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Characteristics of Spoilage-Associated Secondary Cucumber Fermentation

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Secondary fermentations during the bulk storage of fermented cucumbers can result in spoilage that causes a total loss of the fermented product, at an estimated cost of $6,000 to $15,000 per affected tank. Previous research has suggested that such fermentations are the result of microbiological utilization of lactic acid and the formation of acetic, butyric, and propionic acids. The objectives of this study were to characterize the chemical and environmental conditions associated with secondary cucumber fermentations and to isolate and characterize potential causative microorganisms. Both commercial spoilage samples and laboratory-reproduced secondary fermentations were evaluated. Potential causative agents were isolated based on morphological characteristics. Two yeasts, *Pichia manshurica* and *Issatchenkia occidentalis*, were identified and detected most commonly concomitantly with lactic acid utilization. In the presence of oxygen, yeast metabolic activities lead to lactic acid degradation, a small decline in the redox potential (E_h, Ag/AgCl, 3 M KCl) of the fermentation brines, and an increase in pH to levels at which bacteria other than the lactic acid bacteria responsible for the primary fermentation can grow and produce acetic, butyric, and propionic acids. Inhibition of these yeasts by allyl isothiocyanate (AITC) resulted in stabilization of the fermented medium, while the absence of the preservative resulted in the disappearance of lactic and acetic acids in a model system. Additionally, three Gram-positive bacteria, *Lactobacillus buchneri*, a *Clostridium* sp., and *Pediococcus ethanolidurans*, were identified as potentially relevant to different stages of the secondary fermentation. The unique opportunity to study commercial spoilage samples generated a better understanding of the microbiota and environmental conditions associated with secondary cucumber fermentations.

The cucumber pickling industry occasionally reports spoilage of fermented cucumbers associated with increases in brine pH and, in some cases, unpleasant odors from the fermentation tanks. Industrial efforts to manage secondary fermentations that may result in spoilage of the fermented cucumbers include increased monitoring of the pH, total acidity, and sodium chloride (NaCl) concentration and either early processing of the fermented fruits or addition of vinegar to tanks that begin to have a rise in pH. In the event that tanks spoil and cannot be used, losses range from $6,000 to $15,000 per tank.

Early efforts to understand secondary cucumber fermentations concluded that the lactic acid produced during the primary fermentation by lactic acid bacteria (LAB) is consumed, which consequently induces an increase in pH (18). It was also noticed that acetic, propionic, and butyric acids are produced (18). More recent studies of the secondary fermentation of cucumbers under anaerobic conditions concluded that lactic acid can be utilized by *Lactobacillus buchneri* and/or *Lactobacillus parafarraginis*, which can be naturally present in the fermented cucumber brines and fruits (23). It is suspected that the increasing pH leads to the growth of other microorganisms capable of converting lactic acid into acetic, propionic, and/or butyric acids (18, 25).

Modern practices for the commercial fermentation of cucumbers in bulk include the use of open-top tanks and an air-purging routine to prevent cucumber bloating. Gas-purging practices to control bloater damage due to carbon dioxide accumulation in the fermentation tanks were first introduced with the use of nitrogen-purging routines (19). Nitrogen was later replaced with air purging due primarily to its lower cost without sacrifice of the ability to efficiently prevent bloater damage (40). However, the presence of oxygen in commercial fermentation tanks creates an opportunity for oxidative yeasts to grow during or after the primary fermentation (40). The presence of oxidative yeasts in open-top fermentation tanks containing fermented cucumbers located across the United States was demonstrated by Etchells and coworkers in the 1950s (13–15, 17). Shortly after these reports were made, the ability of a selected number of yeasts present in foods to assimilate acetic and lactic acids was discovered (10, 44). More recent studies suggest that yeasts such as *Candida utilis* express two mediated transport systems for lactic acid, one of which appears to be able to transport other monocarboxylic acids, such as acetic acid (7). Since lactic and acetic acids are critical to the preservation of fermented cucumbers during bulk storage, the potential role of yeasts capable of utilizing the organic acids present in fermented cucumbers was evaluated.

Investigations of the development of secondary cucumber fermentations have been limited by the inability to predict the event at the commercial scale and by its sporadic occurrence in a small number of fermentation tanks. In the 2010 cucumber brining season, a considerable number of commercial tanks at a single loca-

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tion spoiled due to secondary fermentations, which resulted in the loss of lactic acid, increased pH, and the discard of the fermented fruits. This outbreak provided a unique opportunity to investigate the development of secondary fermentations in commercial fermentation tanks. The observations made from these investigations are reported here. The objectives of this research were to characterize the chemical and environmental conditions that accompanied secondary cucumber fermentations, characterized by increasing pH and decreasing lactic acid concentrations, and to initiate the isolation and characterization of potential causative microorganisms, including oxidative yeasts.

MATERIALS AND METHODS

Commercial spoilage evaluation. In early August of 2010, a set of commercial brine samples collected from spoiling and standard fermented cucumber tanks were chemically and microbiologically analyzed as described below. Commercial cucumber fermentations were carried out in 40,000-liter open-top tanks containing between 50 and 60% whole cucumbers or precut pieces of the fruits and between 50 and 40% cover brine solutions containing acetic acid, added as concentrated vinegar, and sodium chloride to achieve equilibrated concentrations of 25 mM and 1.03 M (6%), respectively. Cucumbers were packed in fiberglass tanks and immediately covered with wooden boards to prevent them from floating until equilibration between the fruits and cover brine solution components was completed. Air purging was applied to the fermentation tanks during the active lactic acid fermentation to prevent bloater damage. In the peak season (summer months), the primary fermentation occurs within 2 weeks, and during this time the fermentation tanks were air purged intermittently for 8 h (personal communication; unpublished data). The development of spoilage was detected at the commercial scale, based mainly on the measurement of pH values above 3.3 ± 0.2. In addition to the detection of high pHs, the detection of manure-like and cheesy aromas and the presence of bubbles on the surface of the tanks indicated that the process of spoiling had started.

For this study, 40 spoiling and 20 standard fermentation tanks were monitored on the same day. Dissolved-oxygen (dO_2) measurements were done 2 in. below the wooden cover boards with an Oxi 330i portable set meter from WTW Measurements Systems Inc. (Fort Myers, FL) and adjusted for the estimated salt levels in the tanks (6%). A portable AP61 Accumet pH meter equipped with an AP50a electrode (Fisher Scientific, Pittsburgh, PA) was used to collect pH measurements from the cucumber tanks. Redox potential was measured using a submergible redox electrode (model PHEH-65-10; Omega Engineering, Inc., Bridgeport, NJ) connected to a portable conductivity/total dissolved solids (TDS)/pH meter (Omega Engineering, Inc.). The appropriate functioning of the redox potential probe was verified by measuring the redox potentials of both pH 7.0 and pH 4.0 calibration buffers with quinhydrone added to saturation levels. The metal tip of the probe was polished using an alumina powder mixture (1.0 μm; Precision Surfaces International, Houston, TX), as needed. The redox probe was rinsed thoroughly with 70% ethanol to sanitize it between tank measurements. Millivolt measurements were converted to E_h by following the manufacturer's instructions ($E_h = E_{meas} + 207$ mV, at 25°C). Samples for chemical and microbiological analyses were a mixture of aseptically collected cover brine solutions from below the wooden cover boards from three distant spots at the same depth (9 in.) in the tank.

Additional commercial spoilage brine samples (samples 1 to 4) were provided by processors in 2009 and 2010. These samples were incubated at 30°C statically for 15 days to monitor the utilization of lactic acid and acetic acid remaining once they were received. Sample 4, in which lactic acid was completely depleted when received, was spiked with 50 mM lactic acid and then further incubated for 10 days. An accuFet solid-state pH combination electrode (Fisher Scientific) and Accumet AR25 pH meter (Fisher Scientific) were used to measure the pHs of these samples during the incubation period.

