Published in final edited form as:

J Food Sci. 2016 January ; 81(1): M121–M129. doi:10.1111/1750-3841.13158.

Bacterial Ecology of Fermented Cucumber Rising pH Spoilage as Determined by Non-Culture Based Methods

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Abstract

Fermented cucumber spoilage (FCS) characterized by rising pH and the appearance of manure and cheese like aromas is a challenge of significant economical impact for the pickling industry. Previous culture based studies identified the yeasts *Pichia manshurica* and *Issatchenkia occidentalis*, four gram positive bacteria, *Lactobacillus buchneri*, *Lactobacillus parrafaraginis*, *Clostridium* sp. and *Propionibacterium* and one gram-negative genus, *Pectinatus* as relevant in various stages of FCS given their ability to metabolize lactic acid. It was the objective of this study to augment the current knowledge of FCS using culture independent methods to microbiologically characterize commercial spoilage samples. Ion Torrent data and 16S *rRNA* cloning library analyses of samples collected from commercial fermentation tanks confirmed the presence of *L. rapi* and *L. buchneri* and revealed the presence of additional species involved in the development of FCS such as *Lactobacillus namurensis*, *Lactobacillus acetotolerans*, *Lactobacillus panis*, *Acetobacter peroxydans*, *Acetobacter aceti*, and *Acetobacter pasteurianus* at pH below 3.4. The culture independent analyses also revealed the presence of species of *Veillonella* and *Dialister* in spoilage samples with pH above 4.0 and confirmed the presence of *Pectinatus* spp. during lactic acid degradation at the higher pH. *Acetobacter* spp. were successfully isolated from commercial samples collected from tanks subjected to air purging by plating on Mannitol Yeast Peptone agar. In contrast, *Lactobacillus* spp. were primarily identified in samples of FCS collected from tanks

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E. Medina and I. M. Pérez-Díaz conceptualized and planned the experimentation, interpreted data, and wrote the manuscript; E. Medina executed 90% of the experimentation; F. Breidt contributed to the interpretation of the data and managed bioinformatics analysis; J. Hayes and W. Franco executed 10% of the experimentation; N. Butz and M. A. Azcarate-Peril performed and processed Ion Torrent sequencing data.

not subjected to air purging for more than 4 months. Thus, it is speculated that oxygen availability may be a determining factor in the initiation of spoilage and the leading microbiota.

Practical Application—Understanding of the underlying microbiology and biochemistry driving FCS is essential to enhancing the sodium chloride (NaCl)-free cucumber fermentation technology and in preventing losses caused by it from its development in tanks of cucumbers fermented with NaCl.

Keywords

fermented vegetables; cucumbers; Acetic Acid Bacteria

Introduction

Occasional spoilage of sodium chloride (NaCl) fermented cucumbers characterized by rising pH and the appearance of manure and cheese like aromas is a tangible problem for the pickling industry in the U.S. There is one historical instance in which an entire tank yard composed of 800 open top tanks had to be closed in the middle of the pickling season due to this type of spoilage. In the 2010 pickling season alone, the research activities on fermented cucumber spoilage (FCS) prevented losses estimated of more than one quarter of a million dollars. Commercially fermented cucumbers are packed in 40,000 L open top fiberglass tanks containing a cover brine solution with an equilibrated NaCl concentration of 5.8 to 8%. Previous research has suggested that FCS may occasionally proceed during bulk storage as the result of microbiological utilization of lactic acid and formation of acetic, butyric and propionic acids (Fleming and others 1989).

Analysis of commercial spoilage samples from NaCl fermentations and laboratory reproduced spoilage with NaCl identified *Pichia manshurica, Issatchenkia occidentalis, Lactobacillus buchneri*, *L. parrafaraginis*, *L. rapi*, a *Clostridium* sp., and *Pediococcus ethanolidurans* as potentially relevant to different stages of FCS (Franco and others 2012; Johanningsmeier and others 2012; Johanningsmeier and McFeeters 2013). Parallel studies determined that spoilage associated *L. buchneri* and *P. ethanolidurans* are able to produce lactic acid from sugars, but only *L. buchneri* produces acetic acid at the expense of lactic acid under both aerobic and anaerobic conditions regardless of the initial acidic pH of 3.2 in the medium (Franco and Pérez-Díaz 2012; Johanningsmeier and others 2012; Johanningsmeier and McFeeters 2013). The characterization of NaCl cucumber fermentation spoilage bacteria using culture dependent and independent techniques, and an enrichment step revealed that *Propionibacterium* and *Pectinatus* species play a role in converting lactic acid to propionic acid at pH above 4.2 (Breidt and others 2013). Additionally, the analysis of 16S rRNA cloning libraries confirmed that Gram-negative anaerobic bacteria supersede Gram-positive Firmicutes species after the pH rises from around 3.2 to 5, and propionic and butyric acids are produced.

Initiation of lactic acid utilization certainly induces the increment in pH, which allows for the proliferation of microbes that are sensitive to pH below 3.5. Therefore, identification of organisms capable of utilizing lactic acid at pH 3.0 to 3.5 under the conditions present in commercial cucumber fermentations is essential in the design of strategies for the prevention

of the undesired FCS. The ability of spoilage associated yeasts to degrade lactic acid under conditions similar to those present in commercial cucumber fermentations has been confirmed (Franco and Pérez-Díaz 2012). *L. buchneri* and closely related species seem to be unique among several lactic acid bacteria (LAB) in their ability to metabolize lactic acid in fermented cucumber slurry, with concurrent increases in acetic acid and 1,2-propanediol at pH 3.8 in the presence of 2% to 6% NaCl (Johanningsmeier and McFeeters 2013). Although the ability of other spoilage associated bacteria, such as *Clostridium bifermentans*, *Enterobacter cloacae*, *Propionibacterium* spp. and *Pectinatus sottacetonis* to utilize lactic acid has been observed under conditions similar to those present in commercially fermented cucumber tanks containing NaCl, such activity proceeds at pH above 4.5 (Franco and Pérez-Díaz 2012; Breidt and others 2013). Therefore, these microbes are considered as secondary microbiota in FCS.

Even though significant progress has been made in understanding the underlying microbiology and biochemistry driving FCS; a comprehensive study of the dominant microbiota in commercial NaCl FCS using culture independent techniques is lacking. The need to understand FCS is augmented by the implementation of NaCl-free cucumber fermentations at the commercial scale, which is more vulnerable to FCS during long term storage (Pérez-Díaz and others 2014). Thus, the objective of this study was to apply non-culture based techniques, such as the Ion Torrent sequencing technology and analysis of 16S rRNA cloning libraries to obtain a comprehensive view of the bacterial community present in commercial FCS in the NaCl and calcium chloride ($CaCl_2$) systems. The approach taken in this study was the utilization of cucumber fermentations brined with $CaCl_2$ as a tool to consistently reproduce FCS at the commercial scale, and the more resistant NaCl cucumber fermentation system as control; followed by chemical and microbiological analyses to monitor the state of such tanks during experimentation (Figure 1). Particularities of spoiled cucumber fermentations brined with NaCl or $CaCl_2$ were documented by comparing samples of spoiled cucumber fermentations brined with NaCl provided by processors in previous years (2009–2012), with the characteristics of cucumber fermentations brined with $CaCl_2$ studied during the 2013 pickling season.

