Development of a Model System for the Study of Spoilage Associated Secondary Cucumber Fermentation during Long-Term Storage

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Abstract: Calcium chloride fermentations represent an alternative to reduce chloride concentrations in the wastewaters generated from commercial cucumber fermentations, currently performed in cover brine solutions containing 6% to 12% sodium chloride. However, preliminary attempts to commercially ferment the cucumbers in the presence of oxygen led to the development of a secondary cucumber fermentation or spoilage. The development of cucumber secondary fermentation has also been occasionally reported by processors using cover brine solutions containing sodium chloride. This study focused on the development of a model system to characterize $CaCl_2$ and NaCl secondary cucumber fermentations under conditions similar to those present on the commercial scale. Cucumber fruits mixed with cover brine solutions, containing 100 mM $CaCl_2$ or 1.03 M NaCl, and 25 mM acetic acid, were fermented in 2 L fermentation vessels subjected to air-purging at a rate of 5 mL/min. Microorganisms and selected biochemical changes detected in the experimental cucumber fermentations had been previously observed in commercial spoilage samples, suggesting the successful reproduction of the secondary fermentation in the laboratory. Experimental secondary fermentations were characterized by the rapid oxidation of the lactic acid produced during the primary fermentation, which, in turn, increased pH. Lactic acid disappearance seemed to be the result of yeast metabolism that also led to the chemical reduction of the environment to levels at which other bacteria could become established and produce butyric, propionic, and acetic acids. This model system will be applied for the identification of strategies to prevent the initiation of the cucumber secondary fermentation and reduce economic losses in the pickling industry.

Keywords: cucumber secondary fermentation, fermented cucumber, lactic acid utilization, organic acids, yeast

Practical Application: The study of secondary cucumber fermentation has represented a challenge for many years. The successful development of a model system for the study of this phenomenon in the laboratory is instrumental in furthering the study of the event and in optimizing the sodium-chloride-free fermentation at the commercial scale.

Introduction

The study of secondary cucumber fermentation has represented a challenge for many years, primarily because of the sporadic occurrence of the event at the commercial scale in tanks containing cover brine solutions with 1.03 M (6%) NaCl, and the inability to predict the development of the spoilage after the primary fermentation is completed. Additionally, the limited number of studies completed to date have based the characterization of the event under anaerobic conditions, low NaCl, and/or elevated initial brine pH (Kim and Breidt 2007; Johanningsmeier 2011); conditions which do not fully mimic the current commercial fermentation. Commercial practices for cucumber fermentation include air-purging, addition of 6% to 12% sodium chloride, and an initial fermentation pH around 4.2.

Although a number of potential causative agents have been identified from samples of spoilage-associated secondary cucumber fermentations, specific roles have not been linked to any of these isolates under the conditions prevailing in the commercial process. The study of a secondary cucumber fermentation outbreak in 2010 provided an opportunity to document biochemical and microbiological characteristics of commercial fermentations undergoing spoilage (Franco and others 2012). This study suggested that oxidative yeasts, such as *Pichia manshurica* and *Isstachenkia occidentalis,* were involved in the development of the phenomenon and *Lactobacillus buchneri* was frequently detected in secondary fermentations. In an independent study, Johanningsmeier (2011) observed that *Lactobacillus buchneri* was unique in its ability to metabolize lactic acid in fermented cucumber slurry among a group of lactic acid bacteria isolated from spoiled fermented cucumber samples.

The profile of secondary metabolic products associated with the spoilage of fermented cucumbers is similar to the fatty acids observed during the "zapatera" spoilage of table olives (Montaño and others 1992). Zapatera spoilage occurs when there is limited to no sugars present in the fermentation matrix, and is commonly

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characterized by the formation of propionic acid followed by butyric acid among other secondary metabolites (Montaño and others 1992). While propionic and butyric acid production has been associated with Propionibacteria and members of the clostridial genus; these organisms are not able to initiate the secondary fermentation under the conditions prevailing after the primary fermentation is completed (acidic pH, oxidized environments, and high organic acid concentrations). Important outbreak cases associated with zapatera spoilage have been reported for the table olive industry (Fenicia and others 1992; Cawthorne and others 2005), making it imperative to better understand the possible causative agents associated with the initiation of secondary vegetable fermentations, such as the oxidative yeasts and/or spoilage LAB.

The acidic conditions, lack of readily available sugars, and the irregularities in the air-purging routine applied at the commercial scale after the cucumber primary fermentation is completed suggest that the organisms capable of initiating the spoilage of the fermented products are acid resistant, able to utilize organic acids as a source of energy, and are microaerophilic or aerobic. Thus, it was hypothesized that it should be possible to develop the secondary cucumber fermentation in the laboratory by introducing an air-purging routine to fermentation vessels during and after the primary fermentation. The successful development of such model system for the study of the secondary cucumber fermentation in the laboratory is essential to gain a better understanding of the event and in optimizing the table-salt-free cucumber fermentations at the commercial scale.

