reduces the production of carbon dioxide during the fermentation, which has been identified as the culprit in the formation of hollow cavities inside the vegetables, a defect commonly known as bloating (Fleming and Pharr, 1980). Two of the pediococcal species found in the fermentations studied, *acidilactici* and *pentosaceous*, are able to proliferate at temperatures as high as 45 °C in the presence of up to 10% NaCl (deVos et al., 2009). Together the ability to sustain growth at low pH values, relatively high temperatures and salt concentrations makes this genus an ideal candidate for starter cultures for cucumber fermentations, in particular, for tank yards located in the southern part of the USA, where summer temperatures may exceed 37 °C. Additionally, this genus seems to be less prone to bacteriophage infections as compared to the dominant lactobacilli present in cucumber fermentations (Lu et al., 2012). *P. ethanolidurans* was the prevalent pediococci in both of the fermentation studied, but with a cell density lower than those observed for the lactobacilli (Table 1). This species was proposed by Liu et al., in 2006, given the ability to tolerate 6.5% ethanol at pH 3.5, but not in the presence of 4% NaCl. The *P. ethanolidurans* type strain was isolated from a distilled-spirit-fermenting cellar, also a plant based fermentation.

Although, a significant number of *Leuconostoc* spp. are able to produce exopolysaccharides, which helps in survival under stressful conditions similar to those found in cucumber fermentations, the genus was not found to dominate in cucumber fermentations (Table 1 and Fig. 4). *Leuconostoc* spp. are known to be non-acidophilic, even though their growth may proceed at pH 4.5

Table 2
Results of the *recA* amplification test for the discrimination of isolates ambiguously identified as *Lactobacillus plantarum*/*pentosus*.

Cover brine samples source	Fermentation age (d)	No. of isolates ambiguously identified as *Lb. plantarum*/*pentosus* using the partial 16S rRNA gene sequence	No. of isolates identified as *Lb. plantarum* or *Lb. pentosus* using *recA* amplicon size		
			Lb. pentosus	*Lb. plantarum*	No amplicon
Tank 1 (n = 190)	1	39	38	0	1
	3	49	44	5	0
	7	46	42	3	1
	14	56	56	0	0
	% composition		95%	4%	1%
Tank 2 (n = 62)	1	0	–	–	–
	3	3	0	1	2
	7	25	11	13	1
	14	34	34	0	0
	% composition		71%	22%	7%

(deVos et al., 2009). The growth ceases with an internal pH of 5.4–5.7 (McDonald et al., 1991). The optimum growth temperature for *Leuconostoc* spp. is 20–30 °C, well below the ideal range for the competing *Pediococci* spp. (deVos et al., 2009). The growth of these LAB is also stimulated by anaerobic conditions (deVos et al., 2009), which are absent in cucumber fermentation tanks purged with air during the active fermentation period. Most of the members of this genus are capable of utilizing fructose, naturally present in cucumbers, as an electron acceptor producing mannitol (Kandler and Weiss, 1986; Wisselink et al., 2002). Mannitol production was not observed in the fermentations studied. The *Leuconostoc* spp. detected in the cucumber fermentations studied, including *Lc. mesenteroides*, *Lc. fallax*, *Lc. citreum*, and *Lc. holzapfelii*, have been found in other vegetable fermentations such as sauerkraut and kimchi (Barrangou-Poueys et al., 2002; Choi et al., 2003). These species are nonhalophilic and sensitive to NaCl concentrations at/or slightly below 6.5% (deVos et al., 2009; De Bruyne et al., 2007). Although some of these species can grow and reduce pH to 3.9, most of them cannot proliferate in more acidic conditions. *Lc. holzapfelii* was originally isolated from an Ethiopian coffee fermentation and found to be closely related to *Lc. citreum* (De Bruyne et al., 2007). These microorganisms are also capable of decarboxylating L-malic acid to L-lactic acid in the presence of fermentable carbohydrates. Thus, their lack of dominance in cucumber fermentations is desirable, given that it may translate into lower CO_2 production and a reduced incidence of cucumber bloating.

Leuconostoc spp. were detected in Tank 2, where *Pediococcus* spp. were absent. Differences between the two fermentations include initial pH (more acidic in Tank 2), the extent of time the fresh cucumbers were in the tanks prior to the addition of the cover brines, and temperature. *Lc. mesenteroides* prevails in cucumber fermentations at temperatures from 7 to 10 °C (Pederson and Albury, 1950) containing low NaCl levels (2.5–3.75% m/v). Although, some differences in the temperature profiles were found for the two commercial cucumber fermentations studied, it is also possible that the extended incubation of the fresh cucumbers had in Tank 2 prior to brining, favored the proliferation of the more acid and salt sensitive *Leuconostoc* spp. in this tank. The studies by Pederson and Albury (1956) and Costilow et al. (1956) also detected the exclusive presence of *Lc. mesenteroides* or *Pediococcus* spp. Although, differences in temperature and salt levels, may be influencing the cell densities of these two LAB in cucumber fermentations, other factors may be impacting their ability to compete (Pederson and Albury, 1956). Dominance of pediocin producing *Pediococcus* spp. in laboratory scale cucumber fermentations brined with 2% NaCl (m/v) concomitant with the absence of *Lc. mesenteroides* has been observed (Singh and Ramesh, 2008). Regardless of the specific factors influencing the proliferation of these two LAB, both populations represented a minor component of the microbiota dominated by the lactobacilli.

