Influence of Sodium Chloride, pH, and Lactic Acid Bacteria on Anaerobic Lactic Acid Utilization during Fermented Cucumber Spoilage

Suzanne D. Johanningsmeier, Wendy Franco, Ilenys Perez-Diaz, and Roger F. McFeeters

Abstract: Cucumbers are preserved commercially by natural fermentations in 5% to 8% sodium chloride (NaCl) brines. Occasionally, fermented cucumbers spoil after the primary fermentation is complete. This spoilage has been characterized by decreases in lactic acid and a rise in brine pH caused by microbial instability. Objectives of this study were to determine the combined effects of NaCl and pH on fermented cucumber spoilage and to determine the ability of lactic acid bacteria (LAB) spoilage isolates to initiate lactic acid degradation in fermented cucumbers. Cucumbers fermented with 0%, 2%, 4%, and 6% NaCl were blended into slurries (FCS) and adjusted to pH 3.2, 3.8, 4.3, and 5.0 prior to centrifugation, sterile-filtration, and inoculation with spoilage organisms. Organic acids and pH were measured initially and after 3 wk, 2, 6, 12, and 18 mo anaerobic incubation at 25 °C. Anaerobic lactic acid degradation occurred in FCS at pH 3.8, 4.3, and 5.0 regardless of NaCl concentration. At pH 3.2, reduced NaCl concentrations resulted in increased susceptibility to spoilage, indicating that the pH limit for lactic acid utilization in reduced NaCl fermented cucumbers is 3.2 or lower. Over 18 mo incubation, only cucumbers fermented with 6% NaCl to pH 3.2 prevented anaerobic lactic acid degradation by spoilage bacteria. Among several LAB species isolated from fermented cucumber spoilage, *Lactobacillus buchneri* was unique in its ability to metabolize lactic acid in FCS with concurrent increases in acetic acid and 1,2-propanediol. Therefore, *L. buchneri* may be one of multiple organisms that contribute to development of fermented cucumber spoilage.

Keywords: lactic acid degradation, *Lactobacillus buchneri*, reduced salt, spoilage biochemistry, vegetable fermentation

Practical Application: Microbial spoilage of fermented cucumbers during bulk storage causes economic losses for producers. Current knowledge is insufficient to predict or control these losses. This study demonstrated that in the absence of oxygen, cucumbers fermented with 6% sodium chloride to pH 3.2 were not subject to spoilage. However, lactic acid was degraded by spoilage microorganisms in reduced salt, even with pH as low as 3.2. Efforts to reduce salt in commercial brining operations will need to include control measures for this increased susceptibility to spoilage. *Lactobacillus buchneri* was identified as a potential causative agent and could be used as a target in development of such control measures.

Introduction

Cucumber fermentation and storage in bulk tanks is a method for the preparation of pickle products that allows the preservation of cucumber fruits for extended periods of time and results in a unique food product. Occasionally, tanks of commercially fermented cucumbers spoil after the primary fermentation. The unpredictable nature of this spoilage has resulted in increased production costs for the pickling industry, mainly in the form of increased monitoring of fermentation tanks. In cases where the conditions leading to microbial spoilage are not detected early, additional costs are incurred from product losses and disposal of the spoiled product. Fleming and others (1989) observed this spoilage in pilot scale (4500 L) closed tank fermentations carried out in reduced salt brines under anaerobic conditions. However, the organism(s) that initiated lactic acid degradation were not identified. Further study found that spoilage of small cucumbers increased when the pH of samples of fermented cucumbers from commercial tanks (salt concentration 4.8% to 5.5%) was raised from 3.6 to 3.8 to pH 4.0 and decreased when pH was reduced to 3.5 (Fleming and others 2002). However, at reduced sodium chloride (NaCl) concentrations (0% and 2% NaCl), spoilage potential was demonstrated at pH 3.5 within 3 mo of anaerobic incubation (Kim and Breidt 2007). This type of spoilage has been characterized by a normal lactic acid fermentation followed by depletion of lactic acid, rise in pH, and increases in acetic, propionic, and butyric acids and n-propanol during prolonged storage under anaerobic conditions (Fleming and others 1989; Fleming and others 2002). Commercially, the production of volatile compounds and increased pH can compromise both the quality and safety of the product. Attempts to isolate the responsible microorganisms have proven difficult (Fleming and others 1989), and the causative organisms and environmental conditions required for lactic acid utilization in fermented cucumbers have not yet been fully elucidated.

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Although lactic acid is typically considered an end product of fermentation, it can also serve as a substrate of metabolism for many microorganisms, including some species of lactic acid bacteria (LAB). Aerobic utilization of lactic acid as an energy source by *Lactobacillus plantarum* has been demonstrated with acetic acid as the accumulated end product (Murphy and others 1985). This metabolic activity was inhibited by 6 and 8% NaCl and less evident under anaerobic conditions (Bobillo and Marshall 1991). Anaerobically, *L. plantarum* fermented glucose to lactic acid with subsequent co-depletion of lactic and citric acids accompanied by increases in acetic, formic, and succinic acids and carbon dioxide (Lindgren and others 1990). Similarly, *Lactobacillus buchneri* and *Lactobacillus brevis* have been shown to exhibit anaerobic growth with lactate as an energy source, and growth of these bacteria was significantly increased in the presence of glycerol (Viega-da-Cunha and Foster 1992). Lactic acid degradation by *L. buchneri* was also demonstrated in silage, resulting in increased aerobic stability of the silage due to the increases in acetic and propionic acid concentrations (Driehuis and others 1999). Furthermore, *Lactobacillus* species were previously isolated from fermented cucumber spoilage samples (Franco and others 2012; unpublished data). Therefore, it was hypothesized that LAB which are able to survive and maintain metabolic activity in the presence of 4% to 6% NaCl and high acid pH contribute to the lactic acid degradation observed during fermented cucumber spoilage. The objectives of this study were (1) to determine the combined effects of pH and NaCl concentration on the ability of spoilage organisms from reduced NaCl fermentations and commercial sources to degrade lactic acid in fermented cucumber slurry; and (2) to test the ability of LAB isolated from these spoilage sources to initiate lactic acid degradation in fermented cucumbers.