Materials and Methods

Model system

Size 3B (39 to 51 mm diameter) cucumbers were diced into approximately 10 mm cubes and packed with cover brine solutions containing $CaCl_2$ or NaCl and acetic acid added as vinegar, to equilibrated concentrations of 100 mM or 1.03 M and 25 mM, respectively, using a 50 : 50 pack out ratio (w/w). Cucumbers packed in this way were fermented in a 2 L glass water jacketed fermentation vessels of a BioFlo110 Modular Benchtop Fermentor (New Brunswick Scientific Co., Inc., Edison, N.J., U.S.A.) applying air-purging at a rate of 5 mL/min controlled by a Matheson PG-1000 (U001) flowmeter (Matheson Instruments, Montgomeryville, Pa., U.S.A.). It was expected that the presence of oxygen in the fermentation vessel would provide appropriate conditions for the secondary fermentation to proceed relatively fast (2 wk) allowing the effective study of the sequence of events that lead to the development of the spoilage.

Vessels were inoculated with a mixture of 3 *Lactobacillus plantarum* strains, LA445, LA98 (ATCC 14917), and LA 89 (USDA-ARS Food Science Research Unit, Raleigh, N.C., U.S.A., culture collection) to 10^5 CFU/mL. The 3 *L. plantarum* strains used were originally isolated from fermented vegetables, either cucumbers or cabbage (sauerkraut). A constant temperature of 30 °C was maintained in the fermentation vessels using a recirculating chiller (NESLAB Merlin M-75, Thermo Electron Co., Newington, N.H., U.S.A.). Control experiments followed the same experimental design, but nitrogen gas was passed through the headspace of the fermentation vessels to displace oxygen. The control experiments were not air-purged. Parameters such as redox potential (Pt4805-DPAS-SC-K8S/200 redox electrode; Mettler-Toledo, Bedford, Mass., U.S.A.), dissolved oxygen (Inpro 6830/220 electrode; Mettler-Toledo), and pH (pH electrode model 405-DPAS-SC-K8S/225; Mettler-Toledo) were monitored over time. The measured redox potentials were converted to the redox potential against the standard hydrogen electrode (E_h) by adding 203.4 mV (30 °C) according to the manufacturer's instructions. Electrodes for all of these measurements were calibrated, rinsed with 70% ethanol, and secured into fittings on the head plate of each fermentation vessel.

Microbiological analysis

Aseptically collected samples were serially diluted in 0.85% saline solution, and spiral plated using an Autoplate 400 (Spiral Biotech, Norwood, Mass.) on Lactobacilli deMan Rogosa and Sharpe agar (MRS, Becton Dickinson and Co., Franklin Lakes, N.J.) supplemented with 1% cycloheximide (0.1% solution, OXOID, New England) to prevent growth of yeasts and achieve accurate enumeration of lactic acid bacteria. Samples and dilutions were also inoculated onto yeast and molds 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 prevent bacterial growth; violet red bile agar supplemented with 1% glucose (VRBG, Becton, Dickinson and Co.); and differential reinforced clostridia agar (DRCA, Becton) supplemented with 1% cycloheximide (OXOID, New England) for the enumeration of yeast and molds, enterobacteria, and *Clostridium* spp., respectively. Selected samples of the NaCl and the $CaCl_2$ fermentations were additionally spread plated with no dilutions on DRCA and VRBG agar plates to decrease the limit of detection. Purple and pink colonies after 24 h incubation on VRBG plates were recorded as presumptive enterobacteria. Black colonies after 48 h anaerobic incubation on DRCA plates were recorded as *Clostridia* spp.

Distinct colony morphologies visually observed in the agar plates were selected for isolation and identification. Three to four independent clones of representative colonies of each morphology type were picked and streaked in the respective culture medium. Isolated colonies were analyzed microscopically (Optiphot-2; Nikon, Tokyo, Japan) using Gram staining for bacteria or 10% KOH-glycerol fixation for yeasts. The bacteria and yeast isolated were identified using partial 16S and/or 26S *rrna* gene sequencing, respectively. Bacterial chromosomal DNA was obtained using DNeasy genomic extraction and purification kit (DNeasy, Qiagen, Valencia, Calif., U.S.A.), while yeast chromosomal DNA was obtained using MasterPure™ Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, Wis., U.S.A.). The PCR mix contained 2X master mix (Biorad, Hercules, Calif.), chromosomal DNA, and forward and reverse primers, which were 27F (5-AGAGTTTGATCCTGGCTCAG-3′) (Barrangou and others 2002) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Wilson and others 1990) for the bacterial isolates, and NL-1 (5′-GCCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) for yeasts (Kurtzman and Robnett 1997). The identification of the enterobacteria was accomplished by the sequencing of *dnaJ* as described by Nhunga and others (2007) using the degenerate primers DN1-1F (5′-GATYTRCGHTAYAACATGGA-3′) and DN1-2R (5′-TTCACRCCRTYDAAGAARC-3′) for PCR amplification and sequencing. All primers were obtained from Integrated DNA Technologies (Coralville, Iowa, U.S.A.). PCR products were purified using the Qiagen PCR purification kit and sequenced by Eton Bioscience Inc. (Raleigh, N.C.). The sequences obtained were subjected to the basic local alignment search tool (BLAST) (Altschul and others 1990) in the GenBank (Benson and others 2000) 16S *rrna* microbial database to determine the identity of