Members of *Weisella* (the obligate heterofermentative short rods) were detected in both commercial fermentations studied (Table 1 and Figs. 4 and 5). *Weisella* spp. are known to grow at temperatures as low as 15 °C and as high as 45 °C (Vincenzina et al., 2015), suggesting that fluctuations in temperature in outdoor tanks should not significantly impact their ability to proliferate in cucumber fermentations. However, Fig. 4 shows the presence of *Weisella* spp. in relatively low abundance as compared to lactobacilli. Although *Weisella* spp. were found to represent a significant portion of the microbiota in sauerkraut fermentations during the heterofermentative stage, it inconsistently dominated over *Leuconostoc* spp. (Plengvidhya et al., 2007). The four *Weisella* species (*confusa*, *paramesenteroides*, *cibaria* and *hellenica*) found in the fermentations studied are phylogenetically closely related based on the 16S *rRNA* gene sequence. Further physiological studies are needed to understand the growth limitations encountered by these species in cucumber fermentations.

A number of lactobacilli, previously associated with fermented cucumber spoilage or undesired secondary fermentation, were not isolated from samples collected early in the fermentation (Table 1), which included *Lb. alimentarius*, *Lb. graminis*, *Lb. buchneri*, *Lb. paracollinoides*, and *Lb. zymae*, despite their ability to conduct the energetically advantageous heterofermentation. *Lb. buchneri* was found able to degrade the lactic acid produced during cucumber fermentation into 1,2-propanediol (Johanningsmeier and McFeeters, 2013). Other lactobacilli such as *Lb. rapi* were found to convert 1,2-propanediol to acetic acid. *Lb. buchneri* is also able to form undesirable biogenic amines (Molenaar et al., 1993; Sumner et al., 1985) and produce ethylcarbamate precursor from arginine to derive energy (deVos et al., 2009). Most of these lactobacilli are resistant to 6% NaCl or more and have been isolated from other food matrices. Discovery of the intrinsic limiting growth factors for these spoilage-associated-lactobacilli in cucumber fermentations is relevant to the development of effective strategies for their inhibition during bulk-storage.

3.2. Comparative genomic analysis of the prevailing LAB in modern cucumber fermentations

The available genome sequences of the LAB species detected in the cucumber fermentations studied were explored to further understand the features enabling them to thrive and in some cases prevail. Evidently, the robustness of cucumber fermentation relies on the ability of lactobacilli to prevail regardless of a specific ambient temperature. Securing a niche in cucumber fermentations is likely associated with their ability to produce lactic acid and

Table 3
Comparative analysis of the genome sequences statistics for the LAB prevailing in cucumber fermentations.

Genome name	No. of genomes included	Genome size	Gene count	CRISPR count	5S *rRNA* count	16S *rRNA* gene count	23S *rRNA* count	*tRNA* count	Pseudo genes %	TC %
Lactobacillus plantarum		3273640 ± 157023	3230 ± 199	1 ± 2	3 ± 2	3 ± 2	3 ± 2	61 ± 9	1 ± 5	12 ± 2
Lactobacillus pentosus	2	3571435 ± 205220	3299 ± 231	5 ± 0	6 ± 1	3 ± 3	4 ± 1	66 ± 6	0 ± 0	13 ± 0
Lactobacillus brevis	13	2631140 ± 206544	2715 ± 2715	2 ± 2	3 ± 3	3 ± 3	3 ± 3	62 ± 62	0 ± 0	11 ± 2
Lactobacillus buchneri		2582275 ± 171267	2580 ± 275	3 ± 2	3 ± 2	3 ± 2	3 ± 2	60 ± 3	1 ± 1	11± 3
Lactobacillus namurensis	1	2593418	2465	4	1	1	1	57	0	13
Lactobacillus panis	1	2082789	2294	3	5	11	16	61	0	10
Lactobacillus zymae	1	2700824	2540	7	4	1	1	58	0	13
Pediococcus ethanolidurans	2	2266351 ± 14073	2238 ± 30	1 ± 1	2 ± 1	2 ± 1	3 ± 2	52 ± 9	0 ± 0	11 ± 5
Pediococcus pentosaceus	5	1782394 ± 38683	1794 ± 38	0 ± 0	3 ± 2	3 ± 2	4 ± 2	53 ± 3	0 ± 1	12 ± 2
Pediococcus damnosus	1	2231216	2266	2	2	1	1	56	0	12
Pediococcus acidilactici	7	1970138 ± 34044	1993 ± 111	1 ± 0	2 ± 2	2 ± 2	3 ± 2	43 ± 20	1 ± 1	12 ± 2
Leuconostoc mesenteroides	12	1891565 ± 130168	1994 ± 135	1 ± 0	3 ± 1	2 ± 1	2 ± 1	60 ± 9	1 ± 4	12 ± 2
Leuconostoc citreum	6	1843339 ± 106019	1911 ± 129	0 ± 0	2 ± 2	2 ± 2	2 ± 2	57 ± 9	0 ± 0	13 ± 1
Leuconostoc fallax	1	1638971	1604	0	1	1	1	50	0	12
Weissella cibaria	3	2455600 ± 132428	2425 ± 180	1 ± 0	7 ± 3	6 ± 4	6 ± 4	86 ± 10	0 ± 0	13 ± 1
Weissella confusa	2	2247997 ± 50951	2202 ± 50	0 ± 0	2 ± 0	2 ± 0	5 ± 0	65 ± 11	0 ± 0	13 ± 0
Weissella hellenica	2	1866054 ± 70098	1906 ± 86	0 ± 0	8 ± 1	5 ± 0	3 ± 1	70 ± 2	0 ± 0	10 ± 5
Weissella paramesenteroides	1	1962173	2017	0	1	1	1	62	0	14