Material and Methods

Commercial Cucumber Fermentation Tanks

Commercial cucumber fermentations were carried out in 40,000 L open top fiberglass tanks containing 60% 3A (39 to 51 mm diameter) or 2B (27 to 38 mm diameter) whole cucumbers or pieces of variable size fruits; and 40% cover brine solutions containing either 100 mM (0.9%) $CaCl_2$ and 6 mM (0.1%) potassium sorbate (equilibrated concentrations) or a combination of acetic acid, added as concentrated vinegar, potassium sorbate, $CaCl_2$ and NaCl to achieve equilibrated concentrations of 15 (0.09%), 2 (0.03%), and 40 (0.9%) mM and 1.03 M (6%), respectively. Cushion cover brine was added into the tanks, prior to the addition of the fruits of different sizes. Two tanks were packed with the experimental $CaCl_2$ (tanks 3 and 4) cover brine and two tanks were packed with the traditional NaCl cover brine (tanks 1 and 2). Fresh cucumbers came from multiple farmers primarily located in North Carolina, U.S.A. In-tank fruits were immediately covered with wooden boards to prevent

them from floating, after adding the remaining cover brine volume, to allow for the equilibration between the fruits and cover brine solution components without exposing the fresh fruits on the top of the tank to air. The experimental tanks were purged for 4 h in the morning and 4 h in the afternoon during the active lactic acid fermentation to prevent bloater damage. In the peak of the season (summer months), the primary fermentation occurs within 2 wk and during this time the fermentation tanks were air-purged as described above. The tanks were purged for the first 7 d and on days 9, 11 and 13. Tanks were not purged on days 8, 10, 12 and after day 14. Air purging was minimally applied after primary fermentation to allow for mixing of preservatives and prior to the collection of samples. Rain water was not mixed in by air purging circulation.

Cucumber fermentations brined with $CaCl_2$ were inoculated to 10^6 CFU/mL by using 1850 mL of *L. plantarum* LA0045 inoculum (USDA-ARS Food Science Research Unit culture collection) at 10^9 CFU/mL for a 30,400 L total volume. Preparation of starter cultures was done as described by Pérez-Díaz and McFeeters (2011). Tanks packed with the traditional NaCl formulation were not inoculated.

Cover brine samples for microbiological, molecular and chemical analyses were collected on days 1, 2, 3, 7, 14, 21, 30, 60, 75, 90, 105 and 120. On each sampling day, approximately 500 mL of cover brine samples were taken from an average of 1 m below the cover brine surface via a perforated pipe placed next to the air purging system in the tank. Cover brine samples were collected after 100 mL of the cover brines had moved through the sampling tubing and air purging had been applied for 15–20 min. The sampling apparatus consisted of a buffer siphon PVC pump (BSP-1000; CBS Scientific Inc., San Diego, Calif., U.S.A.) connected to a 1.3 cm diameter tygon tubing with a thin wall. The samples were placed in 1.3 kg glass jars and immediately transported to our laboratory for same day processing.

A set of 10 commercial cover brine samples were provided by processors during the 2010, 2012 and 2013 pickling seasons. These samples were collected from tanks with symptoms of spoilage that had been packed with the experimental $CaCl_2$ cover brine formulation or the traditional NaCl cover brine formulation as indicated on the text. The NaCl commercial fermentations were air purged continuously for the 14 to 21 d in which the primary fermentation occurred; and sporadically during storage to allow for the incorporation of rain water into the tank. Samples were aseptically collected from 1 m under the cover boards, collected in sterile 50 mL conical tubes and refrigerated until delivered to our facilities.

Detection of fermented cucumber rising pH spoilage at the commercial scale was based mainly on the measurement of pH values above 3.3 ± 0.2. In addition to pH, the detection of manure-like and cheesy aromas and the surfacing of bubbles in the tanks also indicated tanks may be in the process of spoiling. Suspected secondary fermentation was confirmed by HPLC analysis of organic acids and alcohols as described below.

For the microbial analysis cover brine samples were aseptically collected as described above, serially diluted in 0.85% saline solution, and spiral plated using an Autoplate 400 (Spiral Biotech, Norwood, Mass., U.S.A.). Enumeration of lactic acid bacteria was done using deMan, Rogosa and Sharpe agar (MRS, Becton Dickinson and Co., Franklin Lakes,

N.J., U.S.A.) supplemented with 1% cycloheximide (0.1% solution, Oxoid, New England) to prevent growth of yeasts. MRS agar plates were incubated anaerobically using a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, Mich., U.S.A.) at 30 °C for 48 h. Yeasts were enumerated using yeast and mold agar (YMA; Becton Dickinson and Co.) supplemented with 0.01% chloramphenicol (Sigma-Aldrich, St. Louis, Mo., U.S.A.) and 0.01% chlortetracycline (Sigma-Aldrich) to inhibit bacterial growth. YMA plates were incubated aerobically at 30 °C for 48 h. *Acetobacteraceae* were enumerated by plating in Mannitol, Yeast Extract and Peptone agar (MYP), which contained 25.0 g of mannitol, 5 g of yeast extract, 3 g of peptone, and 15 g of agar per L of distilled water. MYP plates were incubated at 30 °C for 24 h. The pH was determined with a Fisher Accumet pH meter, model 825 MP (Pittsburgh, Pa., U.S.A.).

Identification of acetic acid bacteria by 16S rRNA sequencing was completed from isolated colonies growing on MYP that were catalase positive, showed motility in a microscope preparation and were Gram negative. Such colonies were randomly selected for identification by the partial amplication of the 16S *rRNA* sequencing. DNA isolation from single colonies was done using InstaGene Matrix DNA extraction kit (Bio-Rad, Hercules, Calif., U.S.A.). The PCR mixture contained 2X master mix (Bio-Rad), 10 uL of the resulting total genomic DNA extracted from bacterial isolates, and 0.6 μM of primers RBUP (5-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Wilson and others 1990). The PCR cycle 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 and stored at 4 °C until used. PCR products were sequenced by Eton Bioscience Inc. (Durham, N.C., U.S.A.). Sequence data was 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 and others 1990; Benson and others 1997) using the 16S ribosomal RNA sequence database to determine the identity of the strains (accession # KT074234 to KT074257).

Isolates of acetic acid bacteria (AAB) from MYP plates were picked, mounted on a slide and observed in a microscope in order to detect cell motility. Hydrogen peroxide was added on the colonies and only those showing production of bubbles were classified as catalase positive.