most bacterial strains and the nonredundant nucleotide database for the identification of yeasts and enterobacteria (Zhang and others 2000).

Chemical analysis

Samples of cover brine solutions were aseptically collected, spun for 10 min at 12000 rpm using an Eppendorf 5810R Centrifuge (Eppendorf North America, Inc., Westbury, N.Y., U.S.A.) and the supernatants diluted 10X prior to HPLC analysis. The concentrations of organic acids and sugars in the supernatants were measured using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, Calif.) (McFeeters and Barish 2003). The column was heated to 37 °C and eluted with 0.03N sulfuric acid at a flow rate of 0.6 mL/min. A Thermo Separations UV6000 diode array detector (Spectra System Thermo Scientific, Waltham, Mass.) 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.) connected in series with the diode array detector was used to measure glucose, fructose, and ethanol. External standardization of the detectors was done using 4 concentrations of the standard compounds.

Results and Discussion

The proposed model system successfully reproduced the primary and secondary fermentations observed during the commercial scale process and storage of the fermented fruits. Changes in organic acids, pH, and redox potential were observed in concordance to the evolution of the primary and secondary fermentations and were associated with a number of microorganisms. The culturable microbiota observed from the air-purged $CaCl_2$ and NaCl fermentations presented morphologies representative of those previously isolated from commercial spoilage samples.

The primary fermentation of cucumbers brined as described in the materials and methods section proceeded as expected and was followed by growth of yeasts under both air-purging and anaerobic conditions with both the NaCl and the $CaCl_2$ cover brine solutions. Under air-purged conditions, the completion of primary fermentation was evident by day 6 due to complete sugar utilization, the production of 83.6 ± 6.1 mM lactic acid, and a pH of 3.2 in the vessels containing $CaCl_2$ (Figure 1), and by the formation of 81.5 ± 0.6 mM lactic acid, and same final pH in the fermentations containing NaCl (Figure 1). The fermented product for both cover brines remained stable in the control vessels in which a nitrogen headspace was maintained (Figure 1). However, under air-purged conditions, a secondary fermentation or spoilage was initiated and characterized by the rapid oxidation of the lactic acid produced during the primary fermentation, which, in turn, increased the brine pH. The lactic acid disappearance seems to be the result of yeast metabolism (Figure 2) that also leads to the reduction of the environment (E_h = −100 mV, Ag/AgCl, 3M KCl) (Figure 3) to levels at which bacteria, other than the lactic acid bacteria responsible for the primary fermentation (Figure 4), become established and produce butyric, propionic, and acetic acids (Figure 4).

Yeasts are normally associated with vegetable fermentations as contributors of flavor and nutrition factors (Arroyo-López and others 2008). As the primary fermentation is completed and sugars are depleted, the yeasts populations decrease to levels below 3 log CFU/mL, facilitating the microbial stability of the fermented product (Etchells 1941). However, in the air-purged model system proposed, yeasts populations increased once the sugars were depleted (Figure 2) and remained above 5 log CFU/mL during the active lactic acid utilization. The ability of yeasts to transport and utilize organic acids has been reported previously. A transporter for monocarboxylate acids was reported for the spoilage yeasts *Candida utilis* (Cássio and Leão 1993). In addition, different yeasts isolated from table olive fermentations were reported as able to utilize organic acids (citric, acetic, and lactic) under aerobic incubation (Ruíz-Cruz and González-Cancho 1969). In