possibly other antimicrobial compounds and/or create physicochemical conditions that prevent the survival of competing microorganisms. It is well documented that production of lactic acid by LAB and resistance to acidic pH are the main factors in controlling the growth of undesired microorganisms in food fermentations and likely the succession of specific species. The fundamental question of how LAB take over a fermentation by quickly producing lactic acid remains unanswered.

It is relevant to mention that the 6 Lactobacillales dominating in cucumber fermentations are phylogenetically and evolutionarily related (Makarova et al., 2006; Zhang et al., 2011), suggesting that their core genome sequences possess specific elements that are advantageously or appropriately equipping them to proliferate in cucumber fermentations. Table 3 shows the results of the comparative analysis for the genome sequences belonging to the Lactobacillales prevailing in cucumber fermentations. The *Lb. plantarum* and *Lb. pentosus* genomes published to date present genome sizes bigger than those for the other prevailing LAB (Table 3). It has been proposed that while the *P. pentosaceus* and *Leuconostoc* genomes are prone to ancestral gene loss, *Lb. plantarum* counterbalances such losses via duplication and horizontal gene transfer (Marakova and Koonin, 2007), possibly maintaining biosynthetic pathways needed in vegetable fermentations. Additionally, the dominant species within each of the genera identified, including, in order of prevalence, *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella*, have larger genome sizes as compared to the competing species within their respective genus (Table 3), suggesting that gene loss and metabolic simplification may limit their performance in the cucumber fermentation niche.

In line with the larger *Lb. plantarum* and *Lb. pentosus* genomes are a higher number of total gene counts accompanied by a reduced number of pseudogenes (Table 3) as compared to that found in *Lactobacillus delbrueckii* and other LAB (Makarova et al., 2006). This observation suggests some stability in the genomes of the LAB dominating in cucumber fermentations, which is possibly reflecting the lack of pressure in a system where natural fermentations are favored over the use of starter cultures.

The putative *rRNA* cluster counts are higher in the *Lb. plantarum* and *Lb. pentosus* genomes and some of the *Pediococcus* spp. genomes. The *rRNA* clusters are well distributed from the origin of

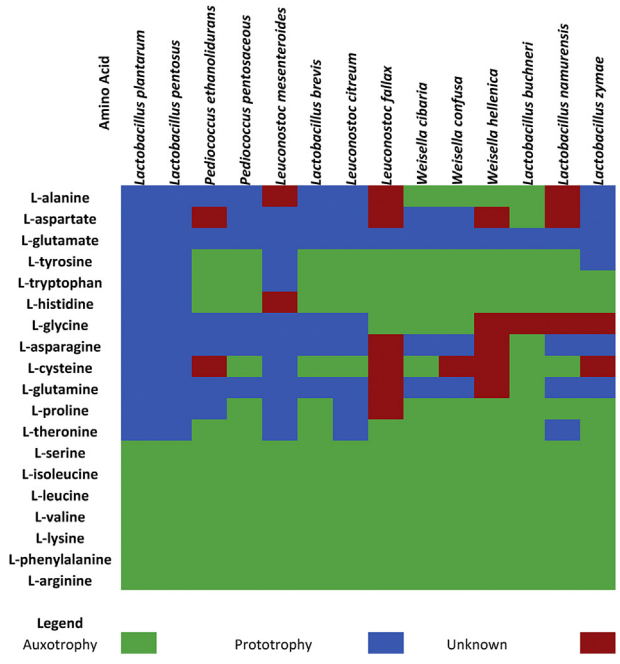

Fig. 6. Summary of the predicted amino acid auxotrophies and prototrophies from the genome sequences corresponding to the lactic acid bacteria prevailing in commercial cucumber fermentations. Green, blue and red boxes represent auxotrophies, prototrophies and unknown status, respectively. (For interpretation of the references to colour in this figure legend , the reader is referred to the web version of this article.)