Chemical analysis. The concentrations of organic acids and sugars were measured by high-performance liquid chromatography (HPLC) analysis using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, CA) for the separation of components (32). The column temperature was held at 37°C, and sample components were eluted with 0.03 N sulfuric acid at a flow rate of 0.6 ml/min. A Thermo Separations UV6000 diode array detector (Spectra System Thermo Scientific, Waltham, MA) set to collect data at 210 nm was used to quantify malic, lactic, acetic, propionic, and butyric acids. A Waters model 410 refractive index detector (Waters Corp., Millipore Corp., Billerica, MA) connected in series with the diode array detector was used to measure glucose, fructose, and ethanol. External standardization of the detectors was done using four concentrations of the standard compounds.

Microbiological analysis. Brine samples were serially diluted in 0.85% saline solution and spiral plated using an Autoplate 400 (Spiral Biotech, Norwood, MA). Enumeration of lactic acid bacteria was done using de Man, Rogosa, and Sharpe agar (MRS agar; Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 1% cycloheximide (0.1% solution; Oxoid) to inhibit the growth of yeasts. MRS agar plates were incubated anaerobically using a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, MI) 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) and 0.01% chlortetracycline (Sigma-Aldrich) to inhibit bacterial growth. YMA plates were incubated aerobically at 30°C for 48 h.

Isolation and identification of microorganisms. A variety of cucumber fermentation spoilage samples were used for the isolation and identification of potential causative microorganisms from MRS agar, YMA, and differential reinforced clostridial agar (DRCA; Becton Dickinson and Co.) (see Tables 2 and 5). Different colony morphologies visually observed in the agar plates were selected for this purpose. 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 (41) for yeasts. To determine the presence of *Clostridium* spp. in sample 4, which had the highest initial pH upon delivery to the laboratory, 25 ml of the spoiled brine was aseptically transferred to 50-ml centrifuge tubes in quadruplicate. Two tubes were incubated at 80°C for 30 min to induce clostridial spore germination, while the other two tubes were not heated. An aliquot of 100 μl from each tube was spread plated in DRCA, and plates were incubated anaerobically at 30°C for 48 h. Black colonies on DRCA plates were tentatively identified as *Clostridium* species and isolated for further analysis. Frozen stocks of all isolates were prepared in MRS agar, YM broth, or differential reinforced clostridial medium (DRCM; Becton Dickinson and Co.), as appropriate, containing 15% glycerol (Fischer Scientific). Isolates to be identified were coded with the letter M to indicate a morphology-based selection, followed by either the letter B, Y, or C to indicate the isolation medium, which was MRS agar, YMA, or DRCA, respectively. The three selected letters are indicative of bacterium (B), yeast (Y), and presumptively clostridial (C) isolates. A number was assigned to differentiate between morphologies from the same group, and a letter was assigned to identify the specific clone. Clones were isolated from independent secondary cucumber fermentations (see Table 3). Thus, "MY1A" indicates clone A with morphology 1 isolated from YMA, so it was presumed to be a yeast isolate.

The bacterial and yeast isolates were identified using partial 16S or 26S rRNA gene sequencing, respectively. Bacterial chromosomal DNA was obtained using a DNeasy genomic extraction and purification kit (Qiagen, Valencia, CA), while yeast chromosomal DNA was obtained using a MasterPure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI). The PCR mix contained 2× master mix (Bio-Rad), chromosomal DNA, and forward and reverse primers, which were 27F (5′-AGA

GTTTGATCCTGGCTCAG-3′) (2) and 1492r (5′-GGTTACCTTGTTAC GACTT-3′) (49) for the bacterial isolates and NL-1 (5′-GCCATATCAA TAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGA CGG) for yeasts (27). All primers were obtained from Integrated DNA Technologies (Coralville, IA). PCR products were purified using the Qiagen PCR purification kit and sequenced by Eton Bioscience Inc. (Raleigh, NC). The sequences obtained were subjected to the Basic Local Alignment Search Tool (BLAST 2.2.26) (1) in the GenBank (3) 16S rRNA microbial database for the bacterial cultures and the nonredundant nucleotide database for the yeast cultures (51) to determine the identities of the isolates. Only alignment matches with 99 or 100% identity, with no gaps, and expect values of 0.0 were considered for identification purposes.

Induction of secondary cucumber fermentations in the laboratory using commercial spoilage samples as the source of spoilage microorganisms. The experimental medium (EM) used for the reproduction of the secondary fermentation was prepared from 2B cucumbers (32 to 38 mm in diameter) acquired from a local processing company. The fruits were washed and packed into 1-gallon glass jars at a 55:45 pack-out ratio (weight of cucumbers to volume of cover brine) with cover brine solution to equilibrate it at 4% NaCl. The brined fruits were inoculated with a *Lactobacillus plantarum* LA 0219 starter culture (USDA-ARS Food Science Research Unit, Culture Collection, Raleigh, NC) to 6 log CFU/g. Fermentation progress was monitored by pH and HPLC analyses of acids and sugars. Upon completion of the lactic acid fermentation, fermented cucumbers were blended into a slurry and stored at −10°C. To prepare media for inoculation with spoilage microorganisms, fermented cucumber slurry was thawed, pressed through cheesecloth to remove large particulates, and spun at 23,400 × *g* for 15 min (Sorvall RC 5B; DuPont Instruments, Wilmington, DE). NaCl concentration was adjusted to 6% to be representative of NaCl levels used commercially, and pH was raised to 3.8 with 6 N NaOH to allow for more rapid development of secondary fermentation (18, 23, 25). The resulting EM was filter sterilized with a 0.2-μm bottle top filter apparatus (Nalgene FAST PES, 0.2-μm pore size, 90-mm-diameter membrane; Daigger, Vernon Hills, IL). Twelve-milliliter aliquots of the filter-sterilized medium were aseptically transferred to either 50- or 15-ml conical centrifuge tubes for aerobic and anaerobic incubation at 25°C, respectively. Incubation under anaerobic conditions was done using a Coy anaerobic chamber (Coy Laboratory Products, Inc.).

Aliquots of commercial spoilage sample 4 (see Table 2) were used as the source of inoculum to reproduce the spoilage in the laboratory. The aliquots were centrifuged for 10 min at 10,000 × *g* using an Eppendorf (Hamburg, Germany) 5810R centrifuge and supernatants decanted. The cell pellets were resuspended in filter-sterilized EM. An 8%, by volume, inoculum was added to each experimental tube containing the EM. For anaerobic study, tubes of EM were placed into the anaerobic chamber 2 days prior to inoculation. Triplicate tubes were inoculated for each treatment, and negative-control tubes, which were not inoculated, were included. EM samples (0.8 ml) were aseptically collected at different time points for microbiological and chemical analyses. Samples were collected at 0, 2, 4, and 8 days postinoculation for both environmental conditions. Afterwards, samples held under aerobic conditions were sampled on average every 4 days, while samples held under anaerobic conditions were sampled on average every 7 to 8 days. The incubations under aerobic and anaerobic conditions proceeded for 32 days and 3.5 months, respectively.

Inhibition of lactic acid degradation by yeasts isolated from the spoilage samples. Fresh cucumber juice was prepared from 2A pickling cucumbers (25.4 mm to 31.8 mm in diameter) obtained from a local processor using an automatic juice extractor (JM400 Juiceman Jr.; Black & Decker, Towson, MD). Particles were removed from the fresh juice using cheesecloth and subsequently spun for 1 h at 10,000 × *g* using a Sorvall RC-5B centrifuge (Dupont Instruments). The supernatant was mixed (50:50) with cover brine solution such that, after equilibration, the mixture had 100 mM $CaCl_2$ and 25 mM acetic acid. Half of the cucumber and cover brine solution mixture was supplemented with 100 ppm allyl isothiocyanate (AITC; Aldrich, St. Louis, MO), and the other half was left without the preservative to serve as a control. AITC, the major component of mustard oil, has been reported to exert a strong antifungal activity at low concentrations (31, 35, 39, 47), while being much less inhibitory toward lactic acid bacteria (46). The mixtures were individually filter sterilized using a 0.22-μm bottle top filter apparatus (Nalgene, Vernon Hills, IL).