Total Genomic DNA Extraction

Commercial spoilage cover brine aliquots of 10 - 400 mL were spun (Sorvall, Thermo Fisher Scientific Inc., Waltham, Mass., U.S.A.) at 10,000 rpm for 10 min at 4 °C, and washed in 1 mL of 0.85% saline solution. Pellets were resuspended in 490 μL sterile saline solution and treated with propidium monoazide (PMA) to eliminate dead bacterial and extracellular DNA (Pan and Breidt 2007). Ten microliters of 2.5 mM PMA (Biotium, Inc., Calif., U.S.A.) were added to 490 μL of the cells resuspension to achieve a final concentration of 57 μM PMA. Treated samples were vortexed for 10 s at high speed and incubated in the dark for 5 min at room temperature. Samples were then placed on ice with the tops open and exposed to a 650-watt halogen lamp, 20 cm above the top of the microcentrifuge tubes, for 5 min to allow

for cross-linking of the dye with the DNA. Samples were spun at 10,000 rpm for 5 min at room temperature, the supernatants were removed and the pellet treated with PMA a second time as described. PMA-treated samples were then stored as cell pellets at −20 °C until DNA extraction was performed.

DNA isolation was done using a MasterPure™ DNA Purification Kit (Epicentre, Madison, Wis., U.S.A.) with proteinase K according to the manufacturer instructions. Purified DNA samples were stored at −20 °C until use.

The 16S rRNA library was constructed by PCR amplification using the primers RBUP and 1492r described above. PCR products were cleaned up using a QIAquick PCR purification kit (Qiagen, Valencia, Calif., U.S.A.) and the resulting 16S rRNA PCR products were cloned in *Escherichia coli* using the TOPO®TA cloning kit for sequencing (Invitrogen, Life Technologies, Grand Island, N.Y., U.S.A.), according to the manufacturer instructions. For rRNA clone analysis, petri plates with the rRNA clones were sent to Eton Bioscience (Eton Bioscience Inc., Research Triangle Park, N.C., U.S.A.). The sequence data obtained from Eton Bioscience, was formatted and analyzed using BioEdit software (www.mbio.ncsu.edu/bioedit). Only bases that have basecaller quality value greater than or equal to 20 were used for the alignments. Sequence similarities were determined using the NCBI BLASTN algorithm (www.ncbi.nlm.nih.gov) with the 16S ribosomal RNA (Bacteria and Archaea) database. All sequences were submitted to the National Center for Biotechnology Information Genbank database (accession # KF998365 - KF998547).

16S rRNA amplicon sequencing analysis using Ion Torrent targeted the V1–V2 fragment of the 16S rRNA. PCR amplification was performed from purified total bacterial DNA isolated from each sample using the forward primer (5′-xxxxxxxxxx*AGAGTTTGATCCTGGCTCAG* - 3′); where the underlined sequence contained barcodes consisting of 10 nucleotides (Supplementary Material, Table S1) and the sequence in italics contained the universal primer 8F. The reverse primer was 338R (5′-TGCTGCCTCCCGTAGGAGT-3′). PCR reactions contained 5–10 ng of DNA template, 2.5 units of HotStar Hi-fidelity DNA polymerase (Qiagen), 1× HotStar Hi-Fidelity PCR buffer containing dNTPs, and 0.6 μM of each primer. Reaction conditions consisted of an initial denaturation for 5 min at 94 °C followed by 35 cycles of 94 °C for 60 s, 57 °C for 60 s, and 72 °C for 60 s, and a final extension of 72 °C for 10 min. Negative controls, not containing template, were amplified for all barcode-primer sets. The PCR products with approximately 400 nucleotides were confirmed and purified by gel electrophoresis in an E-gel system (Invitrogen, Life Technologies). DNA concentrations were quantified using PicoGreen dsDNA reagent (Invitrogen, Life Technologies) on a 96-well plate reader and mixed at equimolar concentrations. All kits were used according to the respective manufacturer's instructions. Purified libraries were submitted to the UNC Microbiome Core Facility (Chapel Hill, N.C., U.S.A.) for sequencing on the Ion Torrent platform.

Amplicon high-throughput sequencing data analysis was conducted from raw Ion Torrent fastq files, which were demultiplexed, quality-filtered, and analyzed using QIIME (Caporaso and others 2010). The 400-bp reads were truncated at any site if more than three sequential bases receiving a quality score of *<20*, and any read containing ambiguous base calls or

barcode/primer errors were discarded, as were truncated reads. Operational Taxonomic Units (OTUs) were assigned using the QIIME implementation of UCLUST (Edgar 2010), with a threshold of 97% pairwise identity, and representative sequences from each OTU selected for taxonomy assignment. Beta diversity estimates were calculated within QIIME using weighted and unweighted Unifrac distances (Lozupone and Knight 2005) between samples at a depth of 989 sequences per sample. From these estimates, jackknifed principal coordinates were computed to compress dimensionality into two- and three-dimensional principal coordinate analysis plots. QIIME was also used to calculate alpha diversity on rarefied OTUs to assess sampling depth coverage using observed species, Shannon and phylogenetic diversity *(PD)* metrics (Table 2).

HPLC analysis

Samples of commercial cover brines were collected as described above and spun at 12000 rpm for 10 min at 21 °C (ambient temperature) to remove residual particulate matter (Eppendorf Benchtop Refrigerated Centrifuge 5810R, Hamburg, Germany). Samples were diluted 10X prior to analysis with distilled water. Organic acids and sugars concentrations were measured using a 30-cm HPX-87H column (Bio-Rad) (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 Fisher Scientific Inc.) set to collect data at 210 nm was used to detect malic, lactic, acetic, propionic, and butyric acids. A Waters model 410 refractive index detector (Waters Corp., Millipore Corp., Billerica, Mass., U.S.A.) connected in series with the diode array detector was used to measure glucose, fructose, and ethanol. External standardization of the detectors was done using four concentrations of the standard compounds.

Results

Attributes of fermentations

The characteristics of the commercial fermentations studied were in agreement with the expectations for a NaCl primary fermentation and for a $CaCl_2$ cucumber fermentation in which spoilage took place. Most lactic acid production was completed in the first 28 d of fermentation, generating post-fermentation pH values of 3.2 ± 0.1 and 3.3 ± 0.1 for the NaCl and $CaCl_2$ fermentations, respectively. Acetic acid was produced to an average of 5.6 ± 7.9 and 13.1 ± 3.4 mM in the sodium and calcium systems, respectively. Maximum lactic acid concentrations of 101.9 ± 7.7 mM were reached after 60 d in all the tanks studied. The traditional NaCl fermentations were stable during long term storage as evidenced by undetectable levels of LAB and yeasts after day 28 of the fermentation. In contrast, FCS proceeded in the fermentations brined with $CaCl_2$ presenting an increase in pH to 4.3 ± 0.3 and a decrease in lactic acid concentration to undetectable levels after 120 d of storage at ambient temperatures (Table 1). Counts of LAB and yeasts remained stable at 4.9 ± 0.1 Log CFU/mL by day 60 in the fermentations brined with $CaCl_2$, however an increase in the counts of LAB from MRS plates was noted by day 120 to 5.6 ± 0.4 Log CFU/mL (Table 1). An increase in acetic acid concentration from 5.0 ± 2.5 mM to 30.3 ± 16.5 mM was also evident after 76 d of fermentation (Table 1). The formation of propionic acid, butyric acid

and propanol was detected after 100 d of storage in the $CaCl_2$ cover brines to 6.3 ± 0.3, 13.2 ± 1 and 15.4 ± 2 mM, respectively.