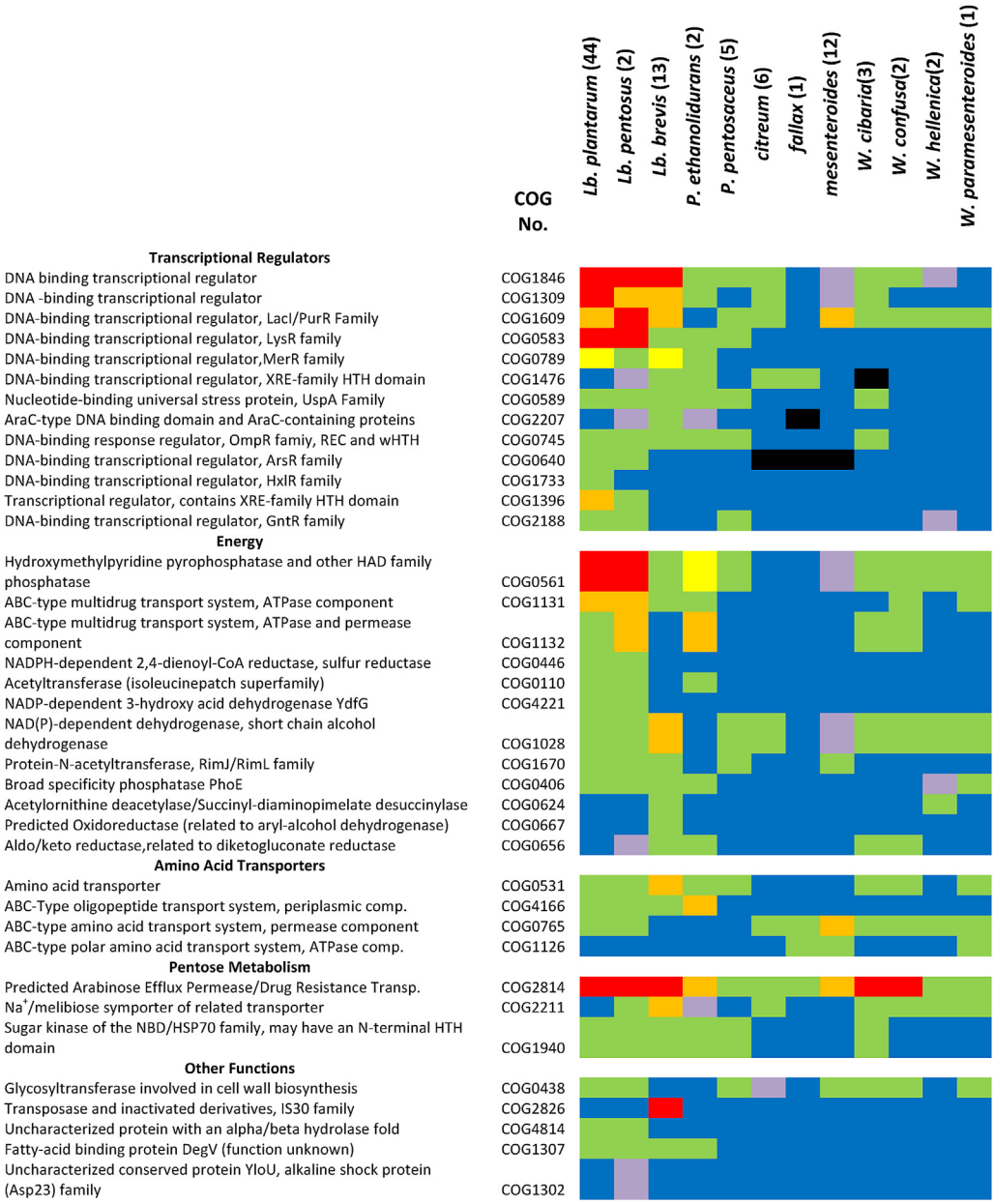

Fig. 7. Abundance profile for COG functions assigned to the genome sequences corresponding to the species found to prevail in commercial cucumber fermentations. Red, green and blue boxes represent high, medium and low abundance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

replication in the *Lb. plantarum* and *Lb. pentosus* genomes. Most of the other lactobacilli and the pediococci concentrate the *rRNA* gene clusters near the origin of replication, while the *leuconostocs* tend to distribute them in the first half of their genome starting from the origin of replication. Differences in the number of *rRNA* gene operons and their locations within the genomes have been associated with bacterial growth rate and the ability to respond to a given environment (Klappenbach et al., 2000). Based on these observations we theorized that *Lb. plantarum* and *Lb. pentosus* may grow faster and adapt to cucumber fermentations more efficiently as compared to other LAB found in the fermentations studied.