Two-liter aliquots of each of the filter-sterilized media containing either 0 or 100 ppm AITC were aseptically transferred into two sterile 3.8-liter glass jars. The jars were sealed with lids that were fitted with a redox electrode (InLab 501/170; Mettler-Toledo, Bedford, MA), an inlet and outlet for the air-purging application, and a rubber septum for sample collection. After the proper functioning of the redox electrode probes were tested, as described above, the probes were rinsed with ethanol (70%) and securely fitted into the experimental jars. The tips of the electrodes were placed in the center of the jar in the cover brine solution. A silicone sealant was applied around the fittings holding the redox electrode and the air inlets to prevent air leakage into the jars. Jars were held at room temperature (24 ± 3°C). Air purging was applied at a flow rate of 5 ml/min, controlled by a model PG-1000 (U001) flow meter (Matheson Instruments, Montgomeryville, PA). Fermentation vessels were inoculated with a starter culture composed of three *L. plantarum* strains, LA 445, LA 98, and LA 89 (USDA-ARS Culture Collection, North Carolina State University, Raleigh, NC), originally isolated from cucumber fermentations, and a mixture of the two yeast isolates presenting morphologies MY1 and MY2 (see Table 4) (USDA-ARS Culture Collection) to 5 log CFU/ml. The starter LAB culture was added to accelerate the fermentation process and ensure a complete primary fermentation under aeration in the presence of large yeast populations. Brine samples were aseptically collected using 21-gauge, 6-in. sterile metal needles and syringes every 24 h for microbiological and chemical analyses as described above. This process was repeated with 2 lots of fresh cucumber juice. Data presented here represent the means of results of two independent replicates.

Statistical analysis. Log microbial plate counts and measured concentrations of organic acids and sugars were analyzed using the analysis of variance (ANOVA) procedure with Duncan's multiple-range test of the Statistical Analysis Systems version 9.0 software (SAS Institute, Cary, NC).

RESULTS

Commercial spoilage samples. A processor reported the outbreak of spoiling cucumber fermentations in commercial tanks in August 2010. The spoiling fermentations were characterized by atypical fermentation odors, but not manure- or cheese-like aromas. About 80% of the spoiling tanks contained a mixture of whole cucumbers of various sizes and cucumber slices and/or spears. Fifty-eight percent of the spoiling tanks exhibited evidence of gas formation, and most of them were packed in July 2010. Sodium chloride (NaCl) concentrations were readjusted in 63% of the total number of tanks to the targeted salometer level of 25 (6%). The concentrations of the salt in fermenting cucumber cover brines before adjustment ranged from salometer levels 17 to 21 (4 to 5%). Lactic acid concentrations were 45 to 85 mM lower than the mean concentration in normal fermentations, 115.5 ± 24.5 mM. The opposite was observed for acetic acid concentrations, which were on average 2-fold higher than the 25 mM observed in standard fermentations (Table 1). The differences in lactic and acetic acid concentrations were indirectly detected by the increased pH of the cover brines in spoiling fermentation tanks, as shown in Fig. 1. The environmental conditions in spoiling tanks were characterized by significantly higher dissolved oxygen (dO_2) and lower redox potential (E_h) measurements than those of standard tanks (Table 1). Moreover, the tank in which both propionic and butyric acids were detected showed the most reduced environment, as indicated by negative E_h readings (Table

TABLE 1 Chemical and microbiological characteristics of commercial spoiling cucumber fermentations sampled in the year 2010[a]

Type of cucumber fermentation	Concn of selected odorous acid(s)	No. of tanks monitored	Chemical properties					Microbial count (log CFU/ml) on:		Microbial count (log CFU/ml) based on characteristic morphologies			
			pH	dO_2 concn (mg/liter)	E_h (mV)	Lactic acid concn (mM)	Acetic acid concn (mM)	MRS agar	YMA	MB1	MB2	MY1	MY3
Standard	No propionic or butyric acids	20	3.3 ± 0.1 A	0.3 ± 0.1 A	351.2 ± 9.5 A	115.5 ± 24.5 A	24.7 ± 4.2 A	0.2 ± 0.0 A	5.7 ± 0.2 A	ND A	ND A	1.4 ± 0.0 A	5.4 ± 1.3 A
Spoiling	No propionic or butyric acids	19	3.9 ± 0.2 B	1.7 ± 0.3 B	277.0 ± 9.5 B,A	69.5 ± 25.4 B	45.3 ± 14.2 B	4.7 ± 0.1 B	5.0 ± 0.0 B	4.4 ± 0.0 B	3.7 ± 0.1 B	3.6 ± 0.0 B	ND B
	4–12 mM propionic acid; no butyric acid	19	3.7 ± 0.2 C	1.8 ± 1.1 C	331.04 ± 7.3 A	58.9 ± 14.9 B	42.8 ± 13.3 B	3.6 ± 0.0 C	4.8 ± 0.0 B	3.2 ± 0.0 C	2.5 ± 0.1 C	3.9 ± 0.1 B	ND B
	6–12 mM butyric acid; no propionic acid	1	4.2 D	0.9 D	263.0 A	33.9 C	48.3 B	5.6 ± 0.0 C	5.4 ± 0.0 A	5.2 ± 0.0 D	4.9 ± 0.1 D	4.1 ± 0.0 C	ND B
	6–12 mM butyric acid; 8 mM propionic acid	1	4.4 E	2.2 E	−139.0 B	50.1 D	53.1 C	NA	NA	NA	NA	NA	NA

[a] NA, not available; ND, not detected. Means within a column followed by the same uppercase letter are not significantly different ($P < 0.05$).

FIG 1 Lactic and acetic acid concentrations in commercial spoiling tanks, ranked according to pH. A total of 66 commercial fermentation tanks were monitored. Eighteen were standard fermentation tanks kept under storage for 1 to 12 months with a stable pH of 3.3 ± 0.1. Twenty-eight spoiling tanks whose results are included in the box plots shown in the figure had pH values ranging from 3.6 ± 0.1 to 3.8 ± 0.1, 17 had a pH ranging from 3.9 ± 0.1 to 4.1 ± 0.1, and only 4 had a pH of 4.2 ± 0.2. An average temperature of 31.6 ± 0.6°C was recorded from the 66 fermentation tanks monitored at the time of sampling. Significant differences ($P > 0.05$) in lactic and acetic acid concentrations were observed when means of concentrations from spoiling tanks were grouped by Duncan's multiple-range test and compared with the means of concentrations from standard fermentation samples. The samples are ranked according to pH. The data are presented in box plots in which the upper and lower divisions of the boxes represent the 75th and 25th percentiles, respectively; the whiskers extend to the 10th and 90th percentiles. The thick horizontal lines within boxes indicate medians, and the black circles represent the outliers.

1). Fifty percent of the spoiling fermentation tank samples had detectable levels of propionic acid (Table 1). In contrast, only two samples had detectable levels of butyric acid, with concentrations ranging between 6 and 12 mM. The presence of butyric acid was detected only in spoiling fermentations with pHs of >4.0. However, the presence of propionic acid was noted in spoiling fermentations with pHs (3.7 ± 0.2) lower than those observed from spoilage samples in which neither propionic acid nor butyric acid was produced (pH 3.9 ± 0.2) (Table 1).