Samples of commercial FCS provided by processors

Commercial FCS samples collected from tanks brined with either the sodium or the $CaCl_2$ salt were delivered by independent processors to our facilities during the seasons of 2010, 2012 and 2013. Air purging was not applied to the NaCl spoiling tanks during the course of spoilage. Acetic acid was detected (~25 mM) in the samples collected from tanks brined with NaCl, at the concentrations added from vinegar. The detection of high pH values (Table 2), manure-like and cheesy aromas, and the presence of bubbles on the surface of these tanks indicated that FCS had started in the corresponding tanks (Franco and others 2012). A NaCl cover brine sample with a pH of 3.22 (sample E), had no detectable counts of LAB, unusually high numbers of yeast counts, relatively high concentration of lactic acid, and spoilage associated metabolites such as propionic acid (12 mM) and propanol (4.58 mM) (Table 2). In contrast a second sample of FCS containing NaCl (sample F) showed a pH of 4.26, relatively high counts of LAB, and relatively low lactic acid concentration. Although, the differences in the characteristics of the FCS samples containing NaCl are primarily attributed to the variability of the pH values of the samples at the moment of analysis; it could also suggest that the course of the initial spoilage stage was different in these tanks.

$CaCl_2$ commercial spoilage samples represented different stages of FCS with a pH range from 3.05 to 4.50 (Table 2). A reduction in lactic acid concentrations was observed in these tanks and depletion of the organic acid was detected in the samples with the higher pH (Table 2). An increase in the acetic acid concentration was detected in all of the $CaCl_2$ spoiled fermentations. Significant levels of propionic and butyric acids and propanol were detected in the samples with the lower acidity and higher pH values. Counts of LAB from MRS were relatively high, regardless of the fermentation pH. However, yeast counts from YMA varied, presumably due to the variations in the levels of dissolved oxygen in the tanks, which was partially influenced by differences in the air purging rate and routine.

Changes in bacterial diversity of cucumber fermentation spoilage

Selected spoilage samples collected from tanks brined with NaCl (Tank 1) or $CaCl_2$ (Tanks 3 and 4) on days 76, 90, 105 and 120 and commercial cover brine samples collected by processors (A, B, E and F) with different pH values, were used for the analysis of population diversity using the Ion Torrent technology. FCS samples from days 76, 90, 105 and 120 were selected given that these days mark the initiation of significant changes in organic acid concentrations or bacterial counts. For example, lactic acid disappearance and increases in pH started after day 60, thus the samples from day 76 should provide information regarding the bacterial community potentially involved in such changes. Similarly, acetic acid production and increases in the MRS counts were more evident by day 90, production of propionic and butyric acids was detected by day 105, and production of propanol evolved after 120 d of bulk storage.

Sequencing reads were obtained from a single run with 14 bacterial PCR amplicons derived from independent samples. A total of 302,295 high quality sequences with an average length

of 400 bp were obtained. An average of 21,592 reads were obtained per sample or amplicon. Differences in the bacterial diversity were observed between the two types of fermentations during bulk storage. General diversity indices, based on the Chao1 and Shannon algorithms, showed a decline in the number of species participating in the spoilage as the pH increased (Table 3). NaCl control tanks presented two main groups of microorganisms belonging to the orders of *Rhodospirialles* and *Rhizobiales* (Figure 2). The relative distribution of these groups varied with the fermentation age. *Rhodospirialles* were particularly abundant (66.23%) in samples from day 76 with *Acetobacteraceae* representing the only family identified. Two genera, *Acetobacter* and *Gluconobacter* predominated in the reads detected. However, the order of *Rhizobiales* predominanted in the samples from day 120 and was composed of the *Aurantimonodaceae* family and the genus *Methylobacterium* with relative abundances of 73.65 and 7.59%, respectively. The fact that the predominant microbes demand an aerobic environment for growth may suggest the relevance of air purging in the dominant persisting microbiota during long term storage.

The family *Acetobacteraceae* was dominantly detected in the samples collected from two tanks brined with $CaCl_2$, regardless of the fermentation age, reaching relative abundance values between 71.55 and 99.9%. *Lactobacillus* spp. were uniquely identified in one sample of $CaCl_2$ spoiled cover brine collected on day 76 of the fermentation with a 25% relative abundance. A significant change in the bacterial diversity was detected on day 120 of the fermentation, when the predominant order found was *Bacteroidales*, which included the genus *Prevotella* with 53.06% relative abundance. Also the family *Veillonellaceae* was identified in the samples from 120 d of the fermentation with a 7.3% relative abundance, which included *Pectinatus* spp. Samples collected at a later time point, had pH values above 4.5, which presumably predominantly allowed for the proliferation of *Prevotella*. At the lower pH values and in the presence of air, members of the *Acetobacteraceae* dominated.

Analysis of the 16S rRNA cloning library

Four samples were selected to represent the different stages of spoilage in pickles for the creation of a 16S *rRNA* cloning library. Three samples represented the initiation of spoilage (day 76) at pH values between 3.0 and 3.4, and 1 sample represented the advance stage of spoilage (day 120) with a pH value of 4.5. Table 4 presents the clones obtained for the commercial spoiled cucumber fermentations. The control sample collected from a microbiologically dormant NaCl commercial fermentation (Tank 1) on day 76, presented 10 (26%) and 9 (23%) clones with sequences of the *Acetobacter* and *Lactobacillus* genuses, respectively; with the majority of species being *Acetobacter peroxydans* and *L. parabrevis*. The rest of the clones had sequence similarity with the *Proteobacteria* and *Firmicutes* phyla. The spoiled $CaCl_2$ fermentation sample (Tank 4) collected after 76 d presented 47 (94%) clones with sequence similarities to the *Lactobacillus* genus, mostly composed of *L. acetotolerans* followed by *L. namurensis*, *L. buchneri* and *L. panis* (Table 4). A clone sequence from this same sample was identified as *A. peroxydans*. Sample D showed a pH of 3.4 and high counts of LAB, yeasts and AAB on day 76 (Table 4). Three main groups have been identified in this commercial brine. Twenty-seven clones were identified as members of *Acetobacter* genus, with *A. peroxydans, A. aceti* and *A. pasteurianus* being the most abundant. The second group was the *Lactobacillus* genus with 12 clones, including *L.*

buchneri and *L. panis. Pectinatus* sp. was also identified in sample D with 10 clones. The $CaCl_2$ commercial sample B collected on day 120 with a pH of 4.5 (Table 2), had 26 (55.3%) clone sequences with similarity to those sequences from the *Prevotella* genus followed by 13 cloned sequences with similarity to the *Dialister* genus. Additionally, 6 and 2 clone sequences with similarities to those of *Pectinatus sp*. and *Acetobacter* spp., respectively, were detected (Table 4).