LAB are known for their numerous nutrient requirements for proficient growth. The presence of proteases and peptidases in selected LAB has been recognized as advantageous for their proliferation in dairy products, more specifically cheese. Cucumbers have limited protein content compared to milk (USDA-ARS National Nutrient Database Standard Reference Release 28), thus it may be possible that different features are needed for LAB prevailing in either system. Auxotrophies for 7 amino acids are predicted in the genome sequences corresponding to all the species detected in cucumber fermentations in this study. The 7 amino acids are L-serine, L-isoleucine, L-leucine, L-valine, L-lysine, L-phenylalanine, and L-arginine (Fig. 7). *Lb. plantarum* and *Lb. pentosus* have complete putative pathways to synthesize 12 amino acids and 7 detectable auxotrophies, followed by *Lc. mesenteroides* with 10 prototrophies (Fig. 6). With the exception of the cysteine *tRNA*, most *tRNAs* genes are present in multiple copies in the *Lb. plantarum*, *Lb. brevis*, *Lc. mesenteroides* and *P. pentosaceous* genomes. Ten amino acids (asparagine, aspartate, cysteine, histidine, isoleucine, methionine, phenylalanine, trypsine, tyrosine, and valine) seem to be uniquely encoded by a single codon in the same four bacterial genomes (Breidt et al., 2013). Together these observations suggest

that *Lb. plantarum* and *Lb. pentosus* enjoy a certain level of independence from the amino acid content of fresh cucumbers, the main source of nutrients in the fermentations. According to the USDA-ARS National Nutrient Database Standard Reference Release 28, the individual amino acid content in raw cucumbers is 2.5–10 fold lower than that found in milk, with actual amounts being less than 0.04% (m/v), with the exception of glutamic acid present to 0.2% (m/v) (https://ndb.nal.usda.gov/ndb/nutrients/index). Thus, it is theorized that the ability to synthesize amino acids is certainly advantageous in cucumber fermentations containing residual amino acid concentrations after the raw vegetables equilibrate with the cover brines.

The majority of the 3199 LAB-specific COG functions (LaCOG) encoded by the LAB genomes belong to translation, transcription and replication (Makarova et al., 2006). Fig. 7 shows that a number of putative DNA-binding transcriptional regulators are abundantly present in the *Lb. plantarum* and *Lb. pentosus* genome sequences, but not in the *Pediococcus* spp., *Leuconostoc* spp. or *Weissella* spp. genome sequences. The repertoire of biosynthetic pathways is narrower for *P. pentosaceous* and *Lb. brevis*, as compared to *Lb. plantarum* and *Lc. mesenteroides* (Makarova et al., 2006), and may be defining their role in cucumber fermentations and enabling *Lc. mesenteroides* to compete with *Lb. plantarum* early in the fermentation if the appropriate temperature and pH exist. The differences in the number of putative genes coding for energy related and pentose metabolism in *Lb. plantarum* as compared to the other prevailing Lactobacillales are also noticeable (Fig. 7).

The LAB genome sequences have been found to encode for a variety of transporters that enable the efficient intake of a number of carbon and nitrogen containing compounds with amino acid uptake systems that dominated over the carbohydrate and peptide transporters (Makarova et al., 2006). Around 13–18% of the LAB genomes encode for transporters. The percentage is higher than those in most other non-LAB bacterial genomes (Schroeter and Klaenhammer, 2009). The number of predicted transporters in the prevailing *Lactobacillales* genome sequences scrutinized in this study ranges from 11 to 13% of the total gene counts (Table 3), with main inter species differences in the abundance of the putative genes coding for amino acid and oligopeptide transporters (Fig. 7). These observations suggest that the LAB of interest in cucumber fermentations have a tendency to maintain biosynthetic pathways in a plant environment with nutritional limitations, thus not investing as much in nutrient uptake systems (Kleerebezem et al., 2003).

Besides glucose and fructose, other carbohydrates such as cellobiose, trehalose, gentiobiose, xylose and lyxose have been detected in cucumber fermentation (Johanningsmeier and McFeeters, 2015). These carbohydrates are utilized during anaerobic spoilage of fermented cucumbers, suggesting that they are not utilized or are underutilized during the primary fermentation by facultative heterofermentative and obligately heterofermentative LAB. Although, *Lb. plantarum*, *Lb. pentosus*, *W. paramesenteroides*, *Lc. mesenteroides*, *Lc. fallax* and *Lc. citreum* have been found to utilize the glucose disaccharides, cellobiose and trehalose, this does not seem to be the case under the conditions present in cucumber fermentations (deVos et al., 2009). Similarly, xylose, a pentose carbohydrate targeted for the discrimination of *Lb. pentosus* from *Lb. plantarum* (Dellaglio et al., 1975; Huang et al., 2010; Kandler and Weiss, 1986), is present at a detectable level after primary fermentation is completed. Most *Lb. brevis* strains, *W. confusa*, *W. cibaria*, *W. paramesenteroides*, *P. acidilactici* and *P. pentosaceous*, are also able to metabolize xylose (deVos et al., 2009). Perhaps the lack of secondary carbohydrates utilization is the result of the presence of residual glucose and fructose in the fermentations studied by Johanningsmeier and McFeeters (2015). The catabolite control protein, CcpA, is by far the most relevant regulator in carbohydrate degradation by LAB (Douillard and deVos, 2014). The cellobiose-specific PTS system in *Lactococcus lactis* is also able to transport lactose tightly regulated by CcpA and requires the presence of cellobiose to be fully induced (Aleksandrzak-Piekarczyk et al., 2011). Further research is needed to reconcile the observed circumstantial discrepancies, likely due to robust transcriptional regulation systems in the prevailing LAB.