Counts of lactic acid bacteria from samples of spoiled fermented cucumbers were, on average, 4 logs higher than counts from samples of standard cucumber fermentations (Table 1). Two

TABLE 2 Chemical and microbiological characteristics of the commercial spoilage samples delivered to the laboratory that were unrelated to the 2010 spoilage outbreak[a]

Commercial spoilage sample delivered to our laboratory from 2009 to 2010	Observation time	pH	Measured concn (mM)				Microbial count (log CFU/ml) on:		Estimated microbial count (log CFU/ml) based on characteristic morphologies		
			Lactic acid	Acetic acid	Propionic acid	Butyric acid	MRS agar	YMA	MB1	MB2	MY1
Control[c]		3.3 ± 0.1	115.5 ± 24.5	24.7 ± 4.2	ND	ND	0.2 ± 0.02	5.7 ± 0.2	ND	ND	1.4 ± 0.02
#1	T_i	5.1	ND	8.3	ND	11	6.2 ± 0.3	5.3 ± 0.4	NA	NA	NA
#2	T_i	3.6 ± 0.1	65.2 ± 1.1	27.3 ± 0.8	3.1 ± 0.1	ND	5.3 ± 0.1	5.5 ± 0.1	0.1 ± 0.1	ND	5.5 ± 0.1
	T_f	6.9 ± 0.1	18.7 ± 1.1	1.9 ± 0.5	0.2 ± 0.1	ND	3.8 ± 0.1	5.5 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	5.5 ± 0.1
#3	T_i	3.6 ± 0.1	63.9 ± 1.3	29.6 ± 0.8	4.1 ± 0.1	ND	6.1 ± 0.1	2.4 ± 0.1	ND	ND	3.6 ± 0.1
	T_f	7.2 ± 0.04	1.2 ± 1.2	9.6 ± 0.5	23.3 ± 0.1	ND	4.6 ± 0.1	6.5 ± 0.1	1.4 ± 0.1	ND	6.5 ± 0.1
#4[b]	T_i	4.7 ± 0.2	ND	38.8 ± 0.1	46.4 ± 0.3	44.0 ± 0.1	5.3 ± 0.1	4.2 ± 0.1	1.6 ± 0.1	2.7 ± 0.1	3.8 ± 0.1
	T_f	5.0 ± 0.1	13.1 ± 1.1	0.6 ± 0.3	58.7 ± 0.3	61.6 ± 0.3	6.8 ± 0.1	4.8 ± 0.1	3.6 ± 0.1	3.7 ± 0.1	3.2 ± 0.1

[a] T_i designates observations obtained from the commercial spoilage samples upon delivery, and T_f designates observations obtained from the commercial spoilage samples after incubation at 30°C for 15 days under aerobic and static conditions. NA, data not available; ND, not detected.
[b] Sample 4 was spiked with 50 mM lactic acid before incubation. After the addition of lactic acid, the sample pH was 3.9.
[c] Expected values for standard fermentation ($n = 20$).

distinct colony morphologies recognized on MRS agar plates inoculated with the spoilage samples were designated MB1 and MB2 (see Table 3). These two bacterial morphologies were more abundant in spoilage samples that did not contain propionic and/or butyric acid than in those that did ($P < 0.05$).

While significant differences in total yeast and mold counts were not observed between standard and spoiled fermentations, it was possible to identify significant differences in the yeast morphologies that predominated in the spoilage samples from those in standard fermentation samples (see Table 4). The MY1 morphology (Table 1) was detected on all YMA plates inoculated with spoiled fermentation samples but in only 9 standard fermentation samples. Numbers of MY1 colonies were higher on plates inoculated with the spoiled fermentation samples by at least 2 log CFU/ml ($P < 0.05$). In addition, the morphology MY2 was observed only on plates inoculated with spoilage samples, but it was detected in noticeably fewer colonies than the morphology MY1 (data not shown). The morphology designated MY3 (Tables 1 and 3) was detected exclusively in standard fermentation samples.

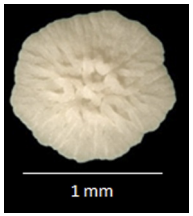
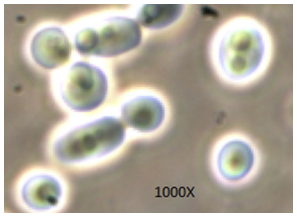
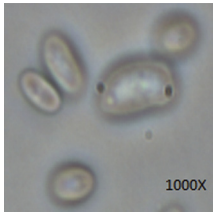
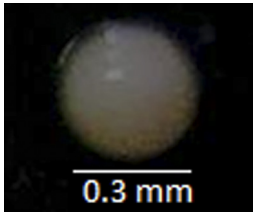
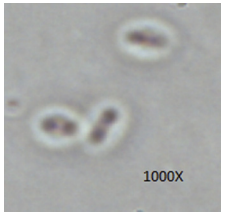
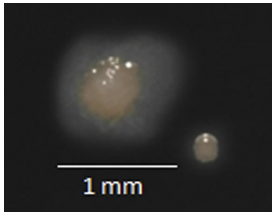
Additional spoilage samples obtained in 2009 and 2010 included those from (i) an experimental tank packed at a commercial facility with fresh cucumbers and a cover brine solution containing no sodium chloride and 100 mM calcium chloride (sample 1), (ii) commercial tanks in which recycled NaCl brines were used for packing fresh cucumbers (samples 3 and 4), and (iii) a commercial tank containing organic cucumbers in which recycled NaCl cover brine solution was used for packing (sample 2) (Table 2). The first indication of spoilage was a cover brine pH significantly higher than the targeted postfermentation pH of 3.3 ± 0.3 (5, 8). Samples 1 and 4 were also characterized by off odors resembling manure and cheese. Samples 2 and 3 developed off odors after incubation in the laboratory. Such odors suggested the development of secondary fermentations and the formation of products associated with spoilage, such as butyric and propionic acids, which was confirmed by HPLC analysis (Table 2). In all samples, lactic and acetic acid concentrations were below the standard postfermentation values (115.5 ± 24.5 mM and 24.7 ± 4.2 mM, respectively) or not detected, as in the case of sample 4 (Table 2).

Yeast and bacterial morphologies detected in spoilage samples collected from the 2010 outbreak were also observed in the four samples unrelated to that outbreak. The MY1 morphology was present in all spoilage samples (1 through 4), and the MY2 morphology was detected in samples 1 and 4 (Table 2). While the MB1 morphology was present in all spoilage samples, MB2 was detected exclusively in samples 1, 2, and 4 (Table 2). Presumptively clostridial isolates showing black colonies in DRCA plates were observed from both nonheated and heat-treated aliquots of sample 4. As expected, the numbers of black colonies in heat-treated samples were significantly higher (2 logs [data not shown]) than those in nonheated ones. The organism (presumptively *Clostridium*) isolated from an aliquot of sample 4 was designated MC1 (Table 3).

Variability of acetic acid concentrations in the spoilage samples might be due to the presence of different organisms. Samples in which the acetic acid concentration was above the standard 25 mM (Table 1) were associated with the presence of the LAB isolate with the MB1 morphology as the most abundant organism. Decreased acetic acid concentrations in samples incubated for an additional period (Table 2) were associated with the presence of yeast counts of about 5 log CFU/ml.

Isolation and identification of potential causative agents. A description of the morphological characteristics and identification of the isolates based on the 16S or the 26S rRNA gene sequencing data are shown in Table 3. Three yeasts (morphologies MY1, MY2, and MY3) were isolated and identified. MY1 and MY2 were the most abundant colony morphologies present in spoilage samples. MY1 colonies are white, crateriform, umbonate in elevation, and about 1 mm in diameter after 48 h of aerobic incubation on YMA plates at 30°C, and they have wrinkles, radial striations, and undulate margins. MY1 colonies can be clearly distinguished from others due to their "volcano-like" morphology. The cells occur in buds, are ellipsoidal, and have ascospores (Table 3). MY1 isolates were identified as *Pichia manshurica*. MY2 colonies are whitish beige and irregular in shape, and they have an umbonate elevation and lobate margin after 48 h of aerobic incubation on YMA plates at 30°C. The colonies have a "fuzzy" surface, with diameters from 1 to 1.5 mm. Under the microscope, cells are ogi-

TABLE 3 Identification of selected isolates from the secondary cucumber fermentation based on colony morphology and rRNA gene sequencing