Materials and Methods

Production of anaerobic spoilage at varying NaCl concentrations and pH

Pickling cucumbers (size 2B) were fermented with 0%, 2%, 4%, and 6% NaCl equilibrated concentrations. *Lactobacillus plantarum* (Culture Collection ID LA0219, USDA-ARS Food Science Research Unit, Raleigh, N.C., U.S.A.) was inoculated to 10^6 CFU/g to each fermentation vessel. Fermentations were conducted in triplicate 1 gal (approximately 3.8 L) sized glass jars with a 55:45 pack-out ratio (weight cucumbers:volume of cover brine solution). Fermentations were monitored by measuring pH and the HPLC analysis of acids and sugars. Fermented cucumbers were blended into slurry and stored at −10 °C. To prepare media for inoculation with spoilage microorganisms, fermented cucumber slurries with 0%, 2%, 4%, and 6% NaCl were thawed and then pressed through 3 layers of cheesecloth to remove large particulates prior to centrifugation in 250 mL bottles at 23400 × *g* for 15 min under refrigeration. For each NaCl concentration, clarified slurry was divided into 4 aliquots. One aliquot had no pH adjustment (pH 3.2). The other 3 aliquots were adjusted to pH 3.8, 4.3, and 5.0 with 6N NaOH. The resulting clarified, pH-adjusted fermented cucumber slurries (FCS) were each filter sterilized with a 0.2 μm bottle top filter apparatus (Nalgene FAST PES, 0.2 μm pore size, 90-mm-dia. membrane, Daigger, Vernon Hills, Ill., U.S.A.). FCS (12 mL) was aseptically transferred into sterile 15 mL conical tubes and placed into the Coy anaerobic chamber for at least 2 d to allow for the removal of oxygen from the media prior to inoculation with spoilage brine. Media were inoculated and stored anaerobically for the entire study period using a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, Mich., U.S.A.). Samples were aseptically withdrawn immediately after inoculation and after 21 d, 2, 6, 12, and 18 mo of incubation at ambient temperature (21 to 25 °C). Samples for chemical analysis were stored at −20 °C. High performance liquid chromatography (HPLC) quantification of glucose, fructose, glycerol, ethanol, propanol, malic, succinic, lactic, acetic, propionic, and butyric acids was used to measure anaerobic lactic acid degradation and formation of possible spoilage metabolites. Targeted pHs were achieved and maintained in uninoculated controls throughout the study. A rise in pH accompanied spoilage in inoculated media as expected.

Spoiled fermented cucumbers from 3 sources were used for anaerobic reproduction of spoilage (Table 1). These spoilage sources, reduced NaCl, commercial 1M, and commercial 2 were handled and used as inocula as described in Figure S1, S2, and S3, respectively. Supplementary data that describe lactic acid utilization in these experimental spoilages are available in Tables S1, S2, and S3 of the Supplementary Information.

Isolation and identification of selected LAB from fermented cucumber spoilage

LAB were isolated from the sources described in Table 2 before and after anaerobic reproduction of spoilage, as outlined in Figure S1 and S3. For experimental spoilage R4, LAB were

Table 1–Spoiled fermented cucumber sources for anaerobic reproduction of spoilage and isolation and identification of lactic acid bacteria (LAB).

Spoilage ID	Source description	Storage
Reduced NaCl	Brine from a 5 gal (19 L) pail of brined cucumbers (2% NaCl) that had fermented normally, but then spontaneously spoiled after several months of storage as described by Johanningsmeier and McFeeters (2011). Residual lactic acid was 10 mM and pH was 4.1. Acetic, propionic and butyric acids were 80 mM, 40 mM, and undetected, respectively. Yeasts were not detected and LAB count on MRS was 1.19×10^6 CFU/mL.	−80 °C with glycerol
Commercial 1M	Spoiled brine from a commercial cucumber fermentation that had been sequentially transferred into 2% NaCl fermented cucumbers that were adjusted to pH 5.0 and held anaerobically at ambient temperature until evidence of spoilage activity was demonstrated (Breidt unpublished data). The resulting spoiled brine was stored in individual aliquots with glycerol at −80 °C.	−80 °C with glycerol
Commercial 2	Cover brine solution from a commercial cucumber fermentation tank that underwent spoilage during the course of this study. In this spoilage brine, lactic acid had been completely depleted, pH was 4.9, and acetic, propionic, and butyric acids were 32 mM, 36 mM, and 52 mM, respectively. NaCl was estimated at 4.3% at the time the sample was received. Yeast count on YMA was 6.13×10^4 CFU/mL and LAB count on MRS was 7.15×10^5 CFU/mL.	4 °C

isolated on Lactobacilli deMan, Rogosa, and Sharpe agar (MRS; Becton Dickinson and Co., Franklin Lakes, N.J., U.S.A.) after 22 wk anaerobic spoilage of FCS (initial pH 3.8) at varying NaCl concentrations. Samples where lactic acid utilization was demonstrated (Table S3) were spiral plated (Autoplate 400, Spiral Biotech, Norwood, Mass., U.S.A.) on MRS and incubated anaerobically for 4 to 6 d at 30 °C. Isolates from these samples were selected for identification based on differences in colony morphology (Johanningsmeier 2011). LAB isolates from original samples and anaerobic spoilage experiments were identified by partial sequencing of 16S rRNA using one of three primer pairs: (1) 8f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) (Wilson and others 1990); (2) Ru2 (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Barrangou and others 2002) and KR2DN (5′-ACT GCT GCC TCC CGT AGG AG-3′); or (3) Ru2 (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Barrangou and others 2002) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) (Wilson and others 1990).

Amplicons were purified and sent to GeneWiz (LaJolla, Calif., U.S.A.) or the North Carolina State Genomic Sciences laboratory (Raleigh, N.C., U.S.A.) for sequencing. All sequences were examined using the original chromatographs and edited with ABI Sequence Scanner (Applied Biosystems, Carlsbad, Calif., U.S.A.). The sequences obtained were subjected to the National Center for

Table 2–Lactic acid bacteria isolated from fermented cucumber spoilages.