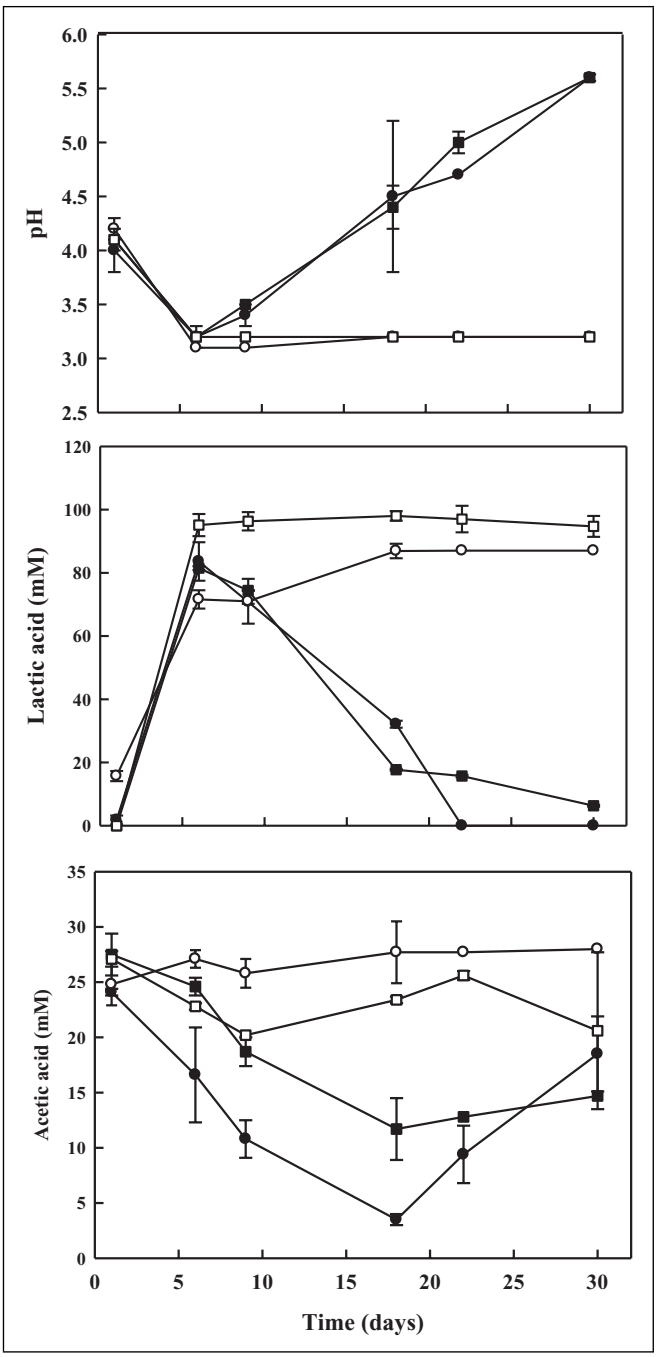

Figure 1–Changes in pH and lactic and acetic acids observed in $CaCl_2$ and NaCl cucumber fermentations with and without air-purging in a 2 L vessel BioFlo110 system. Values represent mean ± standard deviation of 3 independent replicates. Air-purged and nonaerated fermentations are represented with closed (■, ●) and open (□, ○) symbols, respectively. In each case, fermentations containing $CaCl_2$ and NaCl are shown with circles (●, ○) and squares (■, □), respectively.

addition, the yeasts *Pichia manshurica* and *Issatchenkia occidentalis*, isolated from secondary cucumber fermentations, were associated with acetic and lactic acids utilization in a cucumber juice model system (Franco and others 2012)

Two stages were noticed during the cucumber secondary fermentation. The first stage was characterized by a continuous increase in cover brine pH, and the disappearance of lactic and acetic acids (Figure 1), which are characteristics observed from the commercial secondary fermentation as well (Franco and others 2012). During this period, the yeast population increased in both NaCl and $CaCl_2$ fermentation cover brines to populations of ~6 log CFU/mL (Figure 2). As yeast populations increased, the environment was further reduced as evidenced by decreases in redox potential to negative values (Figure 3). Reduction of the environment occurred at a faster rate in $CaCl_2$ fermentation brines as compared to the NaCl cover brines, achieving a value of −228.6 mV by day 9 (Figure 3). In NaCl cover brine solutions, the reduction of the environment was noticed concomitantly with the increase in pH once primary fermentation was completed passed day 9. Yeast population started to decline 9 d postpacking in $CaCl_2$ brines and 18 d in NaCl brines. Although the declining trends in E_h continued up to the end of the experimentation (30 d), yeast counts were, on average, 5.4 and 4.0 log CFU/mL for $CaCl_2$ and NaCl brines, respectively.

In the fermentations brined with 100 mM $CaCl_2$, day 9 marked the beginning of a tertiary fermentation stage, characterized by the production of butyric and propionic acids, an E_h of −228.6 ± 8.8 mV, dissolved oxygen (dO_2) of 5.2 mg/L, pH above 3.4 and increases in the counts for enterobacteria and presumptive *Clostridium* spp. to 1.9 and 2.0 log CFU/mL, respectively (Figure 4). While the numbers of the enterobacteria remained at 5 log CFU/mL until the end of the experimentation, the population of presumptive clostridia increased to 5.9 log CFU/mL concomitantly with the production of butyric acid to ~20 mM (Figure 4). In NaCl fermentations, the tertiary fermentation was observed later, on day 22, with the production of propionic acid only (Figure 4). The production of the propionic acid was concomitant with a significant increase in the population of enterobacteria to more than 4 log CFU/mL. No *Clostridium* species were identified during the course of the experimentation in NaCl-brined fermentation that was also in agreement with the absence of butyric acid (Figure 4).