Morphology identification[a]	Colony appearance	Cell appearance by microscopy	Source (morphology identification)[c]	Species identification[d]	GenBank accession no.	USDA-ARS Culture Collection no.[e]
MY1			Outbreak 2010 (MY1A)	*Pichia manshurica* or *Pichia galeiformis*	JQ086340	Y091
			Spoilage sample 1 (MY1B)	*Pichia manshurica* or *Pichia galeiformis*	JQ086342	Y088
			Spoilage sample 2	Not identified		
			Spoilage sample 3	Not identified		
			Spoilage sample 4 (MY1C)	*Pichia manshurica* or *Pichia galeiformis*	JQ08341	Y092
MY2			Outbreak 2010 (MY2A)	*Issatchenkia occidentalis*	JQ086337	Y093
			Spoilage sample 1 (MY2B)	*Issatchenkia occidentalis*	JQ086338	Y089
			Spoilage sample 3	Not identified		
			Spoilage sample 4 (MY2C)	*Issatchenkia occidentalis*	JQ086639	Y094
MY3			Standard fermentation brines	*Candida etchellsii*	JQ086343	Y092
MB1		Gram positive, non-spore-forming bacilli; cells are spherical	Outbreak 2010 (MB1A)	*Lactobacillus* sp.	JQ086333	LA1192
			Spoilage sample 1 (MB1B)	*Lactobacillus buchneri*	JQ086334	LA1149
			Spoilage sample 2	Not identified		
			Spoilage sample 3	Not identified		
			Spoilage sample 4 (MB1C)	*Lactobacillus* sp.	JQ086335	LA1193

		Outbreak 2010 (MB2A)	*Pediococcus* sp.	JQ086330	LA1194
MB2[b]	Gram-positive, non-spore-forming coccus; cells occur in chains	Spoilage sample 1 (MB2B)	*Pediococcus ethanolidurans*	JQ086332	LA1150
		Spoilage sample 2	Not identified		
		Spoilage sample 4 (MB2C)	*Pediococcus ethanolidurans*	JQ086331	LA1195
MC1	Gram-positive, spore-forming rod	Spoilage sample 4 (MB2B)	*Clostridium* sp.	JQ086336	B431

[a] Microorganisms designated with an MY, MB, or MC number were isolated from YMA, MRS agar, or DRCA plates, respectively.
[b] The small colony represents the isolate MB2. The bigger colony represents an *L. plantarum* colony (USDA-ARS Culture Collection, strain LA 445). The approximate diameter for *L. plantarum* is 1 mm, while MB2 is about 1/10 the size of *L. plantarum*. Both colonies are shown here for comparison of sizes.
[c] The colony morphology was observed in different commercial secondary cucumber fermentation brines.
[d] Based on the 16S or 26S rRNA gene for bacteria or yeasts, respectively.
[e] All isolates are stored at the USDA-ARS Food Science Research Unit, North Carolina State University, Raleigh, NC.

val and have round ascospores (Table 3). MY2 colonies were identified as *Issatchenkia occidentalis*. MY3 colonies, observed and isolated only from standard fermentation samples, are small, whitish beige, shiny, and spherical, and they have a slight elevation and entire margin after 72 h of aerobic incubation on YMA plates at 30°C. Under the microscope, the cells are round to ellipsoidal, with ascospores at the center of the cell. MY3 colonies were identified as *Candida etchellsii*.

The bacterial MB1 isolates, identified as *Lactobacillus buchneri* (MB1B) and *Lactobacillus* sp. (MB1A and MB1C), are Gram-positive, non-spore-forming, spherical bacilli. Colonies on MRS agar plates after 48 h of incubation appeared filamentous and flat, with a diameter of approximately 1 mm. The shape of the colony resembled a star. The MB2 bacterial isolates, identified as *Pediococcus ethanolidurans* (MB2C and MB2B) and *Pediococcus* sp. (MB2A), are Gram-positive, non-spore-forming cocci (Table 3). Under a microscope, the cells occur in chains. After 48 h of incubation on MRS agar, colonies look punctiform and convex and have an entire margin. The colony size was about 1/10 that of an *L. plantarum* colony. The organism presumed to be *Clostridium* that was isolated from sample 4 (MC1) was identified as a *Clostridium* sp. by the partial sequencing of the 16S rRNA gene. The colony was irregular and umbonate in elevation and had a lobate margin. After 48 h of anaerobic incubation, the colony grew immersed in the agar and developed a translucent filament around it. Under a microscope, the cell is a Gram-positive, spore-forming rod.

Induction of a secondary cucumber fermentation in the laboratory. The changes observed during aerobic and anaerobic incubations of fermented cucumber media inoculated with aliquots from sample 4 are shown in Table 4. The pHs of samples incubated under aerobic and anaerobic conditions increased to 8.3 ± 0.1 and 4.3 ± 0.1 at 0.10 and 0.005 pH units/day, respectively, and proceeded together with the reduction in lactic acid concentrations. Lactic acid utilization associated with yeast growth was inhibited by anaerobic conditions. Conversely, total yeast counts from samples incubated under aerobic conditions reached about 7 log CFU/ml during active lactic acid utilization. Both the MY1 and MY2 morphologies were recognized on YMA plates (Table 4). In contrast, total yeast counts decreased with time in samples incubated under anaerobic conditions, and only MY1 was detected in YMA plates toward the end of the incubation period (Table 4).

Total LAB counts increased with time under both incubation conditions, reaching populations above 7 log CFU/ml (Table 4). Initially, total LAB population increased with the utilization of the remaining sugars, primarily glucose and fructose, which in turn decreased the medium pHs to 3.79 and 3.74 under aerobic and anaerobic conditions, respectively. The spoilage LAB, of the MB2 morphology, was not detected at the beginning of the experiment, but it was observed once the medium pH was above 4.0 under anaerobiosis and above 6.8 under aerobic conditions. The MB1 and MB2 LAB were able to persist in samples incubated under aerobic conditions after 32 days and 3.5 months of incubation under anaerobic conditions (Table 4). Acetic and propionic acids from samples incubated under aerobic and anaerobic conditions were detected in similar total amounts by the end of the incubation periods, and their formation was independent of yeast growth. Acetic and propionic acids formed at a rate of 0.55 and 0.53 mM/day under aerobiosis, compared to a more linear rate of 0.24 and 0.14 mM/day under anaerobic conditions. Interestingly, under aerobic conditions which allowed oxidative yeasts to pro-

TABLE 4 Trends in chemical and microbiological changes during secondary cucumber fermentation reproduced in our laboratory, with commercial spoilage sample 4 used as the inoculum[a]

Type, length of incubation	Time point (day)	pH	Microbial count (log CFU/ml)						Acetic acid concn (mM)	Lactic acid concn (mM)	Propionic acid concn (mM)
			YMA			MRS agar					
			Total count	No. of MY1 colonies	No. of MY2 colonies	Total no. of colonies	No. of MB1 colonies	No. of MB2 colonies			
Aerobic, 1 mo	0	3.8 ± 0.1	2.8 ± 0.0	ND	ND	3.8 ± 0.1	2.9 ± 0.1	ND	7.7 ± 0.1	110.7 ± 0.1	ND
	4	4.1 ± 0.1	6.7 ± 0.1	2.2 ± 0.1	ND	4.1 ± 0.1	3.6 ± 0.1	ND	4.5 ± 0.5	90.7 ± 3.4	ND
	8	6.8 ± 0.1	7.4 ± 0.2	1.5 ± 0.2	3.8 ± 1.2	6.8 ± 0.1	5.6 ± 0.1	0.5 ± 0.2	5.6 ± 1.1	61.3 ± 3.0	ND
	16	7.4 ± 0.1	7.2 ± 0.4	2.6 ± 0.4	4.7 ± 0.4	7.4 ± 0.1	5.8 ± 0.5	1.9 ± 0.4	21.7 ± 7.5	18.5 ± 6.2	11.4 ± 17.1
	21	7.8 ± 0.1	6.6 ± 0.6	2.1 ± 0.4	4.3 ± 0.1	7.8 ± 0.1	6.6 ± 0.3	1.9 ± 0.2	27.0 ± 3.3	ND	17.5 ± 10.5
	32	8.3 ± 0.1	4.6 ± 1.2	ND	ND	8.3 ± 0.1	6.1 ± 0.1	2.2 ± 0.1	27.8 ± 5.3	ND	17.7 ± 9.5
Anaerobic, 3.5 mo	0	3.8 ± 0.0	3.2 ± 0.1	ND	ND	3.1 ± 0.1	ND	ND	7.7 ± 0.1	112.2 ± 2.2	ND
	4	3.8 ± 0.1	4.6 ± 0.1	ND	ND	3.0 ± 0.1	ND	ND	7.1 ± 0.1	109.7 ± 0.9	ND
	24	3.8 ± 0.1	2.2 ± 0.1	ND	ND	7.3 ± 0.1	ND	ND	9.4 ± 0.4	114. ± 1.6	ND
	39	3.9 ± 0.1	ND	ND	ND	7.3 ± 0.1	4.0 ± 0.7	3.1 ± 0.5	14.9 ± 1.1	107.5 ± 3.7	3.4 ± 0.4
	56	4.0 ± 0.1	1.3 ± 0.3	1.3 ± 0.3	ND	6.6 ± 0.4	3.1 ± 0.1	2.7 ± 1.1	19.9 ± 1.6	98.3 ± 3.7	6.2 ± 0.7
	106	4.3 ± 0.1	2.3 ± 0.3	1.9 ± 0.8	ND	5.2 ± 0.3	3.8 ± 1.2	3.8 ± 0.2	30.5 ± 2.0	74.3 ± 3.6	14.5 ± 1.1