Spoilage source	Organism ID (Prevalence)	Culture collection ID[a] for representative isolate(s) (accession#[b])	Isolation medium	Temperature	Time (d)
Reduced NaCl fermented cucumber spoilage	*Lactobacillus buchneri (8/14)*	LA1147 (JQ249035); LA1173 (JQ249034); LA1175 (JQ249037); LA1178 (JQ249040); LA1181 (JQ249043); LA1182 (JQ249044); LA1184 (JQ249046)	M17 lactic & MRS	25 °C	7
Reduced NaCl fermented cucumber spoilage	*Lactobacillus brevis (3/14)*	LA1174 (JQ249036); LA1179 (JQ249041); LA1180 (JQ249042)	M17 lactic & BHI	25 °C	7
Reduced NaCl fermented cucumber spoilage	*Pediococcus ethanolidurans (2/14)*	LA1176 (JQ249038); LA1183 (JQ249045)	M17 lactic & MRS	25 °C	7
Reduced NaCl fermented cucumber spoilage	*Lactobacillus plantarum/pentosus (1/14)*	LA1177 (JQ249039)	M17 lactic	25 °C	7
R2: Anaerobic spoilage in 6% NaCl FCS with Reduced NaCl spoilage source	*Lactobacillus buchneri (12/20)*	LA1138 (FJ867641)	MRS	30 °C	6
R2: Anaerobic spoilage in 6% NaCl FCS with reduced NaCl spoilage source	*Pediococcus ethanolidurans (8/20)*	LA1139 (FJ867642)	MRS	30 °C	6
R4: Anaerobic spoilage in FCS (0 to 6% NaCl) with reduced NaCl source reduced NaCl source	*Lactobacillus buchneri (9/13)*	LA1151 (JQ249047); LA1152 (JQ249048); LA1154 (JQ249052); LA1155 (JQ249053); LA1156 (JQ249054); LA1157 (JQ249055); LA1158 (JQ249056); LA1159 (JQ249057); LA1160 (JQ249058)	MRS	30 °C	4 to 6
R4: Anaerobic spoilage in FCS (0 to 6% NaCl) with reduced NaCl source	*Lactobacillus parafarraginis (2/13)*	LA1153 (JQ249049); LA1168 (JQ249050)	MRS	30 °C	4 to 6
R4: Anaerobic spoilage in FCS (0 to 6% NaCl) with reduced NaCl source	*Lactobacillus rapi (2/13)*	LA1169 (JQ249051) LA1169 (JQ249051)	MRS	30 °C	4 to 6
Commercial spoilage 2	*Lactobacillus casei/paracasei (7/23)*	LA1141 (JQ249067)	MRS	30 °C	2
Commercial spoilage 2	*Lactobacillus harbinensis (6/23)*	LA1144 (JQ249070); LA1145 (JQ249071)	MRS	30 °C	2
Commercial spoilage 2	*Lactobacillus plantarum/pentosus (5/23)*	LA1142 (JQ249068)	MRS	30 °C	2
Commercial spoilage 2	*Pediococcus ethanolidurans (2/23)*	N/A	MRS	30 °C	2
Commercial spoilage 2	*Lactobacillus camelliae (1/23)*	LA1146 (JQ249072)	MRS	30 °C	2
Commercial spoilage 2	*Lactobacillus coryniformis (1/23)*	LA1143 (JQ249069)	MRS	30 °C	2
Commercial spoilage 2	*Pediococcus parvulus (1/23)*	LA1140 (JQ249066)	MRS	30 °C	2
R4: Anaerobic spoilage in FCS (0 to 6% NaCl) with commercial spoilage 2	*Lactobacillus buchneri (5/6)*	LA1161 (JQ249060); LA1163 (JQ249062); LA1164 (JQ249063); LA1166 (JQ249064); LA1167 (JQ249065)	MRS	30 °C	4 to 6
R4: Anaerobic spoilage in FCS (0 to 6% NaCl) with commercial spoilage 2	*Lactobacillus harbinensis/perolens (1/6)*	LA1162 (JQ249061)	MRS	30 °C	4 to 6

[a]USDA-ARS Food Science Research Unit, Raleigh, NC.
[b]GenBank® database record accession number (http://www.ncbi.nlm.nih.gov/genbank/).

Biotechnology Information (NCBI) basic local alignment search tool (BLAST 2.2.26; Altschul and others 1990) using the GenBank (Benson and others 2011) 16S microbial database to determine the identities of the isolates. All BLAST matches reported had 99% to 100% identity with the query sequence.

Evaluation of the ability of LAB spoilage isolates to utilize lactic acid

Lactic acid utilization by LAB spoilage isolates was tested by inoculating FCS (pH 3.8, 6% NaCl) with approximately 10^6 CFU/mL of each organism. LAB isolates were grown in either MRS broth or modified cucumber slurry medium (mCS). The modified cucumber slurry medium was prepared from fresh size 2B cucumbers blended into slurry and processed as described above for fermented cucumber slurry. After centrifugation, NaCl, Bacto™ Yeast Extract, Bacto™Peptone (Becton Dickinson and Company, Sparks, Md., U.S.A.) and water were added to give final concentrations of 67% fresh cucumber slurry, 4% NaCl, 1% yeast extract, and 1% peptone. Yeast extract and peptone were added to enhance the ability of some of the more fastidious LAB isolates to grow in pure culture. The resulting growth medium (mCS) was sterile-filtered with a 0.22 μm bottle top filter and stored at 4 °C. LAB isolates (Table 2) were grown anaerobically at 30 °C until visual turbidity was observed. Cells were spun down, resuspended in FCS, and inoculated into FCS at ambient temperature in the anaerobic chamber. Inoculated FCS was aseptically sampled at time intervals throughout the incubation period to measure pH and determine the extent of lactic acid degradation by HPLC analysis.