Plating on MYP was completed to confirm the presence of AAB in FCS samples collected from fermentations brined with $CaCl_2$ during the 2013 pickling season (C, D, G, H, I and J). AAB were detected in three out of six tanks at ~4 log CFU/mL; while below detection levels, or numbers close to that, were observed in the additional tanks. The colonies observed on MYP plates were all Gram negative bacilli, positive for catalase and motile. Twenty-four isolates subjected to partial sequencing of the 16S *rRNA*, were identified as acid intolerant Gram positive *Lysinibacillus sp*. or Gram negative *A. pasterianus* with 99% and 97–98% similarities, respectively, over 100% and 98% of the query sequences, respectively, according to the alignment with the NCBI 16S ribosomal RNA sequences for bacteria and archae databases (Sasaki and others 1997). The absence of AAB in selected tanks may be attributed to the addition of the preservative, sodium benzoate, to the cover brines once spoilage symptoms were observed, and prior to the collection of the samples.

Discussion

This study identified *A. pasteurianus* as a major player in the initiation of fermented cucumber spoilage in the presence of oxygen, and confirms the role of selected LAB, primarily in the absence of oxygen. Microorganisms of the *Lactobacillus* and *Acetobacter* genera were almost always present in variable relative abundance depending on the age of fermentation and the pH of the cover brines. The variability in the dominant bacterial groups identified in the samples studied suggests that either *Acetobacter* spp. or selected lactobacilli may lead the development of spoilage. Additionally, the identification of *Prevotella* and *Dialister* species in spoilage samples from the later stage of spoilage with a higher pH, suggest that these organisms may be implicated in lactic acid utilization at pH values higher than 3.5.

While the Ion Torrent data generally described the diversity of the bacterial community to the family level; the data obtained from the partial sequencing of 16S *rRNA* cloning library confirmed and taxonomically deepened this information identifying relevant microbes to the species level. The Ion Torrent analysis revealed that the Genus *Lactobacillus* dominated in two spoilage samples, while the Family *Acetobacteriaceae* dominated in eight other samples (Figure 1). Moreover, *Acetobacteriaceae* was the only family detected by the Ion Torrent analysis in five out of the eight samples tested (Figure 2); which suggest that members of this family could drive FCS in the absence of other relevant microbes. The analysis of the 16S *rRNA* cloning library augmented this picture by revealing specific lactobacilli species potentially involved in the development of spoilage, such as *namurensis*, *acetotolerans*, *buchneri*, *panis*, and *rapi*. Additionally, such analysis revealed that species of *Acetobacter*, such as *peroxydans*, *aceti*, and *pasteurianus*, were present in the three samples analyzed with pH values below 3.4. However, as stated above, it must be highlighted that significantly

lower microbial counts were observed in the control samples as compared to the experimental spoilage samples on MRS and YMA, suggesting that the microbial load in the NaCl control tanks was minimal and unlikely to reflect significant changes in the biochemistry of the fermentation at the time the samples were collected. Additionally, the sample volume used to extract total genomic DNA from the NaCl control tank samples was 50-fold that of the $CaCl_2$ fermentation samples. Thus, the relative abundance corresponding to *Acetobacter* from the NaCl fermentation samples could have been insignificant when compared to that of the $CaCl_2$ fermentation samples.

Given the parallel in the dominance of *Lactobacillus* spp. in samples of FCS and the absence of air purging in the corresponding originating tanks, it is speculated that oxygen availability may be a determining factor in the initiation of spoilage and the leading microbiota. Spoilage fermentation samples E and F, in which lactobacilli were found to dominate, were collected by processors from commercial tanks that had been inaccessible for more than 4 months due to construction in their nearby location (personal communication with processor). During such storage time between the months of May and September, air purging was not applied to these two tanks, which implies that reduce oxygen amounts were present in the corresponding tanks. It is presumed that the facultative anaerobic lactobacilli could have been potentiated in the absence of air purging, and that aerobic bacteria, such as *Acetobacteraceae* and *Aurantimonadaceae*, were suppressed and, thus, not detected in these tanks.

It is relevant to mention that the lactobacilli species identified in spoilage samples by the analysis of the 16S *rRNA* cloning library were not detected in the sample representing the NaCl control tank. This observation suggests that these species may be playing an important role in the development of spoilage under specific conditions that promotes their proliferation, but are not necessarily universally present in all fermentation tanks. Conditions promoting their proliferation, should they be part of the natural cucumber microbiota, may include anaerobiosis in the context described above, nutrient limitation, unusually high end of fermentation pH (3.5–3.8), and reduced salt. Johanningsmeier and others (2012) reported that lactic acid degradation occurs in fermented cucumber slurry containing *L. buchneri* at pH 3.2 if less than 5.8% NaCl concentrations was present in the cover brine. Lactic acid disappearance was observed in the same system at pH 3.8 and above, regardless of the salt concentration.

In contrast, the use of open-top tanks and air-purging to control bloater damage due to CO_2 accumulation in the fermentation tanks prevents anaerobiosis in the commercial tanks, which could favor the proliferation of AAB in the earliest stages of secondary fermentation when the pH is still acidic. Thus, the disappearance of lactic acid and consequent formation of acetic acid may be partially or fully attributed to AAB, primarily *A. pasterianus*.

Although AAB were detected in cucumber fermentations with both cover brine types, the production of acetic acid was only noticeable in the $CaCl_2$ fermentations (Figure 2). Most *Acetobacter* spp. are sensitive to the presence of NaCl in the medium and pH values below 4.5, conditions met in commercial cucumber fermentations brined with NaCl. De Ley and others (1984) reported that 37% and 2% of *A. liquefaciens*, *A. aceti*, *A. pasteurianus* and *A.*

hansenii are resistant to 0.5% and 2% NaCl, respectively. While the optimum pH for growth of *Acetobacter* spp. has been identified between 6.3 and 5.4, they seem to tolerate pH as acidic as 3.6 (De Ley and others 1984). The combination of an end of fermentation pH of 3.3 and 6% NaCl maybe a factor in the lack of proliferation of *Acetobacter* spp. in the high salt cucumber fermentations, as suggested by the data presented here. The proposed $CaCl_2$ cucumber fermentation contains 0% NaCl and reaches pH as low as 3.0. While the absence of NaCl in this new system could translate into a higher susceptibility to the development of FCS, a final pH of 3.0 could be a hurdle for selected *Acetobacter* spp. *Acetobacter* spp. are known for colonizing lactic acid fermented foods given their ability to catabolize lactic acid to acetic acid (Gossele and others 1984). The fact that *Acetobacter* spp. were detected in most of the samples tested, including those obtained from controlled NaCl tanks, suggests that *Acetobacter* spp. are naturally present in cucumber fermentations, but are outcompeted by those LAB spearheading primary fermentation.