[a] ND, not detected. Values for each time point are averages of results from three independent replicates.

liferate, concentrations of 2.1 ± 0.2 mM acetic acid were utilized between days 0 and 8. The presence of propionic acid coincided with the appearance of the MB2 spoilage LAB (Table 4).

Inhibition of lactic acid disappearance caused by *Pichia manshurica* and *Issatchenkia occidentalis*. As expected, the inoculated *L. plantarum* strains dominated the primary fermentation in the fresh cucumber juice medium. Fermentation proceeded normally in the presence of 0 or 100 ppm AITC within the first week, as evidenced by decreases in the medium pH that were concomitant with increases in lactic acid concentrations (Table 5) and LAB populations (Fig. 2A). However, the rate of lactic acid production and the acidification of the medium were delayed in the presence of 100 ppm AITC. Supplementation of the fresh cucumber juice medium with 100 ppm AITC prevented the disappearance of lactic acid after 20 days of aerobic incubation. The growth of *Pichia manshurica* (MY1A) and *Issatchenkia occidentalis* (MY2A) was evident in the jars not supplemented with AITC. Growth corresponded with lactic and acetic acid degradation (Table 5 and Fig. 2B). Significant changes in pH (Table 5) and redox potential (Fig. 2C) were also detected in the jars not supplemented with AITC. Yeast populations in the medium containing AITC decreased gradually by 3 logs as the incubation proceeded (Fig. 2B). The fermentation environment remained oxidized in the presence of AITC, as indicated by a positive E_h measurement (Fig. 2C), and the final lactic and acetic acid concentrations (100 mM and 24 mM, respectively) were within concentrations expected for standard cucumber fermentations (5).

DISCUSSION

The characterization of secondary fermentations that lead to spoilage of fermented cucumbers has been limited due to the sporadic occurrence of the event at the commercial scale and the inability to predict the process. To our knowledge, there have been three spoilage outbreak events at three distant and independent locations, affecting approximately 100 commercial tanks in the last 2 years. An opportunity to study the problem presented itself during a 2010 spoilage outbreak that occurred near our research facilities and allowed the characterization of commercial brine samples at different stages of spoilage. Based on these observations, it was possible to notice that lactic acid degradation was associated not only with increases in brine pH but also with changes in the conditions inside the fermentation tanks, such as the chemical reduction of the matrix and increased dissolved-oxygen concentrations, compared with those of standard fermentations. Potential causative agents isolated from commercial brines suggest that oxidative yeasts can be related to aerobic initi-

TABLE 5 Inhibition by addition of AITC of lactic acid degradation caused by MY1 and MY2 isolates[a]

Time (day)	pH		Lactic acid concn (mM)		Acetic acid concn (mM)	
	With AITC	Without AITC	With AITC	Without AITC	With AITC	Without AITC
0	4.1 ± 0.01	4.2 ± 0.02	ND	ND	23.0 ± 0.05	23.6 ± 0.05
4	3.5 ± 0.01	3.2 ± 0.01	37.2 ± 0.05	61.7 ± 0.9	23.1 ± 0.03	20.2 ± 0.3
7	3.3 ± 0.01	3.2 ± 0.00	51.4 ± 0.04	66.6 ± 0.01	22.9 ± 0.2	20.1 ± 0.3
15	3.2 ± 0.00	3.1 ± 0.02	61.9 ± 0.5	72.6 ± 1.7	22.4 ± 0.2	13.9 ± 0.2
20	3.1 ± 0.02	6.3 ± 0.01	65.9 ± 0.5	29.8 ± 0.9	23.5 ± 0.3	1.6 ± 0.03
30	3.1 ± 0.04	6.9 ± 0.03	68.5 ± 1.2	6.32 ± 0.2	24.2 ± 0.6	1.3 ± 0.1
60	3.2 ± 0.01	6.9 ± 0.03	70. ± 1.1	ND	24.3 ± 0.5	ND

[a] MY1 colonies were *Pichia manshurica*, and MY2 colonies were *Issatchenkia occidentalis*. With AITC, cucumber juice medium supplemented with 100 ppm AITC; without AITC, cucumber juice medium not supplemented with AITC; ND, not detected. Values at each time point are the means of results of two replicates.

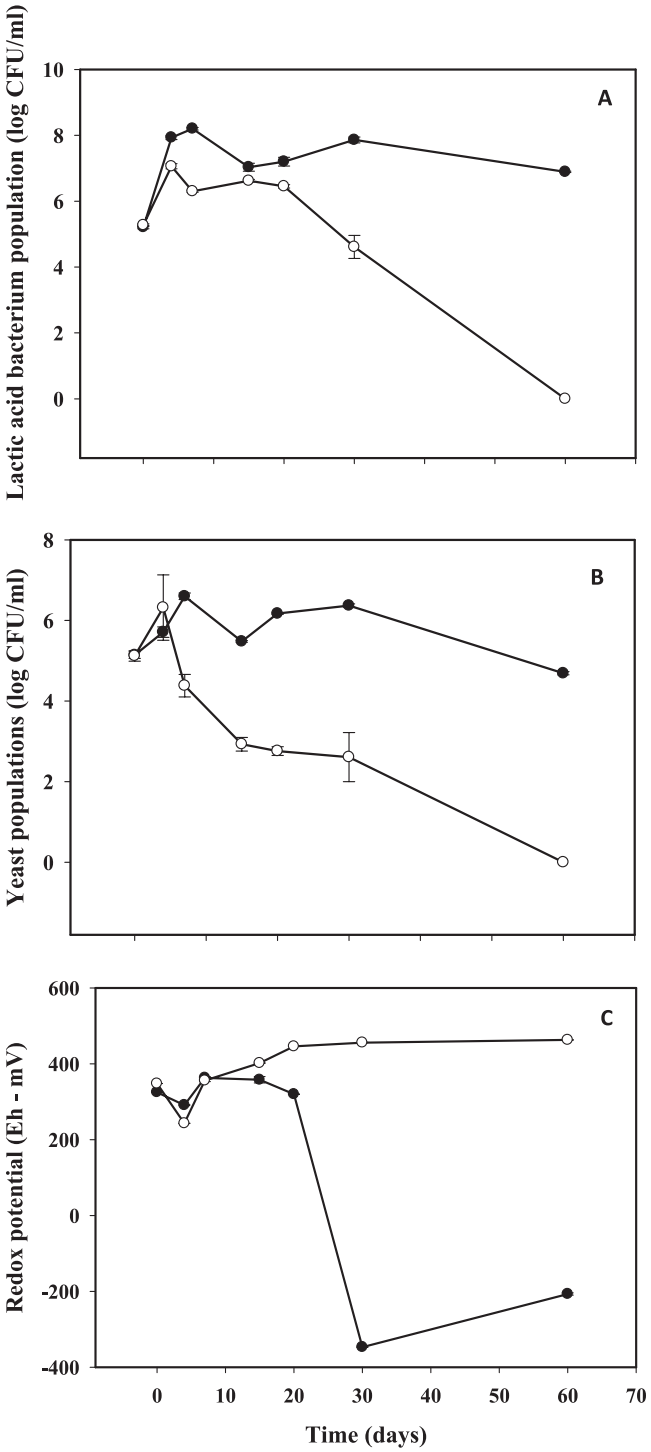


FIG 2 Microbiological and environmental changes observed during the inhibition of lactic acid degradation caused by *Pichia manshurica* and *Issatchenkia occidentalis*. Changes in lactic acid bacterium populations (A), yeast populations (B), and redox potentials (C) observed during air-purged incubation of cucumber juice media supplemented with (○) and without (●) AITC and inoculated with both spoilage yeasts (*Pichia manshurica* and *I. occidentalis*) and *L. plantarum*.