High performance liquid chromatography analysis of fermentation substrates and products

HPLC analysis was conducted with minor modification of the method published by McFeeters and Barish (2003). Briefly, components of samples were separated on an Aminex HPX-87H resin column (300 × 7.8 mm, Bio-Rad Laboratories, Hercules, Calif., U.S.A.) with 0.03 N H_2SO_4 eluent at a flow rate of 0.6 mL/min. The column temperature was held at 37 °C to separate propionic acid, a potential spoilage metabolite, from an unknown component that frequently occurs in fermented cucumbers. Malic, succinic, lactic, acetic, propionic, and butyric acids were detected with ultraviolet light at 210 nm. Glucose, fructose, ethanol, glycerol, and 1,2-propanediol were quantified in the same analysis using a refractive index detector connected in series.

pH measurement

Measurements of pH were done at ambient temperature with an Accumet AR25 pH meter equipped with a gel-filled combination pH electrode (Fisher Scientific, Pittsburgh, Pa., U.S.A.) that was calibrated with certified standards of pH 2.00, 4.00, and 7.00 (Fisher Scientific).

Results and Discussion

Prior attempts to reproduce fermented cucumber spoilage under controlled laboratory conditions and isolate the responsible microorganisms have been challenging. There is a diversity of organisms present in vegetable fermentations, so the potential for multiple stages in the spoilage process exists. In this study, controlled reproduction of lactic acid degradation (spoilage) in FCS was achieved using spoilage cultures obtained from 3 spoilage sources. LAB were isolated and identified from reduced NaCl and commercial spoilage brines and from spoilage produced experimentally using these sources as inoculum in filter-sterilized FCS. Anaerobic lactic acid degradation was observed in FCS inoculated with the reduced NaCl spoilage regardless of NaCl concentration at pH 3.8, 4.3, and 5.0 with the greatest extent of spoilage occurring at pH 3.8 (Figure 1). At pH 3.2, lactic acid degradation occurred in 0%, 2%, and 4% NaCl, but not in 6% NaCl FCS. The rate and extent of lactic acid degradation in FCS at pH 3.2 was significantly greater with 0% and 2% NaCl than in 4% NaCl (Figure 1). Although lactic acid depletion was much slower in 4% NaCl at pH 3.2, a gradual decrease in lactic acid concentration was observed between 6 and 12 mo incubation (Table S1). The increased susceptibility to spoilage at reduced NaCl concentrations that was observed is consistent with previous reports (Fleming and others 2002; Kim and Breidt 2007) and provides evidence for the first time that lactic acid can be degraded under anaerobic conditions in fermented cucumbers with a pH as low as 3.2. Kim and Breidt (2007) demonstrated that spoilage microorganisms could degrade lactic acid in reduced NaCl fermented cucumber slurries at pH 3.5 but not at pH 3.0. The current study shows that the pH limit for anaerobic lactic acid utilization in reduced NaCl fermented cucumbers is 3.2 or lower. This pH is within the range of commercially fermented cucumbers, which typically have a pH of 3.3 ± 0.1 (Franco and others 2012). Decreases in lactic acid were accompanied by increases in acetic acid, propionic acid, and propanol for all NaCl and pH conditions. The only condition where anaerobic lactic acid degradation (spoilage) was completely prevented was in 6% NaCl fermented cucumbers at pH 3.2 (Figure 1). Under these conditions, no lactic acid was degraded even upon extended incubation to 18 mo.

A commercial spoilage sample (commercial spoilage 1M) previously characterized by Breidt and others (unpublished data) was used for comparison of these results to prior studies of fermented cucumber spoilage in our laboratory. Interestingly, this spoilage culture responded differently to varying conditions of NaCl and pH than the reduced NaCl spoilage culture (Table S1). Lactic acid utilization occurred more rapidly at pH 5.0 and 4.3 and butyric acid was produced in some (pH 5.0, 2% and 4% NaCl) but not all NaCl and pH combinations. These differences may have been due to selection of different microorganisms (as evidenced by different spoilage metabolites at pH 5.0) and possibly a preconditioning effect (as evidenced by the longer lag time but formation of similar metabolites at pH 3.8). This particular commercial spoilage culture had been serially passaged through pH 5.0 and 2% NaCl fermented cucumber media prior to freezing and storage. In contrast, the reduced NaCl spoilage culture was obtained directly from fermented cucumber brine (2% NaCl) that had undergone spontaneous spoilage and had not experienced any freezing or other processes that might have selected for or against microorganisms present as spoilage occurred. These differential results indicate that multiple organisms capable of metabolizing lactic acid are likely to be present in fermented cucumber brines. It is reasonable to expect that spoilage brines from different sources may contain different organisms and increasing the pH experimentally can allow organisms to produce metabolic products that may not normally occur in commercial spoilage situations. For example, *Propionibacterium* spp. are known to convert lactic acid to acetic and propionic acids, but this metabolism has only been reported at pH 4.5 and above (Plastourgos and Vaughn 1957; Hsu and Yang 1991; Rehberger and Glatz 1998). Similarly, *Clostridium* spp. are known to produce butyric acid, and this has been demonstrated in fermented cucumbers only when the pH was

experimentally raised to pH 5.0 and above with NaOH (Fleming and others 1989; Franco 2011). Therefore, such organisms may be present in commercial fermentation environments and play a role in the latter stages of fermented cucumber spoilage after the pH has risen, but these organisms would not be able to initiate lactic acid utilization under the conditions that typically prevail prior to spoilage (2% to 6% NaCl and pH 3.2 to 3.8). We have provided evidence that fermented cucumber spoilage can be reproduced in the laboratory in 6% NaCl FCS at pH 3.8. This set of conditions is within the range of NaCl and pH that may be encountered in commercial fermentation tanks near the onset of spoilage. Therefore, FCS at pH 3.8 was used for continued experimental production of spoilage and testing LAB spoilage isolates for the ability to initiate lactic acid degradation in fermented cucumbers.