Application of culture independent methods for the study of the bacterial community present in FCS was instrumental in identifying *Acetobacter* spp. as a relevant causative agent. It has been reported that unless AAB are cultivated in a medium with acetic acid, they may lose their resistance to the organic acid (Holt and others 2000). Within the vinegar industry it is customary to use an ongoing fermentation to inoculate a new batch, given the difficulties in preserving viability in frozen stocks (personal communication with vinegar processors). Azuma and others (2009) reported that the *A. pasteurianus* genome is extremely unstable, has experience intensive reduction, and that cells may have up to six plasmids, which partially explain the difficulties in growing this bacterium *in vitro*. The standard microbiological testing of cucumber fermentation cover brine samples in our laboratory do not include the appropriate media or incubation conditions to successfully detect and isolate *Acetobacter* spp. Thus, previous studies of FCS neither enriched nor selected for *Acetobacter* spp. After understanding that this bacterium was present in commercial cucumber fermentations from their DNA signature in the Ion Torrent and 16S *rRNA* cloning library analyses, we were able to recover viable colonies on MYP for culture based characterization. However, attempts to reproduce the spoilage in the laboratory by inoculating *Acetobacter* spp to fulfill Koch postulates and to study their sensitivity to selected preservatives were hindered by lack of growth of the *Acetobacter* isolates in a cucumber juice medium containing 0 or 10 mM acetic acid (data not shown). Certainly, this aspect needs to be further evaluated.

The culture independent approach taken in this study not only revealed a relevant role for *Acetobacter* spp. in the initiation of secondary fermentation, but also added *Veillonella* spp. and *Dialister* spp. to the list of bacteria potentially active in the later stage of the spoilage and confirmed the participation of *Pectinatus* spp. *Dialister* species have been collected from various human clinical samples and never from fermented foods. *Dialister propionicifaciens* possesses some physiological characteristics in common with the members of the genus *Veillonella*, sharing the same metabolic profile, with the production of acetic, propionic and lactic acids (Jumas-Bilak and others 2005). The genus *Prevotella* is related to humans and has been isolated from oral cavity, upper respiratory and urogenital tracts. The major fermentation products are acetic and succinic acids and, occasionally, lower levels of isobutyric and isovaleric acids are produced (Shah and Collins 1990). *Pectinatus* spp. are

commonly isolated from unpasteurized beer spoilage causing the formation of off-flavors derived from organic acids and sulphuric compounds and are considered strict anaerobes (Juvonen and Suihko 2006). The recently proposed *Pectinatus* species, *Pectinatus sottacetonis*, was isolated from a commercial pickle spoilage tank in a previous study (Breidt and others 2013) and characterized by biochemical, physiological and molecular biological methods (Caldwell and others 2013). The isolate produces acetic acid and propionic acid from fructose and glucose as major metabolites at pH values close to neutrality. However, neither *Pectinatus* nor *Dialister* or *Prevotella* spp. are able of converting lactic acid to propionic acid at pH below 4.8.

Conclusion

Bacterial diversity identified from commercial spoilage samples using high throughput technology as Ion Torrent and 16S rRNA cloning is comprehensively representative of the microbiota present in FCS. Spoilage involves cooperative metabolism among microorganisms, whose contributions are defined by the cover brine pH. The presence of AAB and *Lactobacillus* spp. seems to play a relevant role in the initiation of fermented cucumbers spoilage presumably depending on the amounts of dissolved oxygen present in the tanks when spoilage is initiated. They can convert lactic acid to acetic acid during the first stage of the secondary fermentation initiated at pH below 3.4. Members of the genus *Pectinatus*, *Veillonella*, and *Dialister propionifacien* can utilize lactic acid in the later stage of SCF when the pH is above 4.5. Further studies are needed to define strategies to control the proliferation and metabolic activity of microorganisms involved in SCF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

A Postdoctoral Fellowship from the Spanish Government (MECD) fully funded Dr. E. Medina. We thank Mr. Seth Fornea with the USDA-ARS Food Science Research Unit, for technical help with HPLC analysis. We also thanks Dr. Joscelin Díaz with the Department of Food, Bioprocessing and Nutrition Sciences for assistance with statistical analysis.

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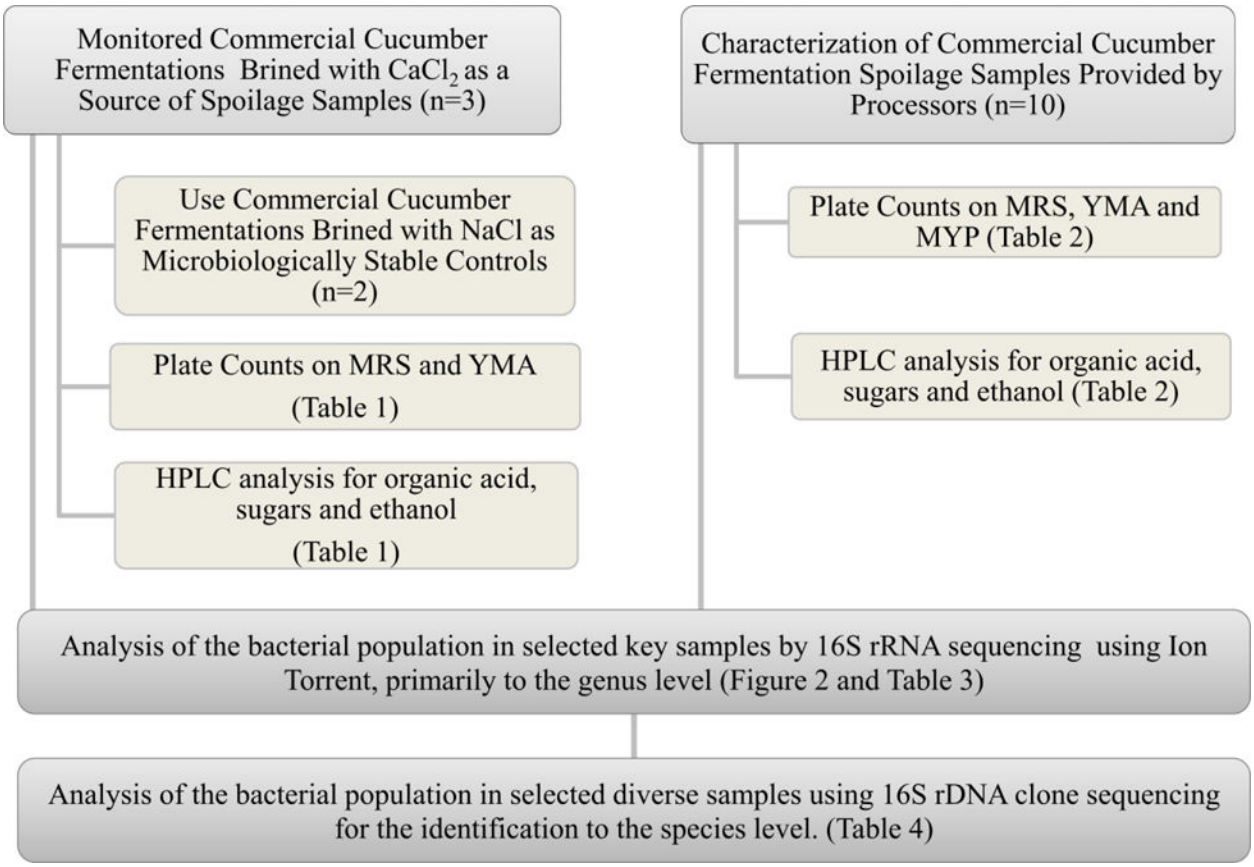