ation of secondary fermentations. Additionally, bacterial isolates seem to be associated with different stages of secondary fermentation. Secondary fermentation of cucumbers, which is partially described by the data presented here, is a complex event demanding further studies to determine the identities of all the possible causative agents and their respective contributions. Furthermore, it is still necessary to identify, test, and develop tools for the early detection of the development of a cucumber secondary fermentation and strategies to prevent its occurrence. It was the intent of this research to identify some of the factors that contribute to the full development of secondary cucumber fermentations, establishing a baseline that should help in gaining a better understanding of this event.

The 2010 secondary fermentation outbreak revealed that the types of yeasts present in the fermentation tanks were more relevant than the total yeast counts. Oxidative yeasts presenting morphologies similar to those characteristic of *P. manshurica* and *I. occidentalis* were fully or partially responsible for the disappearance of lactic acid and an increase in brine pH. The study of this outbreak also confirmed that selected lactic acid bacteria, different from those that carry out the primary fermentation, are capable of proliferating during the secondary fermentation. These spoilage LAB coexisted with the spoilage yeasts. Of particular interest is the presence of *L. buchneri* in the studied samples. The ability of this lactic acid bacterium to degrade lactic acid has been demonstrated in silage (12, 22), in sourdough (50), and recently in fermented cucumbers (23). Acetic acid is one of the end products of that metabolism (37), which is consistent with the increased concentrations of acetic acid in the spoiled commercial fermentations presenting high counts of MB1 colonies. Although 1,2-propanediol has been identified as a metabolic product of lactic acid degradation by *L. buchneri* (37), previous characterization of the metabolites in spoiled fermented cucumber samples did not detect this alcohol (23–25). This alcohol can subsequently be converted into propionic acid by other microorganisms belonging to the lactobacillus family. *Lactobacillus diolivorans* isolated from silage was able to degrade 1,2-propanediol and produce propionic acid in modified MRS agar and sourdough (26, 50). The fact that propionic acid was detected in commercial and laboratory-reproduced spoilage samples suggests that microorganisms other than MB1 organisms have a role in spoilage. Further studies are necessary to determine whether this spoilage involves cooperative metabolism among microorganisms or the presence of other unidentified organisms capable of converting lactic acid into propionic or butyric acid.

Variability in acetic acid concentration in the studied spoilage samples might be due to the presence of different organisms in independent commercial fermentations. Samples in which acetic acid concentrations were above the standard 25 mM (Table 1) were correlated with the MB1 LAB isolate being the predominant organism. Decreased acetic acid concentrations in samples subjected to an extended incubation after their delivery to our laboratory (Table 2) were associated with the presence of yeast counts of about 5 log CFU/ml. The ability of the yeast isolates *Pichia manshurica* (MY1) and *Issatchenkia occidentalis* (MY2) to utilize lactic and acetic acids was evident after inoculating these yeasts in a fresh cucumber juice model system (Table 5). Conversely, the inhibition of yeast metabolic activity when the medium was supplemented with 100 ppm allyl isothiocyanate (AITC) allowed the fermented product to remain stable, thus corroborating that the isolated yeasts were a contributing factor in the utilization of both organic acids.

Significant differences in LAB populations were observed between the spoilage outbreak samples and the spoilage samples

brought to the laboratory. The MB1 morphology predominated among the LAB population detected in the spoilage outbreak samples (Table 1), suggesting a relevant role for the bacteria presenting this morphology in the progression of the spoilage. It is contradictory to such observations that the MB1 colony counts from spoilage samples transported to the laboratory were significantly lower than the LAB counts from the same samples, even after the spoilage samples were subjected to a prolonged incubation period in the laboratory, in which only minimal growth of MB1 organisms proceeded (Table 2). This observation may suggest that the numbers of the bacterial population presenting the MB1 morphology may have been compromised during the handling of the samples before delivery to the laboratory or that the increased oxygen concentration during the collection of samples favored an incremental increase in the oxidative-yeast population capable of causing the spoilage and possibly outcompeting MB1 organisms. Alternatively, these observations may suggest that (i) the presence of MB1 organisms is not essential for the development of spoilage, (ii) the growth of MB1 organisms may depend on the presence of fructose in the culture medium, as is the case for a number of heterofermentative lactic acid bacteria, or (iii) other lactic acid bacteria not yet isolated or identified may have an important role in secondary cucumber fermentations and spoilage.

The data collected during the reproduction of spoilage in the laboratory suggest that a secondary fermentation caused by *L. buchneri* in the absence of the oxidative spoilage yeasts is, at the commercial scale, preferable to spoilage induced by the oxidative yeasts. This is due to the possibility of slower lactic acid utilization and the conversion of some lactic acid into acetic and propionic acids, which would maintain a more acidic pH with time. The presence of oxidative yeasts and aerobic conditions would accelerate the removal of lactic acid, leading to a rapid rise in pH and gas formation that could cause bloating of whole cucumbers.

Once a higher pH develops, due to microbial lactic acid utilization, a variety of putrefactive microorganisms capable of producing fetid odors, loss in color, and softening of the cucumber tissue may occur (4). For instance, during the secondary fermentation reproduced in the laboratory, colony morphologies consistent with *L. buchneri* and *P. ethanolidurans* became established once the medium's pH was above 4.0 under anaerobic conditions and 6.8 under aerobiosis (Tables 3 and 4). Butyric acid was observed in commercial samples that had the most reduced (E_h, 263 and −139 mV) and highest (>4.2) pH (Table 1) fermentation matrices, suggesting that the organisms related with the production of this short-chain fatty acid might be anaerobic or microaerophilic. Selected clostridial species have been reported as capable of producing butyric acid (18, 25, 34, 48); thus, it is possible that the natural clostridial population, including the *Clostridium* spp. isolated from sample 4, produced the butyric acid that was detected in the laboratory-reproduced spoilage under aerobic conditions and that ranged between 3.6 ± 1.8 and 5.1 ± 0.6 mM.

Although changes in pH are currently used at the commercial scale to monitor the stability of fermented cucumbers, redox potential measurements may be used as another tool for the detection of secondary cucumber fermentation. Commercial spoilage fermentations showed decreased E_h values compared to those of standard fermentations (Fig. 3), confirming that the fermentation conditions might be affected by the different microbiota present in the preserved fruits. It has been reported that different microorganisms affect differently the fermentation redox potential. For instance, during the growth of *L. plantarum* in cucumber juice, the E_h remained positive, while the growth of *Enterobacter aerogenes* reduced the environment to negative values (36). Interestingly, when the MY1A and MY2A oxidative yeasts were inoculated in fermented cucumber juice medium, the environment became reduced as the yeasts grew (Fig. 3), suggesting that important changes in redox potential could be associated with the yeasts' activity. However, significant changes in redox potential were not detectable before pH increased to 4.0 or above, after a significant amount of lactic acid had been utilized. Therefore, the best indicator of secondary fermentation based on current knowledge is a rise in pH.