LAB were present in significant numbers during anaerobic spoilage of 6% NaCl FCS adjusted to pH 3.8 and inoculated with reduced NaCl spoilage and commercial spoilage 2. Initially, numbers of LAB were lower for the commercial spoilage treatment but increased to 7.3 log CFU/mL prior to the initiation of lactic acid degradation. Although the number of LAB were higher in the reduced NaCl spoilage inoculum, total LAB counts increased only 1.5 log from 6.1 to 7.6 log CFU/mL and reached the highest numbers 2 d after inoculation. The utilization of residual glucose appeared to be related to the increases in LAB (Figure 2). The

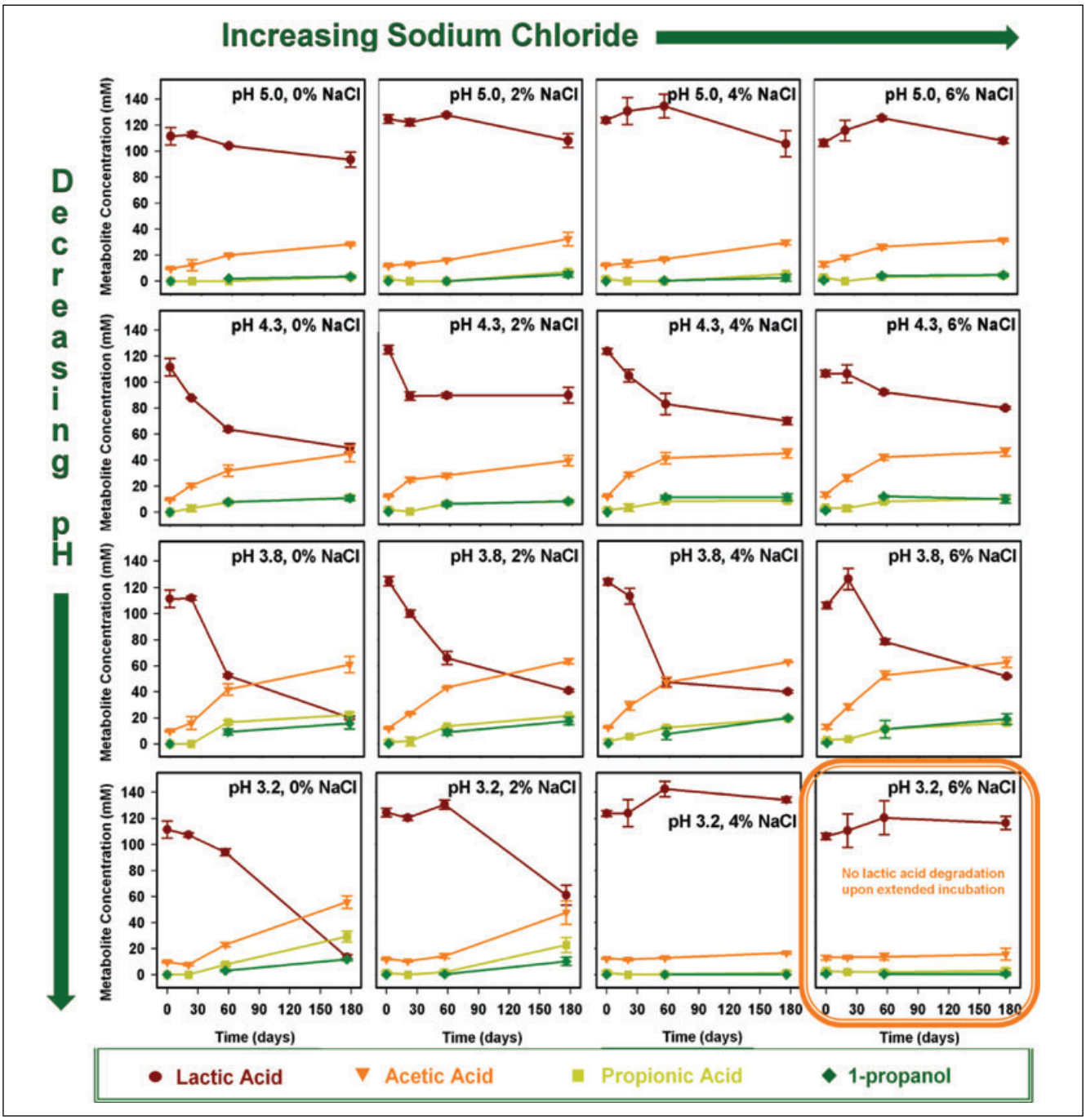

Figure 1–Effects of initial pH and NaCl concentration on anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms from reduced NaCl cucumber fermentation.

approximately 3 mM residual glucose present in FCS was depleted in 8 d by the reduced NaCl spoilage organisms and 24 d by the commercial spoilage organisms. Regardless of the differences during the initial incubation period, anaerobic lactic acid degradation began approximately 24 d after inoculation for both spoilage cultures (Figure 3). The rate of lactic acid utilization by the reduced NaCl spoilage inoculum was greater than for the commercial spoilage source. Nevertheless, decreases in lactic acid concentration were accompanied by increases in acetic and propionic acids (Figure 3) and rise in pH to 4.57 ± 0.01 and 4.33 ± 0.05 for the reduced NaCl and commercial spoilage reproductions, respectively. LAB remained viable during the course of lactic acid utilization (127 d), but did not increase in total number (Figure 2).