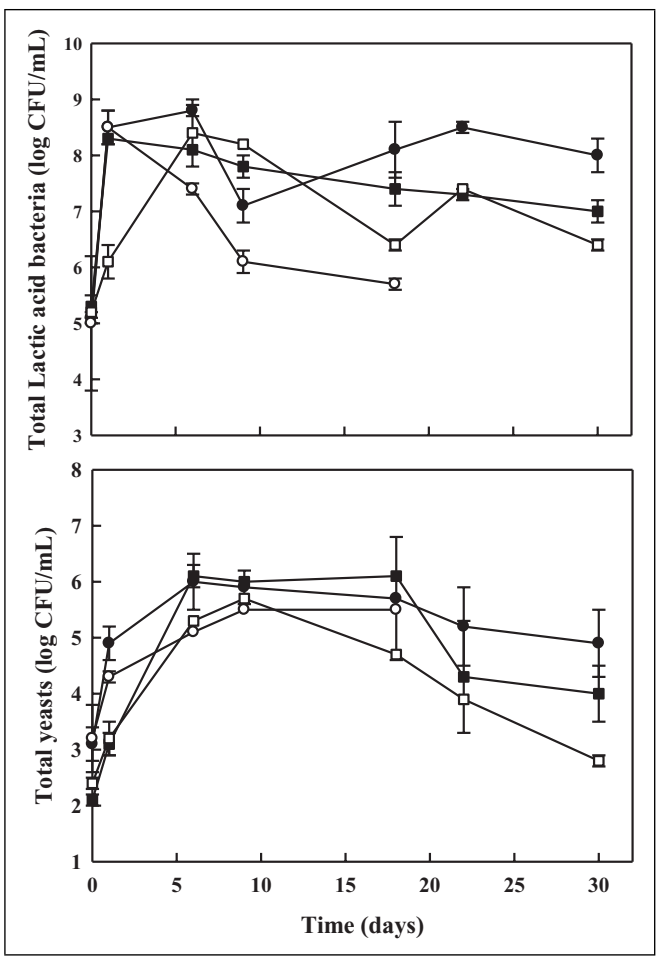

Figure 2–Changes in colony counts for LAB and yeasts in $CaCl_2$ and NaCl cucumber fermentations with and without air-purging in a 2 L vessel BioFlo110 system. Values represent mean ± standard deviation of 3 independent replicates. Air-purged and nonaerated fermentations are represented with closed (■, ●) and open (□, ○) symbols, respectively. In each case, fermentations containing $CaCl_2$ and NaCl are shown with circles (●, ○) and squares (■, □), respectively.

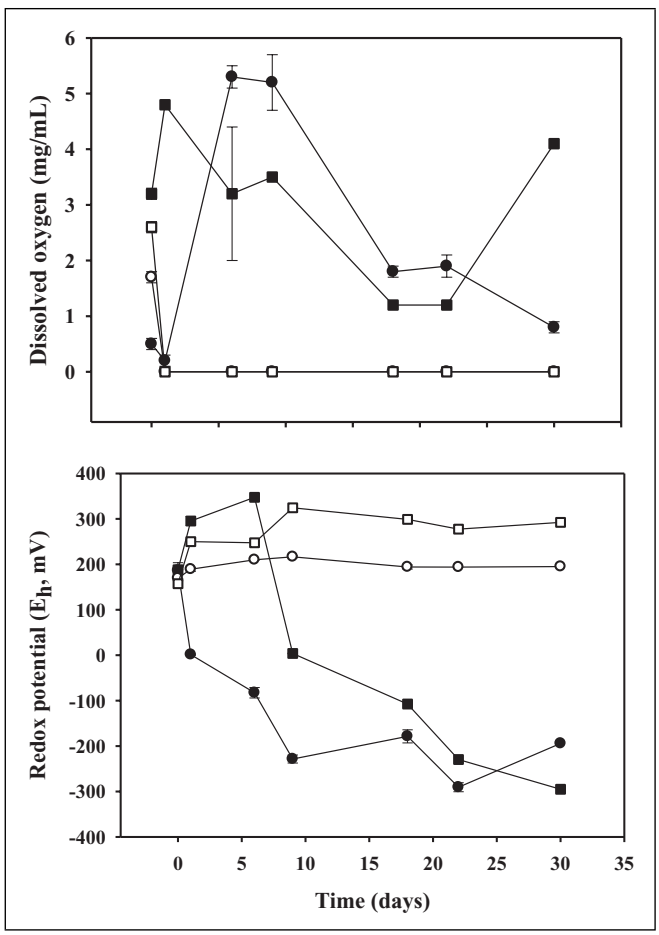

Figure 3–Changes in redox potential (Eh) and dissolved oxygen in $CaCl_2$ and NaCl cucumber fermentations with and without air-purging in a 2 L vessel BioFlo110 system. Values represent mean ± standard deviation of 3 independent replicates. Air-purged and nonaerated fermentations are represented with closed (■, ●) and open (□, ○) symbols, respectively. In each case, fermentations containing $CaCl_2$ and NaCl are shown with circles (●, ○) and squares (■, □), respectively.