Figure 1.
Work flow schematic for the experimental approach.

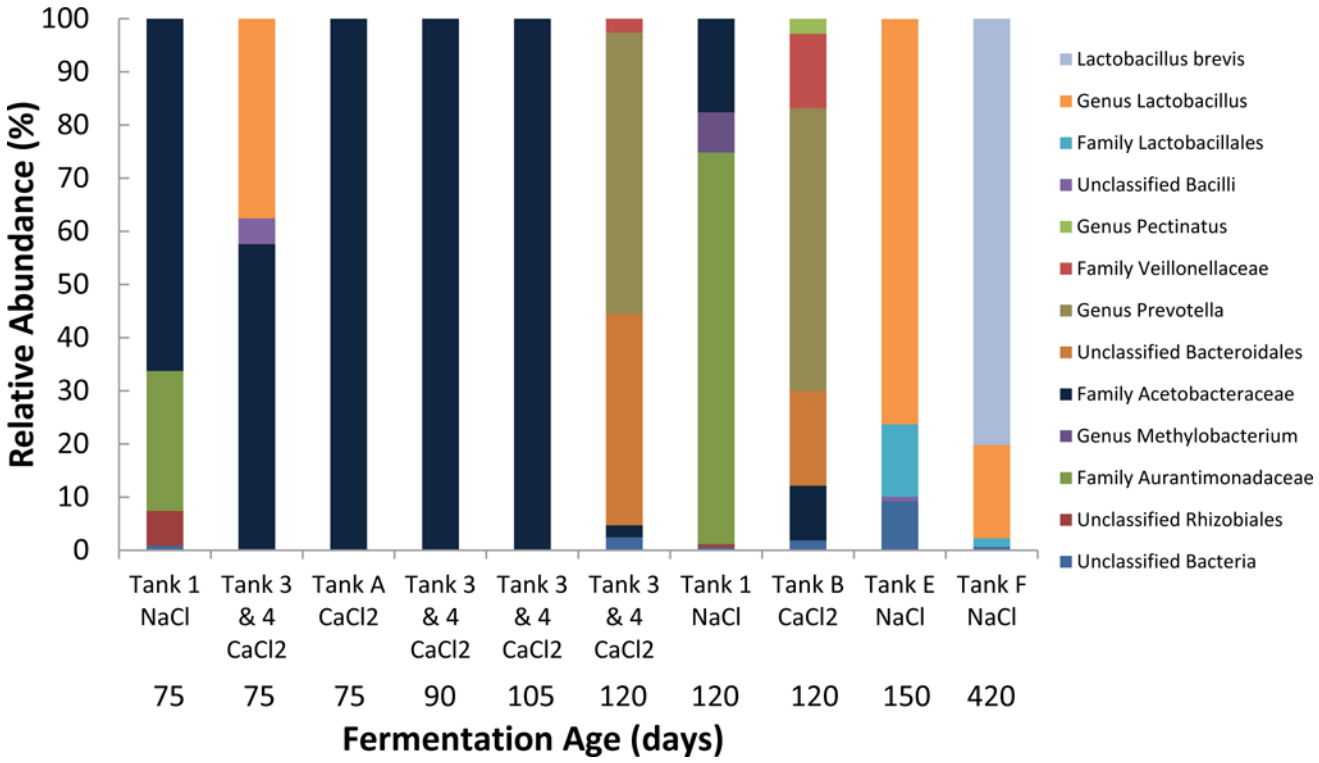

Figure 2.
Bacterial Taxonomic compositions in cucumber fermentation spoilage samples.

Table 1

Changes in pH, organic acid concentrations (mM) and microbial populations (log CFU/mL) in cover brine samples of commercial fermented cucumber rising pH spoilages. Average and standard deviations of two commercial fermentations brined with NaCl and two brined with $CaCl_2$, respectively, are shown.*

				Log of CFU/mL		Organic Acids Concentration (mM)	
Cover Brine Type	**Tanks Identification**	**Fermentation Age (d)**	**pH**	**YMA**	**MRS**	**Lactic**	**Acetic**
$CaCl_2$	3 & 4	60	3.4 ± 0.3^{AB}	3.3 ± 0.6	4.9 ± 0.1	91.6 ± 24.6^{A}	21.6 ± 7.3^{AB}
		76	3.7 ± 0.5^{AB}	3.3 ± 0.1	4.6 ± 0.2	56.6 ± 39.5^{BA}	30.3 ± 16.5^{AB}
		90	3.9 ± 0.7^{AB}	3.6 ± 0.2	5.1 ± 0.2	35.0 ± 49.5^{B}	44.5 ± 20.7^{B}
		105	4.0 ± 0.6^{B}	3.1 ± 0.3	5.6 ± 0.4	18.0 ± 25.4^{C}	54.8 ± 6.1^{C}
		120	4.3 ± 0.3^{C}	NA	NA	BDL^{D}	53.2 ± 7.5^{C}
NaCl	1 & 2	60	3.1 ± 0.1^{A}	BDL	BDL	101.9 ± 7.7^{A}	6.8 ± 9.6^{AD}
		76	3.1 ± 0.1^{A}	BDL	BDL	85.8 ± 7.0^{BA}	5.5 ± 7.8^{D}
		90	3.1 ± 0.1^{A}	3.6 ± 0.1	BDL	84.5 ± 8.1^{BA}	4.4 ± 6.2^{AD}
		105	3.1 ± 0.1^{A}	BDL	BDL	85.6 ± 5.5^{BA}	5.8 ± 8.2^{AD}
		120	3.1 ± 0.1^{A}	BDL	BDL	84.8 ± 9.1^{BA}	3.3 ± 4.7^{D}

Glucose and fructose were not detected in these samples. BDL: Below detection limits. NA: Not Available

*Data adapted from Pérez-Díaz and others, *Submitted*.