Several LAB were isolated from spoiled fermented cucumbers and experimental spoilage produced in sterile-filtered, FCS adjusted to pH 3.8 (Table 2). Organisms were primarily from the *Lactobacillus* genus. *L. plantarum*, *L. brevis*, and *Pediococcus* spp. have been previously documented as part of the normal flora of fermented cucumbers (Etchells and Jones 1946; Pederson and Ward 1949; Pederson and Albury 1950, 1956; Costilow and others 1956). *L. plantarum* and *L. brevis* were isolated from both the reduced NaCl spoilage brine and the commercial spoilage sample in this study. Although these organisms have been shown to degrade lactic acid under certain conditions (Murphy and others 1985; Lindgren and others 1990; Bobillo and Marshall 1991; Viega-da-Cunha and Foster 1992), there was no evidence of lactic acid degradation by *L. plantarum* and *L. brevis* spoilage isolates in FCS (pH 3.8 with either 2% or 6% NaCl) even during extended incubation for 146 d under aerobic or anaerobic atmospheres (data not shown). Therefore, we concluded that these organisms do not contribute to the initiation of fermented cucumber spoilage, since they did not utilize lactic acid in FCS under conditions typical of those encountered in commercial cucumber fermentations. *Pediococcus ethanolidurans* is a newly proposed *Pediococcus* specie that was first isolated from a distilled spirits cellar in China and shown to grow under stressful environmental conditions (Liu and others 2006). This bacterium has also recently been identified in brine samples from commercial cucumber fermentations that were spoiling (Franco and others 2012). In addition, a number of *Lactobacillus* species less commonly associated with cucumber fermentations were found, including *L. buchneri*, *L. camelliae*, *L. casei*, *L. coryniformis*, *L. harbinensis*, *L. parafarraginis*, and *L. rapi*. Of these, *L. buchneri*, *L. casei*, and *L. coryniformis* have been the most well studied and have been frequently associated with food fermentations. *L. camelliae* is a homofermentative organism genetically related to *L. casei* that produces only the L-isomer of lactic acid and was originally isolated from fermented tea leaves in Thailand (Tanasupawat and others 2007). This organism did not grow in mCS with 4% NaCl, so it was not tested for lactic acid degradation in FCS. Since we were unable to test *L. camelliae* under laboratory conditions, we cannot comment on its importance or lack thereof in commercial fermented cucumber spoilage. *L. harbinensis* has been isolated from traditional Chinese fermented vegetables, brewery environments, Korean rice wine (Jianbo and others 2008) and spoiled carbonated beverages and was proposed as a new species related to the *L. casei-Pediococcus* group by Miyamoto and others (2005). *L. parafarraginis* was isolated from distilled shochu residue compost and was found to be closely related to *L. buchneri* (Endo and Okada 2007). Similarly, *L. rapi* was recently isolated from sunki, a traditional Japanese pickle fermented without NaCl, and described as a new species in the *L. buchneri* group of the lactobacilli (Watanabe and others 2009). In all, 11 different LAB species were identified among 76 isolates from fermented cucumber spoilage samples, including multiple strains of *L. buchneri*.

Despite the diversity of LAB that was present in spoiled fermented cucumber samples, *L. buchneri* was unique in its ability to degrade lactic acid in FCS at pH 3.8 and 6% NaCl under anaerobic conditions (data not shown). Decreases in lactic acid accompanied increases in acetic acid and 1,2-propanediol in FCS (2% NaCl, pH 3.8) inoculated with the *L. buchneri* strain isolated from reduced NaCl spoilage (Figure 4). These chemical changes were consistent with the utilization of lactic acid by *L. buchneri* in acidified laboratory medium without NaCl observed by Oude Elferink and others (2001). However, 1,2-propanediol was not detected in the experimental production of spoilage with mixed spoilage cultures, indicating that one or more other organisms were

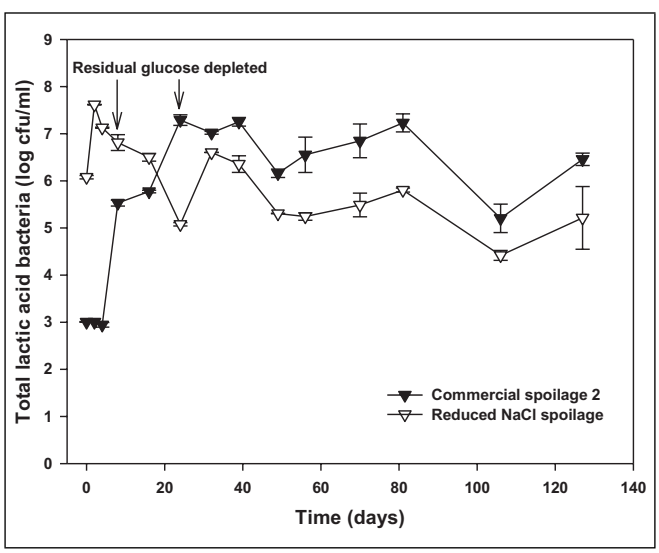

Figure 2–Presence of lactic acid bacteria during anaerobic spoilage of fermented cucumber slurry (6% NaCl, pH 3.8) inoculated with spoilage organisms from reduced NaCl and commercial spoilage 2 brines.

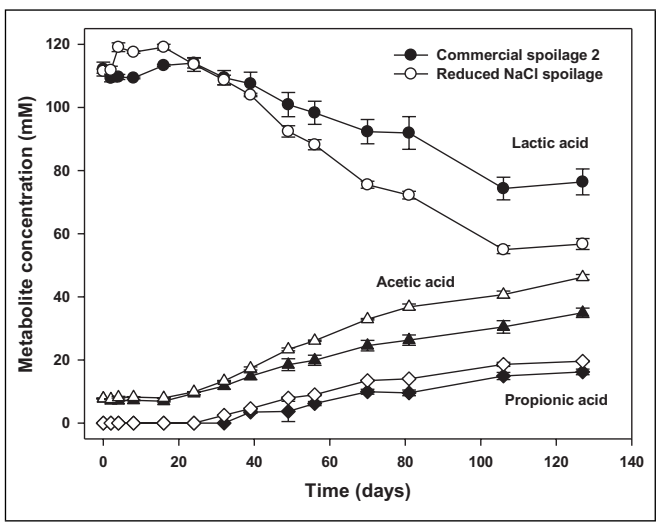

Figure 3–Metabolite changes during anaerobic spoilage of fermented cucumber slurry (6% NaCl, pH 3.8) inoculated with spoilage organisms from reduced NaCl and commercial spoilage 2 brines (circles = lactic acid; triangles = acetic acid; and diamonds = propionic acid. Commercial spoilage represented by filled symbols. Reduced NaCl spoilage represented by open symbols.).

involved. In silage, lactic acid was converted to acetic acid, propionic acid, and propanol when *L. buchneri* was added as a starter culture adjunct (Driehuis and others 1999). However, *L. buchneri* does not produce propionic acid and propanol in pure culture. Accordingly, Krooneman and others (2002) isolated *Lactobacillus diolivorans* from treated silage and proposed that it was responsible for converting 1,2-propanediol produced by *L. buchneri* to propionic acid and propanol. Therefore, the LAB species isolated from the commercial spoiled brine and *P. ethanolidurans*, isolated with *L. buchneri* from experimental spoilage, were inoculated into FCS in combination with *L. buchneri*, but no propionic acid nor propanol were formed. The same amount of 1,2-propanediol accumulated in FCS inoculated with *L. buchneri* regardless of the presence of the other LAB species (data not shown). *L. rapi* and *L. parafarraginis* were isolated for the first time from anaerobic spoilage of FCS in recent experiments. These isolates are currently under investigation, and preliminary evidence suggests that these lactobacilli may also have a role in secondary cucumber fermentation.