Colonies showing morphologies similar to that of *L. plantarum* were observed predominantly during primary fermentation in all the fermentation vessels scrutinized. Although the LAB population remained close to 8 and 7 log CFU/mL in the air-purged $CaCl_2$ and NaCl fermentations, respectively, it is important to mention that upon initiation of the secondary fermentation, the LAB population observed on MRS agar plates presented a multiplicity of emerging colony morphologies (Table 1). Morphologies MB1 and MB2, which are representative of *Lactobacillus buchneri* and *Pediococcus ethanolidurans* among other LAB (Table 2), predominated in the air-purged vessels (Table 1) and were observed concomitantly with propionic acid production (Figure 4). Selected LAB, including *L. buchneri*, has been shown to initiate the spoilage of fermented cucumber media (Johanningsmeier 2011); however, lactic acid consumption proceeds at a slower rate in the absence of yeasts and/or under anaerobic conditions (Franco and others 2012).

A multiplicity of yeast morphologies was also observed from the studied fermentations. The morphologies designated as MYI, and MY2 were exclusively detected from the fermentation vessels subjected to air-purging and have been associated with *P. manshurica* and *I. occidentalis*, respectively (Table 2). Morphology MY4, isolated from anaerobic and aerobic fermentations in this study, was previously isolated from standard (normal) commercial fermentations exclusively, and seems to be representative of *Candida tropicalis*. The inocula from aerobic and anaerobic fermentations containing NaCl brines also produced morphology MY5, which presents a white and smooth colony, with an entire margin, and elevated in the center in YMA plates after 48 h of incubation at 30 °C. The colony can clearly be distinguished from others due to its "mount-like" morphology. The cells occur in buds and have an ellipsoidal shape with the presence of ascospores. The isolated colony was identified as *Pichia membranifaciens* (MY5; Table 2).

Aside from targeting LAB and yeasts, the detection of clostridia and enterobacteria was attempted in the samples subjected to air-purging due to their ability to produce propionic and butyric acids. Three enterobacter morphologies were detected on the VRBG plates inoculated with the samples from the air-purged vessels. Two of these isolates were identified by the partial sequencing of the *dnaJ* as *Enterobacter* sp. and *Citrobacter* sp. (Table 2). The third isolate was identified by the 16S *rrna* partial sequencing as *Pseudomonas nitroreducens* (Table 2). Enterobacteria are usually inhibited as lactic acid is produced and pH decreases during the primary fermentation process (Etchells and others 1945). However, recent studies have indicated that enterobacteria, such as *E. cloacae*, are a source of contamination and potential spoilage problems in naturally fermented table olives (Bevilacquia and others 2009). The isolate targeted from the DRCA plates inoculated with the spoilage samples was confirmed as a *Clostridium sp*. based on the partial sequencing of its 16S *rrna* (Table 2). Members of this bacterial genus have been reported as responsible for lactic acid conversion into butyric acid (Gililland and Vaughn 1943; Fleming and others 1989; Yang and others 2009) primarily at pH above 5.0 under anaerobic conditions (Fleming and others 1989). However, the data from $CaCl_2$ air-purged experiments indicate that butyric acid is produced at reduced environmental conditions (negative E_h values) but lower pH values. Further studies are necessary to test the ability of this presumptive clostridial isolate to produce these changes at a lower pH.

Although a previous study on the characterization of commercial spoilage samples suggested an association between increased pH and decreased redox potential (Franco and others 2012); a correlation among E_h trends and spoilage with time was not clearly established. The air-purged model system showed that the reduction of the fermentation matrix containing $CaCl_2$ could be noticed before the changes in pH were obvious. However, in the fermentations brined with NaCl, a change in redox potential was observed only when the fermentation pH was near the critical safety limit of 4.6 (Figure 1 and 3). Thus, these data indicate that redox potential measurements may be used as a tool to predict the development of spoilage in sodium-chloride-free cucumber fermentations.

The data presented on Figure 2 and 3 show an association between the increase in yeast populations and the reduction of the fermentation matrix. This association was corroborated by the observation of positive E_h values in the control vessels, in which the inhibition of the spoilage yeasts led to the stabilization of the fermented product. The reduction of the fermentation matrix by yeasts maybe facilitating the onset of subsequent events during the secondary fermentation, such as growth of anaerobic microbes. Furthermore, the continuing reduction of the fermentation matrix after the initiation of the secondary fermentation may be associated with the establishment of enterobacteria, which have been reported as capable of reducing the cucumber fermentation matrix (Olsen and Pérez-Díaz 2009).