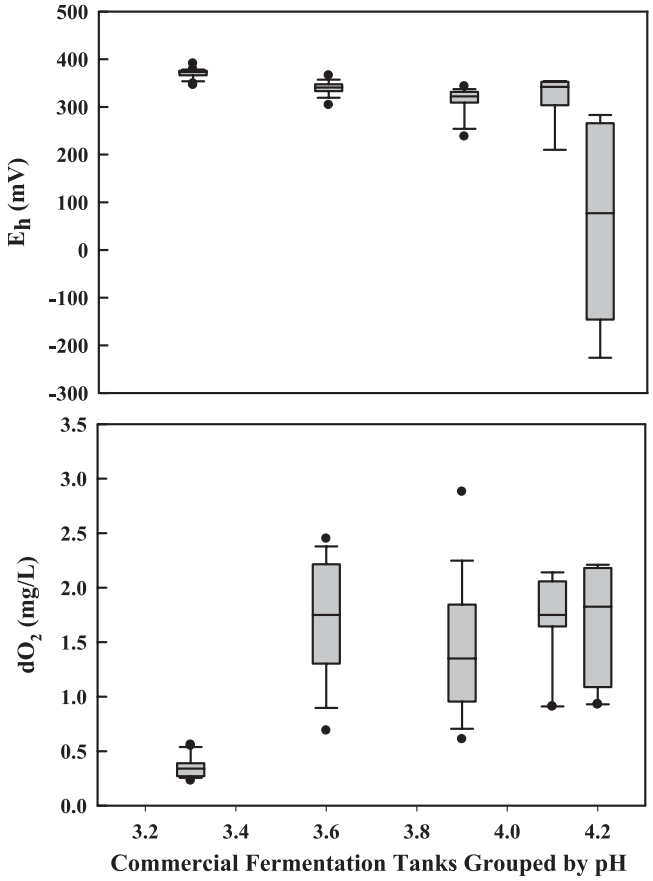


FIG 3 Redox potential and dissolved-oxygen measurements in standard and spoiled commercial cucumber fermentations. A total of 66 tanks were monitored, of which 18 were standard fermentation cucumber tanks in which cucumbers had been in storage between 1 and 12 months, with a stable pH of 3.3 ± 0.1. Although results from 18 spoiling tanks at pH 3.6 ± 0.1 to 3.9 ± 0.1 were included in calculations of the medians, only 8 and 5 tanks were monitored at pHs 4.1 ± 0.1 and 4.2 ± 0.2, respectively. An average temperature of 31.61 ± 0.61°C was recorded from the 66 fermentation tanks monitored on the sampling day. A significant difference between the E_hs at pHs between 3.3 to 4.1 and 4.2 was determined by Duncan's multiple-range test ($P > 0.05$). The samples were ranked according to pH. The data are presented in box plots in which the upper and lower divisions of the boxes represent the 75th and 25th percentiles, respectively; the whiskers extend to the 10th and 90th percentiles. The thick horizontal lines within boxes indicate medians, and the black circles represent outliers.

Of particular interest was the observation that yeast colonies presenting morphologies similar to that of *Candida etchellsii*

(MY3A) were exclusively observed in, exclusively isolated from, and predominantly present in standard fermentation samples. Additionally, the dissolved oxygen in spoiling cucumber fermentation tanks was significantly higher than in standard fermentation tanks (Fig. 2A). Although, the spoilage yeast isolates studied here (MY1 and MY2) were able to utilize oxygen and reduce the environment (Fig. 3C), the potential role of *C. etchellsii* as an oxygen "chelator," a competitor (42), or a killer yeast (20, 21, 29, 45) remains to be studied. However, the fact that higher dO_2 measurements characterized the spoiling fermentation tanks may suggest that a beneficial oxidative organism(s) may be absent from fermentations prone to undergo a secondary cucumber fermentation. Alternatively, the higher dO_2 measurements may suggest that the spoiling tanks were, for an unknown reason, subjected to higher oxygen exposure, which encouraged the proliferation of the oxidative yeasts associated with the spoilage.

Although lower NaCl concentrations were reported as important for the development of spoilage in fermented cucumber pickles (23, 25), data gathered from commercial spoilage samples indicated that fermentations carried out with 0 to 6% NaCl and 0.2 to 1.1% $CaCl_2$ are susceptible to spoilage. Certain spoilage yeasts and LAB are known for being halotolerant (11), which, combined with the ability to utilize organic acids as a source of carbon, gives these organisms a unique competitive advantage in an environment characterized by high NaCl and lactic acid concentrations.

The isolation and identification of yeasts as potential initiators of a secondary fermentation under aerobic conditions is important to the pickle industry. The yeast population naturally present on cucumber fruits is generally controlled once the primary fermentation is completed due to the lack of readily available energy sources (14). The exception to this situation occurs with the film-forming yeasts, which can be inhibited by the spraying of a concentrated potassium sorbate solution on the surfaces of the tanks in cloudy weather or by including the preservative in the formulation of the cover brine solutions used for the fermentations (16). However, our findings showed that oxidative yeasts, such as *P. manshurica* and *I. occidentalis*, are capable of producing important changes during storage of the fermented product, such as the disappearance of lactic acid and an increase in pH. Certain practices currently followed in the pickle industry might favor the development of these organisms on the fresh fruit prior to the fermentation process. Incubation of the fresh fruits at warmer ambient temperatures (72 to 85°F; 22 to 29°C) for extended periods of time during transport and/or arrival at the processing plant during the peak of the cucumber tanking season, along with less-than-ideal sanitation and handling practices, could increase the populations of the microbiota, including the yeast populations, present in the fresh fruit. Total yeast and mold counts detected in fresh cucumber samples collected from the tank yard analyzed during the 2010 season were 2 log CFU/ml higher than the standard counts of 2.82 ± 0.95 CFU/ml (38). Based on our observations, it seems that tanks containing pieces and cuts of the fresh fruits are more susceptible to yeast spoilage than tanks containing the whole fruits. Yeasts, which are generally larger in size than bacteria, are not able to penetrate the cucumber skins (9); consequently, the brined cucumber pieces represent a matrix with more readily available nutrients than the whole fruits.

Although further studies are needed to identify strategies for the prevention of cucumber secondary fermentations, a number of recommendations follow. Steps that could theoretically reduce the incidence of secondary cucumber fermentation leading to spoilage in a commercial environment include better process controls (including the reduction of the holding times for fresh cucumbers prior to tanking, elimination of a washing step for cucumber pieces after they are cut, and prevention of the contact of such pieces with the processing surfaces prior to tanking) and the utilization of processing equipment with a sanitary design. Acidification of the fermented cucumbers with vinegar should be carefully evaluated, as the utilization of acetic acid by spoilage yeasts (Table 5) may aggravate an incipient spoilage. It is also recommended that oxygen availability in the tanks is reduced either by covering the tanks and/or limiting air purging, as the lack of air should limit the metabolic activity of the spoilage associated with oxidizing yeasts. Limiting the oxygen availability has resulted in decreases in specific growth rates for the spoilage yeasts *Zygosaccharomyces bailii* and *Candida utilis* in defined and complex media (43). Additionally, spoiled brines should be isolated to prevent the enrichment of the yeasts in future fermentations. If spoiled brines will be recycled, it is recommended that the cover brine solutions are treated to reduce yeast populations.

Conclusions. Considering the availability of oxygen during the lactic acid fermentation and bulk storage of commercially fermented cucumbers, it is reasonable to suggest that organisms other than the ones previously reported under anaerobic conditions might be responsible for the initiation of the spoilage process. The characterization of several commercial spoilage samples and our experimental observations suggest that oxidative yeasts, such as *Pichia manshurica* and *Issatchenkia occidentalis*, can initiate the spoilage process. The metabolic activities of these yeasts result in changes in the environment that favor other microflora to continue the spoilage process. Further studies are needed to characterize these changes and their contribution to secondary cucumber fermentations.

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