Only 1 of 8 *L. buchneri* isolates from the original reduced NaCl fermented cucumber spoilage demonstrated measurable anaerobic lactic acid degradation in FCS (pH 3.8, 2% NaCl) within 56 d. This suggests that the ability to metabolize lactic acid in fermented cucumbers may be a strain-related characteristic. Although *L. buchneri* was not directly isolated from the commercial spoilage sample in this study, it was detected by nonculture based identification of microorganisms (unpublished data) and isolated upon further characterization of this sample by Franco and others (2012). *L. buchneri* was also isolated from FCS that had been inoculated with the commercial spoilage culture and showed decreased lactic acid concentration during 22 wk of anaerobic incubation (Table 2). Based on subsequent work, we believe that the shorter MRS incubation time used for the commercial sample precluded the isolation of *L. buchneri* from the original spoilage brine due to its relatively slow growth rate on MRS compared to the other species that were present. The repeated isolation and identification of *L. buchneri* in spoilage samples in this study, observation of *L. buchneri* in more than 55 commercial cucumber fermentations undergoing spoilage (Franco and others 2012), and its demonstrated ability to use lactic acid in acid pH, 6% NaCl FCS under anaerobic conditions indicate that *L. buchneri* may be a significant contributor to fermented cucumber spoilage.

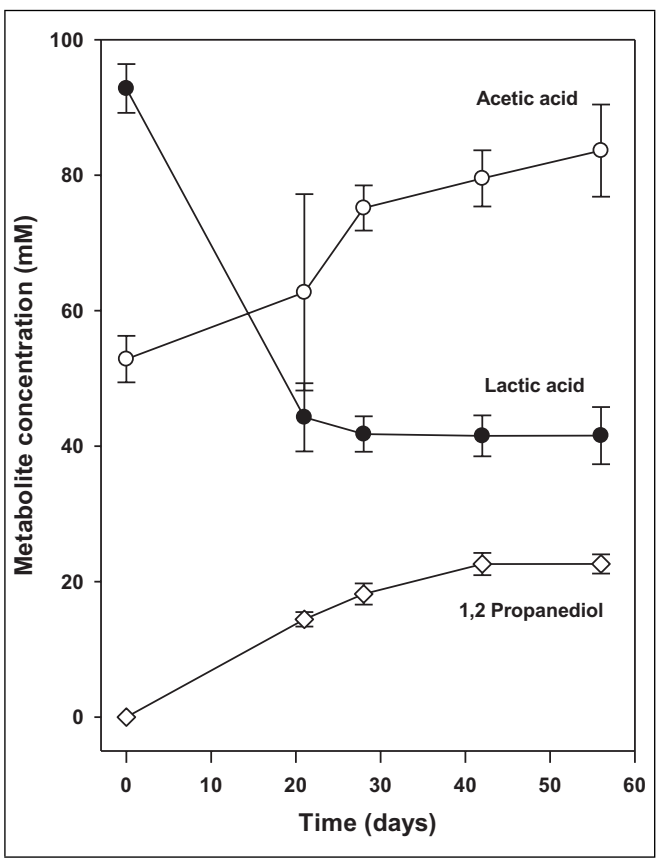

Figure 4–Anaerobic lactic acid utilization in fermented cucumber slurry (2% NaCl, pH 3.8) by a *Lactobacillus buchneri* strain isolated from spoiled fermented cucumbers.

Conclusions

Among several lactic acid bacteria species isolated from spoilage fermentations, *L. buchneri* was unique in its ability to initiate lactic acid degradation in fermented cucumbers and may play an important role in fermented cucumber spoilage. In the absence of oxygen, cucumbers fermented with 6% sodium chloride to a terminal pH of 3.2 were not subject to spoilage. However, lactic acid was degraded by spoilage microorganisms in cucumbers fermented with 0%, 2%, and 4% NaCl, even with a terminal pH as low as 3.2. Ongoing efforts to reduce sodium in commercial brining operations will need to include measures for controlling the increased susceptibility to spoilage of cucumbers fermented and stored with lower NaCl concentrations. *L. buchneri* may be an appropriate target for development of such control measures.

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References

Altschul SF, Gish W, Miller W, Meyers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403–10.

Barrangou R, Yoon S-S, Breidt F, Fleming HP, Klaenhammer TR. 2002. Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial sauerkraut fermentation. Appl Environ Microbiol 68:2877–84.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. 2011. GenBank. Nucleic Acids Res 39:D32–7.

Bobillo M, Marshall VM. 1991. Effect of salt and culture aeration on lactate and acetate production by *Lactobacillus plantarum*. Food Microbiol 8:153–60.

Costilow RN, Coughlin FM, Robach DL, Ragheb HS. 1956. A study of the acid-forming bacteria from cucumber fermentations in Michigan. J Food Sci 21:27–33.

Driehuis F, Oude Elferink SJWH, Spoelstra SF. 1999. Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. J Appl Microbiol 87:583–94.

Endo A, Okada S. 2007. *Lactobacillus farraginis* sp. nov. and *Lactobacillus parafarraginis* sp. nov., heterofermentative lactobacilli isolated from a compost of distilled shochu residue. Intl J Syst Evol Microbiol 57:708–12.

Etchells JL, Jones ID. 1946. Characteristics of lactic acid bacteria from commercial cucumber fermentations. J Bacteriol 52:593–9.

Fleming HP, Daeschel MA, McFeeters RF, Pierson MD. 1989. Butyric acid spoilage of fermented cucumbers. J Food Sci 54(3):636–9.

Fleming HP, Humphries EG, Thompson RL, McFeeters RF. 2002. Acidification of commercially fermented cucumbers in bulk tanks to increase microbial stability. Pickle Pak Sci 8(1):38–43.