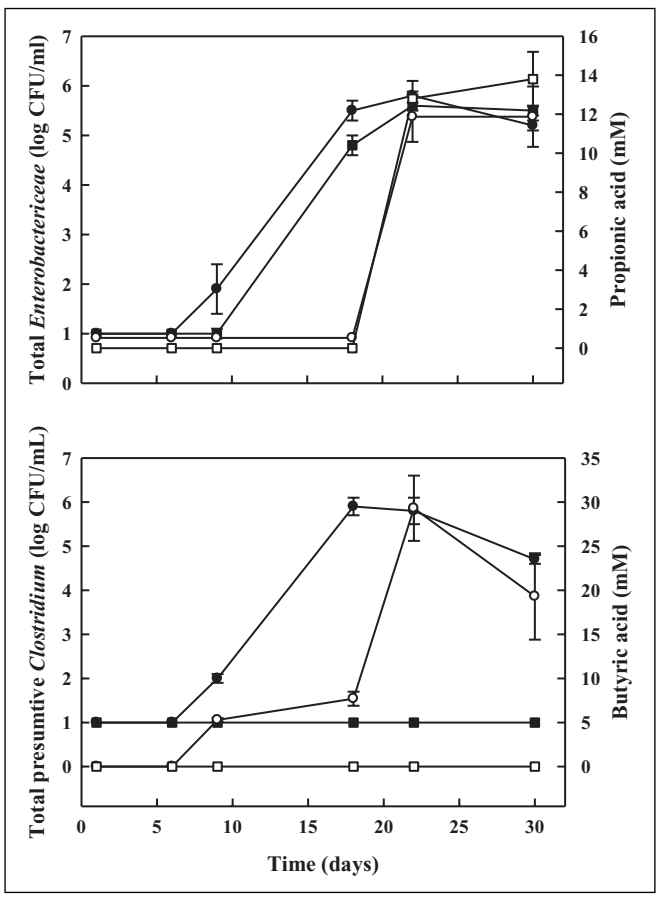

Figure 4–Trends of colony counts for Enterobacteria and *Clostridium* spp. and secondary products concentrations detected in $CaCl_2$ and NaCl cucumber fermentations with air-purging in a 2 L vessel BioFlo110 system. In each panel, the fermentations containing $CaCl_2$ and NaCl brines are shown with circles (●, ○) and squares (■, □), respectively. Values for microbial counts (■, ●) and propionic acid (○) and butyric acid (□) concentrations represent the mean ± standard deviation of 3 independent replicates.

Dissolved oxygen levels decreased significantly during the lactic acid utilization stage, even when the airflow was kept constant at 5 mg/L (Figure 3). Oxygen uptake during and after primary fermentation has traditionally been attributed to yeast populations that become established in high numbers in air-purged fermentations (Potts and Fleming 1979). A continuous removal of air from the fermentation matrix was additionally expected in the event the population of oxidative yeasts were actively growing and playing a role in the development of spoilage associated with secondary fermentation. Thus, the observed reduction in dissolved oxygen further confirms a relevant role of oxidative yeasts in the initiation of secondary cucumber fermentation, more specifically in lactic acid utilization.

Although the characteristics of the secondary fermentations reported here are similar to those described by Franco and others (2012) for a commercial spoilage outbreak; significant differences between the fermentations brined with $CaCl_2$ and those brined with NaCl were observed. The fermentation vessels containing sodium chloride produced an additional 9 mM of propionic acid and 30 mM less butyric acid as compared to the fermentations containing calcium chloride. This is in agreement with previous observations made by Franco and others (2012) consisting of the detection of propionic acid in 50% of the commercial scale secondary fermentations in NaCl brines, and the lack of butyric acid formation in more than 90% of the spoiled fermentation tanks monitored during a spoilage outbreak. In light of the more robust production of propionic acid in the fermentations containing NaCl as compared to those fermentations containing $CaCl_2$, it is then understood that the presence of the former salt in the fermentation vessel may be favoring certain microorganisms capable of utilizing lactic acid and convert it to propionic acid such as selected lactic acid bacteria (Johanningsmeier 2011), and possibly enterobacteria. While butyric acid producers, such as *Clostridium* spp., tolerate a wide range of NaCl concentrations (Spielberg 1944), it seems that the combination of the preservative with low pH and carbon limiting sources is effective in decreasing the chances of the bacterium to produce butyric acid in cucumbers fermented with NaCl brines. About 3% NaCl has been reported as an inhibitor of toxin production and other metabolic activities in *Clostridium botulinum*, but it is not a limiting factor for outgrowth and cell multiplication (Boyd and Southcott 1971).