Table 2

Changes in pH value, organic acid concentrations (mM) and microbial population (log CFU/mL) in cover brine samples of commercial fermented cucumber rising pH spoilages provided by processors.

Cover Brine Type	Sample ID	Fermentation Age (d)	pH	Log CFU/mL			Organic acids concentration (mM)				
				MRS	YMA	MYP	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Propanol
$CaCl_2$	A	75	4.03	4.6	2.6	N/A	28.64	41.98	19.81	BDL	BDL
	B	120	4.50	5.2	4.9	N/A	BDL	58.48	24.86	7.46	7.54
	C	78	3.05	5.2	5.5	4.3	90.07	11.14	BDL	BDL	BDL
	D	72	3.40	6.4	4.9	4.1	29.36	31.83	3.74	BDL	8.42
	G	57	4.01	6.1	2.8	4.0	28.61	48.08	3.49	BDL	11.92
	H	49	4.04	6.2	2.4	BDL	30.53	47.61	BDL	BDL	15.21
	I	48	3.88	6.1	1.3	1.6	46.09	43.07	3.63	BDL	11.05
	J	64	3.90	6.7	4.8	BDL	25.96	53.91	3.93	BDL	15.61
	E*	150	3.22	BDL	5.6	N/A	91.00	31.00	12.00	BDL	4.58
NaCl	F*	420	4.26	6.4	5.4	N/A	39.00	22.34	6.00	BDL	BDL

Glucose and fructose were not detected in these samples. BDL: Below detection limits. N/A: not available.

*Data from Breidt and others 2013.

Table 3

Summary sheet with Chao1 and Shannon alpha diversity metrics for the Ion Torrent sequencing data analysis.

Fermentation Age (d)	Cover Brine Salt	Tank	Reads	OTUs	Chao1	Shannon
76	NaCl	Tank 1	19999	301	224.8 ± 26.9	2.8 ± 0.0
76	$CaCl_2$	Tanks 3 & 4	26192	301	203.1 ± 25.9	2.2 ± 0.1
76	$CaCl_2$	Tank A	40080	365	207.4 ± 24.5	1.7 ± 0.1
90	$CaCl_2$	Tanks 3 & 4	38372	357	199.5 ± 24.6	1.7 ± 0.1
105	$CaCl_2$	Tanks 3 & 4	36080	360	220.6 ± 34.3	1.6 ± 0.1
120	$CaCl_2$	Tanks 3 & 4	23343	235	220.6 ± 24.0	2.2 ± 0.0
120	NaCl	Tank 1	25572	254	173.1 ± 56.8	2.4 ± 0.1
120	$CaCl_2$	Tank B	12920	172	157.9 ± 20.6	3.7 ± 0.0
150	NaCl	Tank E	6407	317	369.4 ± 29.1	3.8 ± 0.0
420	NaCl	Tank F	7465	239	257.0 ± 29.1	4.0 ± 0.0

Table 4

The 16S rRNA clone sequence BLASTN results from spoilage brine samples.

Sample/tank ID	**1**	**4**	**D**	**B**		
Fermentation age (d)	**75**	**75**	**72**	**120**		
pH	**3.08**	**3.33**	**3.4**	**4.5**		
Tentative ID[a]	**Number of clones sequenced**				**% similarity**[b]	**Phylum; Class, Order**
Granulicella sp.	2				97	*Acidobacteria; Acidobacteriales; Acidobacteriaceae*
Delftia sp.	2				99 to 100	*Proteobacteria; Betaproteobacteria; Burkolderiales*
Methylobacterium komagatae	2				98 to 99	*Proteobacteria; Alphaproteobacteria; Rhizobiales*
Sphingomonas sp.	2				98	*Proteobacteria; Alphaproteobacteria; Sphingomonadales*
Achromobacter sp.	1				99	*Proteobacteria; Betaproteobacteria; Burkolderiales*
Aquicella lusitana	1				96	*Proteobacteria; Gammaproteobacteria; Legionellales;*
Comamonas testosteroni	1				100	*Proteobacteria; Betaproteobacteria; Burkolderiales*
Zoogloea resiniphila	1				99	*Proteobacteria; Betaproteobacteria; Rhodocyclales*
Legionella yabuuchiae	1				97	*Proteobacteria; Gammaproteobacteria; Legionellales*
Rhodopirellula baltica	1				86	*Planctomycetes; Planctomycetia; Planctomycetales*
Prosthecobacter fluviatilis	1				97	*Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales*
Pullulanibacillus naganoensis	1				96	*Firmicutes; Bacilli; Bacillales*
Bacillus sp.	2	2			95 to 99	*Firmicutes; Bacilli; Bacillales*
Lactobacillus sp.	1		8		99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus parabrevis	6				98 to 99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus brevis/hammesii	1				98	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus namurensis		6			98 to 99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus acetotolerans		34	1		92 to 99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus buchneri	1	5	1		96 to 99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus panis		1	1		94 to 99	*Firmicutes; Bacilli; Lactobacillales*
Lactobacillus rapi			1		99	*Firmicutes; Bacilli; Lactobacillales*
Acetobacter peroxydans	9	1	23	2	95 to 100	*Proteobacteria; Alphaproteobacteria; Rhodospirillales*
Acetobacter aceti	1		2		98 to 99	*Proteobacteria; Alphaproteobacteria; Rhodospirillales*
Acetobacter pasteurianus			2		94 to 99	*Proteobacteria; Alphaproteobacteria; Rhodospirillales*

Sample/tank ID	**1**	**4**	**D**	**B**		
Fermentation age (d)	**75**	**75**	**72**	**120**		
pH	**3.08**	**3.33**	**3.4**	**4.5**		
Tentative ID[a]	**Number of clones sequenced**				**% similarity**[b]	**Phylum; Class, Order**
Pectinatus sp.			10	6	89 to 99	*Firmicutes; Clostridia; Clostridiales*
Prevotella sp.			1	26	86 to 94	*Bacteroidetes; Bacteroidia; Bacteroidales*
Dialister microaerophilus				8	93 to 95	*Firmicutes; Negativicutes; Selenomonadales*
Dialister propionicifaciens				4	90 to 93	*Firmicutes; Negativicutes; Selenomonadales*
Dialister succinatiphilus				1	94	*Firmicutes; Negativicutes; Selenomonadales*

[a] The tentative identification of the species based on the BLASTN alignment using NCBI Bacterial and Archaeal database

[b] The percentage sequence similarities for each cloned sequence with the closest match in the NCBI 16S Bacterial and Archaeal database