Franco W. 2011. Effect of aeration in calcium chloride cucumbers fermentation [DPhil dissertation]. Raleigh, NC: North Carolina State University, library OCLC Number 76172424590. 163 p.

Franco W, Pérez-Díaz IM, Johanningsmeier SD, McFeeters RF. 2012. Characteristics of spoilage-associated secondary cucumber fermentation. J Appl Environ Micro 78(4):1273–84.

Hsu S-T, Yang S-T. 1991. Propionic acid fermentation of lactose by *Propionibacterium acidipropionici*: effects of pH. Biotechnol Bioeng 38:571–8.

Jianbo J, Kim S-Y, Jin Q, Eom H-J, Han NS. 2008. Diversity analysis of lactic acid bacteria in *Takju*, Korean rice wine. J Microbiol Biotechnol 18(10):1678–82.

Johanningsmeier SD. 2011. Biochemical characterization of fermented cucumber spoilage using non-targeted, comprehensive, two-dimensional gas chromatography-time-of-flight mass spectrometry: anaerobic lactic acid utilization by lactic acid bacteria [DPhil dissertation]. Raleigh, NC: North Carolina State University. library OCLC Number 741165812. 202 p.
Johanningsmeier SD, McFeeters RF. 2011. Detection of volatile spoilage metabolites in fermented cucumbers using nontargeted comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS). J Food Sci 76(1):C168-77.
Kim J-H, Breidt F. 2007. Development of preservation prediction chart for long term storage of fermented cucumber. J Life Sci 17(2):1616–21.
Krooneman J, Faber F, Alderkamp AC, Oude Elferink SJHW, Driehuis F, Cleenwerck I, Swings J, Gottschal JC, Vancanneyt M. 2002. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. Intl J Syst Evol Microbiol 52:639–46.
Lindgren SE, Axelsson LT, McFeeters RF. 1990. Anaerobic L-lactate degradation by *Lactobacillus plantarum*. FEMS Microbiol Lett 66:209–14.
Liu L, Zhang B, Tong H, Dong X. 2006. *Pediococcus ethanolidurans* sp. nov., isolated from the walls of a distilled-spirit-fermenting cellar. Intl J Syst Evol Microbiol 56:2405–8.
McFeeters RF, Barish AO. 2003. Sulfite analysis of fruits and vegetables by high-performance liquid chromatography (HPLC) with ultraviolet spectrophotometric detection. J Agric Food Chem 51:1513–7.
Miyamoto M, Seto Y, Hao DH, Teshima T, Sun YB, Kabuki T, Yao LB, Nakajima H. 2005. *Lactobacillus harbinensis* sp. nov., consisted of strains isolated from traditional fermented vegetables 'Suan cai' in Harbin, northeastern China and *Lactobacillus perolens* DSM 12745. Syst Appl Microbiol 28:688–94.
Murphy MG, O'Connor L, Walsh D, Condon S. 1985. Oxygen dependent lactate utilization by *Lactobacillus plantarum*. Arch Microbiol 141:75–9.
Oude Elferink SJWH, Krooneman J, Gottschal JC, Spoelstra SF, Faber F, Driehuis F. 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. Appl Environ Microbiol 67(1):125–32.
Pederson CS, Albury MN. 1950. Effect of temperature upon bacteriological and chemical changes in fermenting cucumbers. Geneva, N.Y.: New York State Agricultural Experiment Station. Bull No 744. 31 p.
Pederson CS, Albury MN. 1956. Variations in bacterial flora of low salt cucumber brines. Appl Microbiol 4(5):259–63.
Pederson CS, Ward L. 1949. The effect of salt upon the bacteriological and chemical changes in fermenting cucumbers. Geneva, N.Y.: New York State Agricultural Experiment Station. Bull No 288. 29 p.
Plastourgos S, Vaughn RH. 1957. Species of *Propionibacterium* associated with Zapatera spoilage of olives. Appl Environ Microbiol 5(4):267–71.
Rehberger JL, Glatz BA. 1998. Response of cultures of *Propionibacterium* to acid and low pH: tolerance and inhibition. J Food Prot 61(2):211–6.
Tanasupawat S, Pakdeeto A, Thawai C, Yukphan P, Okada S. 2007. Identification of lactic acid bacteria from fermented tea leaves (*miang*) in Thailand and proposals of *Lactobacillus thailandensis* sp. nov., *Lactobacillus camelliae* sp. nov. and *Pediococcus siamensis* sp. nov. J Gen Appl Microbiol 53:7–15.
Viega-da-Cunha M, Foster MA. 1992. Sugar-glycerol cofermentations in Lactobacilli: the fate of lactate. J Appl Bacteriol 174(3):1013–9.
Watanabe K, Fujimoto J, Tomii Y, Sasamoto M, Makino H, Kudo Y, Okada S. 2009. *Lactobacillus kisonensis* sp. nov., *Lactobacillus otakiensis* sp. nov., *Lactobacillus rapi* sp. nov., and *Lactobacillus sunkii* sp. nov., heterofermentative species isolated from sunki, a traditional Japanese pickle. Intl J Syst Evol Microbiol 59:754–60.
Wilson KH, Blitchington RB, Greene RC. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J Clin Microbiol 28:942–1946.

Supporting Information

The following supporting information is available for this article.

Figure S1. Experimental flow chart for reproduction of anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms from reduced NaCl fermented cucumbers.

Figure S2. Experimental flow chart for reproduction of anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms from a commercial fermentation tank (1M).

Figure S3. Experimental flow chart for reproduction of anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms from commercial spoiled fermentation brine.

Table S1. Changes in organic acids during anaerobic lactic acid degradation in cucumbers fermented with 0%, 2%, 4%, or 6% NaCl adjusted to pH 3.8, 4.3, or 5.0 (spoilage R1).

Table S2. Biochemical changes during anaerobic lactic acid utilization in FCS (6% NaCl, pH 3.8) by *L. buchneri* and spoilage organisms from reduced NaCl and commercial cucumber fermentations (spoilage R2).

Table S3. Biochemical changes in FCS (pH 3.8) with varying NaCl concentrations after 22 weeks anaerobic incubation with spoilage organisms (spoilage R4).

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