Table 1–Morphologies observed in MRS and YMA plates during $CaCl_2$ (Panel A) and NaCl (Panel B) cucumber fermentations with and without air-purging in a 2 L vessel BioFlo110 fermentation system. Colony morphologies listed here are described in Table 2.

	Air-purged fermentation				Anaerobic fermentation	
Time (days)	Morphologies observed in MRS	Morphologies observed in YMA	Morphologies observed in VRBG	Morphology observed in DRCA	Morphology observed in MRS	Morphologies observed in YMA
Panel A: $CaCl_2$ fermentation						
1	MB0[†]	MY2, MY4	ME1	N/D	MB0	MY4
6	MB0	MY2, MY1*, MY4*	ME1, ME3	N/D	MB0	MY4
9	MB0	MY4, MY2, MY1	ME1, ME2	MC1	MB0	N/D
18	MB0, MB1, MB2	MY4, MY1, MY2	ME2, ME1, ME3	MC1	MB0	N/D
22	MB1, MB2, MB0*	MY4, MY1, MY2	ME2, ME1, ME3	MC1	N/A	N/A
30	MB1, MB2, MB0*	MY4, MY1	ME2, ME1, ME3	MC1	N/A	N/A
Panel B: NaCl fermentation						
1	MB0	MY4	ME1	N/D	MB0	MY4
6	MB0	MY4, MY5, MY2, MY1*	ME1, ME3	N/D	MB0	MY4, MY5
9	MB0, MB2	MY5, MY4	ME2, ME1	N/D	MB0	MY4, MY5
18	MB2, MB0	MY5, MY4	ME2, ME1, ME3	N/D	MB0	MY4
22	MB2, MB1, MB0*	MY5, MY4, MY2	ME2, ME1, ME3	N/D	MB0	MY4
30	MB2, MB1, MB0*	MY4, MY5	ME2, ME1, ME3	N/D	MB0	MY4

[†]MB0: Morphology was similar to *L. plantarum* and were not scrutinized as part of this study.
*Only few colonies presenting the designated morphology were observed on agar plates.
NA: Data not available; ND: Not detected.
Morphologies are presented in order of abundance from the highest to the lowest.

Table 2–Identification of selected isolates from cucumber secondary fermentation reproduced in the laboratory with cover brine solution containing 100 mM $CaCl_2$.

ID[1]	Identification by the partial *rRNA* sequencing	Genebank accession number	Isolate culture collection number	Reference
MY1	*Pichia manshurica* or *galeiformis*	JQ086340, JQ086342, JQ086341	Y091, Y088, Y092	Franco and others 2012
	Pichia manshurica	JQ394818	Y098	This study
MY2	*Issatchenkia occidentalis*	JQ086337, JQ086338, JQ086639	Y093, Y089, and Y094	Franco and others 2012
	Issatchenkia occidentalis	JQ394819	Y099	This study
MY4	*Candida tropicalis*	JQ394822	Y096	This study
MY5	*Pichia membranifaciens*	JQ394823	Y097	This study
MB1	*Lactobacillus buchneri*	JQ086334	LA1149	Franco and others 2012
	Lactobacillus sp.	JQ086333, JQ086335	LA1192, LA1193	
	Lactobacillus sp.	JQ394820	not available	This study
MB2	*Pediococcus ethanolidurans*	JQ086332, JQ086331	LA1150, LA1195	Franco and others 2012
	Pediococcus sp.	JQ086330	LA1194	
	Pediococcus sp.	JQ394821	LA1197	This study
MC1	*Clostridium* sp.	JQ086336	B431	Franco and others 2012
	Clostridium sp.	JQ394824	B517	This study
ME1	*Citrobacter* sp.	JQ762617	B514	This study
ME2	*Enterobacter* sp.	JQ762618	B515	This study
ME3	*Pseudomonas nitroreducens*	JQ762616	B516	This study

Conclusion

Based on the information gathered in this study, we suggest the following succession of events during the fermentation of cucumber pickles fermented in $CaCl_2$ and NaCl brines under air-purged conditions: (i) primary fermentation dominated by *L. plantarum* completes, converting glucose into lactic acid with the concomitant decrease in brine pH, (ii) unidentified triggers, possibly including oxygen availability, induce the abnormal growth of selected yeasts, possibly *I. occidentalis,* and *P. manshurica,* after the primary fermentation, (iii) the yeasts metabolic activity results in not only organic acids (lactic and acetic acids) utilization and the corresponding increase in brine pH, but also in the further reduction of the environment as dissolved oxygen is utilized; (iv) the reduced fermentation matrix and increased pH leads to the growth of other microorganisms, such as selected LAB, clostridial species, and enterobacteria, that are able to further utilize lactic acid and produce either propionic, butyric, and/or acetic acids.

Given the similitude within a number of vegetable fermentation matrixes, the findings reported here could benefit other industries, such as those of table olives and kimchi, in understanding deterioration occurrences during the processing and/or storage of fermented products.

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