Classification and identification of LAB have certainly been a challenge. A number of phenotypic and genotypic tests have been proposed for the accurate characterization of unknown isolates. The techniques applied in this study rely on the 16S *rRNA* gene sequence to estimate the diversity of LAB and the microbiota in commercial cucumber fermentations. Application of techniques for the rapid identification of microorganisms using other genotypic targets and/or miniaturized high throughput phenotypic assays may present a different dynamics of commercial cucumber fermentations.

4. Conclusion

The observations made in this study, obtained applying the techniques available to date, suggest that *Lb. plantarum* and *Lb. pentosus* are genetically advantageously equipped to thrive in cucumber fermentations and outcompete other fermentative and/or spoilage associated LAB. Such lactobacilli possess comparatively larger genomes, higher copy numbers for *rRNA* gene clusters, transcriptional regulation systems and amino acid transporters. *Lb. plantarum* and *Lb. pentosus* are suspected to be less dependent on the environmental nutritional content, given their ability to synthesize more amino acids.

Acknowledgement

The authors thank AthoGen located in Carlsbad, CA, USA for the Rapid Research Acceleration Program which sponsored the PCR-NMR analysis.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.11.025.

References

Aleksandrzak-Piekarczyk, T., Polak, J., Jezierska, B., Renault, P., Bardowski, J., 2011. Genetic characterization of the CcpA-dependent, cellobiose-specific PTS system comprising CelB, PtcB and PtcA that transports lactose in *Lactococcus lactis* IL1403. Int. J. Food Microbiol. 145 (1), 186–194.

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.

Anukam, K.C., Macklaim, J.M., Gloor, G.B., Reid, G., Boekhorst, J., Renckens, B., van Hijum, S.A., Siezen, R.J., 2013. Genome sequence of *Lactobacillus pentosus* KCA1: vaginal isolate from a healthy premenopausal woman. PloS One 8 (3), e59239.

Barrangou-Poueys, R., Yoon, S.-S., Breidt Jr., F., Fleming, H.P., Klaenhammer, T.R., 2002. Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial sauerkraut fermentation. Appl. Environ. Microbiol. 68 (6), 2877–2884.

Benson, D.A., Boguski, M.S., Lipman, D.J., Ostell, J., 1997. GenBank. Nucleic Acids Res. 25, 1–6.

Breidt, F., McFeeters, R.F., Pérez-Díaz, I.M., Lee, C., 2013. Fermented vegetables. In: Doyle, M.P., Buchanan, R.L. (Eds.), Food Microbiology: Fundamentals and Frontiers, fourth ed. ASM Press, Washington, D.C., pp. 841–855

Cerning, J., Renard, C., Thibault, J., Bouillanne, C., Landon, M., Desmazeaud, M., Topisirovic, L., 1994. Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. Appl. Environ. Microbiol. 60 (11), 3914–3919.

Chen, Y.S., Wu, H.C., Lo, H.Y., Lin, W.C., Hsu, W.H., Lin, C.W., Lin, P.Y., Yanagida, F., 2012. Isolation and characterization of lactic acid bacteria from jiang-gua (fermented cucumbers), a traditional fermented food in Taiwan. J. Sci. Food Agric. 15 (92), 2069–2075.

Choi, I.-K., Jung, S.-H., Kim, B.-J., Park, S.-Y., Kim, J., Han, H.-U., 2003. Novel *Leuconostoc citreum* starter culture system for fermentation of kimchi, a fermented cabbage product. Antonie Leeuwenhoek 84, 247–253.
Costilow, R.N., Bedford, C.L., Mingus, D., Black, D., 1977. Purging of natural salt-stock pickle fermentations to reduce bloater damage. J. Food Sci. 42 (1), 234–240.
Costilow, R.N., Coughlin, F.M., Robach, D.L., Ragheb, H.B., 1956. A study of the acid-forming bacteria from cucumber fermentations in Michigan. J. Food Sci. 21 (1), 27–33.
De Bruyne, K., Schillinger, U., Caroline, L., Boehringer, B., Cleenwerck, I., Vancanneyt, M., De Vuyst, L., Franz, C.M.A.P., Vandamme, P., 2007. *Leuconostoc holzapfelii* sp. nov., isolated from Ethiopian coffee fermentation and assessment of sequence analysis of housekeeping genes for delineation of *Leuconostoc* species. Int. J. Syst. Evol. Microbiol. 57, 2952–2959.
Dellaglio, F., Bottazzi, V., Vescovo, M., 1975. Deoxyribonucleic acid homology among *Lactobacillus* species of the subgenus *Streptobacterium* Orla-Jensen. Int. J. Syst. Bacteriol. 25, 160–172.
deVos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H., Whitman, W.B. (Eds.), 2009. Bergey's Manual of Systematic Bacteriology, vol. 3. The Firmicutes.
Donot, F., Fontana, A., Baccou, J.C., Schorr-Galindo, S., 2012. Microbial exopolysaccharides: main examples of synthesis, excretion, genetics and extraction. Carbohydr. Polym. 87 (2), 951–962.
Douillard, F.P., deVos, W.M., 2014. Functional genomics of lactic acid bacteria: from food to health. Microb. Cell Fact. 13 (Suppl 1), S8.
Etchells, J.L., Goresline, H.E., 1940. Methods of examination of fresh cucumber pickle. Veg. Prods. J. 19 (11), 331–335.
Etchells, J.L., Jones, I.D., 1946. Characteristics of lactic acid bacteria from commercial cucumber fermentations. J. Bacteriol. 52 (5), 593–599.
Fleming, H.P., Pharr, D.M., 1980. Mechanism for bloater formation in brined cucumbers. J. Food Sci. 45 (6), 1595–1600.
Fleming, H.P., Thompson, R.L., Bell, T.A., Hontz, L.H., 1978. Controlled fermentation of sliced cucumbers. J. Food Sci. 43 (3), 888–891.
Hamilton, I.R., Johnston, R.A., 1960. Studies on cucumber softening under commercial salt-stock conditions in Ontario1 II. Pectolytic microorganisms isolated. Appl. Environ. Microbiol. 9, 128–134.
Harutoshi, T., 2013. Exopolysaccharides of lactic acid bacteria for food and colon health. In: Kong, M. (Ed.), Biochemistry, Genetics and Molecular Biology-lactic Acid Bacteria. ISBN 978-953-51-0955-6. Chapter 22.
Huang, C.H., Lee, F.L., Liou, J.S., 2010. Rapid discrimination and classification of the *Lactobacillus plantarum* group based on a partial dnaK sequence and DNA fingerprinting techniques. Ant. Van Leeuwenhoek 97, 289–296.
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., McFeeters, R.F., 2015. Metabolic footprinting of *Lactobacillus buchneri* strain LA1147 during anaerobic spoilage of fermented cucumbers. Int. J. Food Microbiol. 215, 40–48. http://dx.doi.org/10.1016/j.ijfoodmicro.2015.08.004.
Jones, I.D., Veldhuis, M.K., Etchells, J.L., Veerhoff, O., 1940. Chemical and bacteriological changes in dill pickle brines during fermentation. Food Res. 5 (5), 533–547.
Jung, J.Y., Lee, S.H., Jeon, C.O., 2014. Kimchi microflora: history, current status, and perspectives for industrial kimchi production. Appl. Microbiol. Biotechnol. 98 (6), 2385–2393.
Kandler, O., Weiss, N., 1986. Genus *Lactobacillus*. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore, pp. 1209–1234.
Klappenbach, J., Dunbar, J., Schmidt, T., 2000. rRNA operon copy number reflects ecological strategies of bacteria. Appl. Environ. Microbiol. 66, 1328–1333.
Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbring, H.M., Fiers, M.W.E.J., Stickema, W., Lankhorst, R.M.K., Bron, P.A., Hoffer, S.M., Groot, M.N.M., Kerkhoven, R., de Vries, M., Ursing, B., de Vos, W.M., Siezen, R.J., 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U. S. A. 100, 1990–1995.
Lee, S.H., Jung, J.Y., Jeon, C.O., 2015. Source tracking and succession of kimchi lactic acid bacteria during fermentation. J. Food Sci. 80 (8), M1871–M1877.
Liu, L., Zhang, B., Tong, H., Dong, X., 2006. *Pediococcus ethanolidurans* sp. nov., isolated from the walls of a distilled-spirit-fermenting cellar. Int. J. Syst. Evol. Microbiol. 56, 2405–2408.
Lu, Z., Pérez-Díaz, I.M., Hayes, J.S., Breidt, F., 2012. Bacteriophage ecology in a commercial cucumber fermentation. Appl. Environ. Microbiol. 78 (24), 8571–8578.
Marakova, K.S., Koonin, E.V., 2007. Evolutionary genomics of lactic acid bacteria. J. Bacteriol. 189 (4), 1199–1208.
Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.-H., Díaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt Jr., F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D., 2006. Comparative genomics of the lactic acid bacteria. PNAS 103 (42), 15611–15616.
Markowitz, V.M., Chen, M.A., Chu, K., Szeto, E., Palaniappan, K., Pillay, M., Ratner, A., Huang, J., Pagani, I., Tringe, S., Huntemann, M., Billis, K., Varghese, N., Tennessen, K., Mavromatis, K., Pati, A., Kyrpides, N.C., 2014a. IMG/M4 version of the integrated metagenome comparative analysis system. Nucleic Acids Res. 42, D568–D573.
Markowitz, V.M., Chen, M.A., Palaniappan, K., Chu, K., Szeto, E., Pillay, M., Ratner, A., Huang, J., Woyke, T., Huntemann, M., Anderson, I., Billis, K., Varghese, N., Mavromatis, K., Pati, A., Ivanova, N.N., Kyrpides, N.C., 2014b. IMG 4 version of the integrated microbial genomes comparative analysis system. Nucleic Acids Res. 42, D560–D567.
McDonald, L.C., Hassan, H.M., Fleming, H.P., Daeschel, M.A., 1991. Use of continuous culture for internal pH determination of lactic acid bacteria. Food Microbiol. 8 (2), 137–142.
McDonald, L.C., Fleming, H.P., Hassan, H.M., 1990. Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Appl. Environ. Microbiol. 56 (7), 2120–2124.
McDonald, L.C., Shieh, D.-H., Fleming, H.P., McFeeters, R.F., Thompson, R.L., 1993. Evaluation of malolactic-deficient strains of *Lactobacillus plantarum* for use in cucumber fermentations. Food Microbiol. 10 (6), 489–499.
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., Coon, W., Palnitkar, M.P., Velting, M., Fahringer, N., 1977. Reuse of Fermentation Brines in the Cucumber Pickling Industry-Executive Summary. National Service Center for Environmental Publications. EPA/600/12.
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.
Molenaar, D., Bosscher, J.S., ten Brink, B., Driessen, A.J., Konings, W.N., 1993. Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. J. Bacteriol. 175 (10), 2864–2870.
Nanniga, N., 2010. Did van Leeuwenhoek observe yeast cells in 1680?. In: Small Things Considered. American Society for Microbiology. http://schaechter.asmblog.org/schaechter/2010/04/did-van-leeuwenhoek-observe-yeast-cells-in-1680.html.
Passos, F.V., Fleming, H.P., Ollis, D.F., Felder, R.M., McFeeters, R.F., 1994. Kinetics and modeling of lactic acid production by *Lactobacillus plantarum*. Appl. Environ. Microbiol. 60 (7), 2627–2636.
Paramithiotis, S., Kouretas, K., Drosinos, E.H., 2014. Effect of ripening stage on the development of the microbial community during spontaneous fermentation of green tomatoes. J. Sci. Food Agric. 94 (8), 1600–1606.
Pederson, C.S., Albury, M.N., 1950. Effect of temperature upon bacteriological and chemical changes in fermenting cucumbers. N. Y. State Agric. Exp. Stn. Bull. 744.
Pederson, C.S., Albury, M.N., 1956. Variations in bacterial flora of low salt cucumber brines. Appl. Microbiol. 4, 259–263.
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.
Plengvidhya, V., Breidt, F., Lu, Z., Fleming, H.P., 2007. DNA fingerprinting of lactic acid bacteria in sauerkraut fermentations by methods. Appl. Environ. Microbiol. 73 (23), 7697–7702.
Rosen, S., Fabian, F.W., 1953. The importance of biotin, niacin and pantothenic acid in cucumber fermentation. Food Technol. 7, 244–247.
Schroeter, J., Klaenhammer, T., 2009. Genomics of lactic acid bacteria. FEMS Microbiol. Lett. 292, 1–6.
Singh, A.K., Ramesh, A., 2008. Succession of dominant and antagonistic lactic acid bacteria in fermented cucumber: insights from a PCR-based approach. Food Microbiol. 25, 278–287.
Sumner, S.S., Speckhard, M.W., Somers, E.B., Taylor, S.L., 1985. Isolation of histamine-producing *Lactobacillus buchneri* from Swiss cheese implicated in a food poisoning outbreak. Appl. Environ. Microbiol. 50 (4), 1094–1096.
Torriani, S., Felis, G.E., Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum, L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with recA gene-derived primers. Appl. Environ. Microbiol. 67, 3450–3454.
Vahlteich, H.W., Haurand, C.H., Perry, C.A., 1935. Modern science applies itself to cucumber salting. Food Indus 7, 334–336.
Vincenzina, F., Quero, G.M., Cho, G.S., Kabisch, J., Meske, D., Neve, H., Bockelmann, W., Franz, C.M.-A.-P., 2015. The genus *Weisella*: taxonomy, ecology and biotechnological potential. Front. Microbiol. 6, 155.
Wilson, K.H., Blitchington, R.B., Greene, R.C., 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. 28, 942–1946.
Wisselink, H.W., Weusthuis, R.A., Eggink, G., Hugenholtz, J., Grobben, G.J., 2002. Mannitol production by lactic acid bacteria: a review. Int. Dairy J. 12, 151–161.
Wouters, D., Grosu-Tudor, S., Zamfir, M., De Vuyst, L., 2013. Bacterial community dynamics, lactic acid bacteria species diversity and metabolite of traditional Roanian vegetable fermentations. J. Sci. Food Agric. 93 (4), 749–760.
Zanoni, P., Farrow, J.A.E., Phillips, B.A., Collins, M.D., 1987. *Lactobacillus pentosus* (Fred, Peterson, and Anderson) sp. nov., nom. rev. Int. J. Syst. Bateriol 37 (4), 339–341.
Zhang, Z.G., Ye, Z.Q., Yu, L., Shi, P., 2011. Phylogenomic reconstruction of lactic acid bacteria: an update. BMC Evol. Biol. 11, 1.