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Prevention of microbes-induced spoilage in sodium chloride–free cucumber fermentations employing preservatives

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Abstract: This study evaluated preservatives to stabilize sodium chloride (NaCl)–free-cucumber fermentations. The brining of air-purged laboratory cucumber fermentations with 100.0 mM calcium chloride ($CaCl_2$) and 25.0 mM acetic acid resulted in immediate rises in pH, the chemical reduction of the medium, and malodors. Supplementation with 3.0 mM sodium benzoate or 3.0 mM potassium sorbate enabled a decline in pH, a continuous oxidative state of the medium, and delayed rising pH spoilage. However, lactic and acetic acids eventually disappeared in fermentations supplemented with preservatives. The amount of preservatives needed to suppress growth of brined-cucumber-spoilage microbes was determined in Fermented Cucumber Juice Medium (FCJM). Supplementation of FCJM with 10.0 mM sodium benzoate was inhibitory for the spoilage yeasts, *Issatchenkia occidentalis* and *Pichia manshurica*, and the lactobacilli, *Lentilactobacillus buchneri* and *Lentilactobacillus parafarraginis*, but not of *Zygosaccharomyces globiformis*. Potassium sorbate inhibited the spoilage yeasts at 15.0 mM in FCJM but not the lactobacilli. Supplementation of FCJM with 20.0 mM fumaric acid had a bactericidal effect on the spoilage-associated lactobacilli. As expected, NaCl-free-commercial cucumber fermentations brined with 100 mM $CaCl_2$, no acetic acid, and 6 mM potassium sorbate resulted in complete fermentations, but supported rising pH, microbially induced spoilage during long-term storage. Post-fermentation supplementation with 12 mM sodium benzoate, 10 mM fumaric acid, a combination of the two, or 10 mM fumaric acid and 2 mM AITC prevented microbial activity during long-term bulk storage.

KEYWORDS
fermentation, lactic acid bacteria (LAB), microbial inactivation, preservatives, vegetables

Practical Application: Several preservative-based strategies for stabilizing NaCl-free cucumber fermentation in a commercial production setting were developed, enabling the implementation of a processing technology that reduces wastewater volumes and environmental impact.

1 | INTRODUCTION

Commercial fermentation and long-term storage of fermented cucumbers typically utilizes a sodium chloride (NaCl) cover brine that equilibrates with the fruit at 1–2 M (5.8–11.6%). Sodium chloride induces a selection of halotolerant microbes like *Lactiplantibacillus plantarum* to perform fermentation (Pérez-Díaz et al., 2017, Pérez-Díaz et al., 2020) and protects the fermented fruit from spoilage-associated secondary fermentation (Breidt et al, 2013; Franco et al., 2012; Johanningsmeier et al., 2012; Kim & Breidt, 2013).

While pickling generates over half a billion dollars in the US economy (Pickle Packers International), cucumber fermentation at the commercial scale has significant environmental impacts, primarily through the effects of sodium and chloride in effluent wastewater, the energy cost of wastewater treatment, and the production of thousands of tons of salty sludge as solid waste on an annual basis (McFeeters and Pérez-Díaz, 2010; Pérez-Díaz et al., 2015).

To reduce the total amount of sodium and chlorides released as a result of production, researchers demonstrated the viability of replacing the NaCl cover brine typically used in cucumber fermentation with a calcium chloride ($CaCl_2$) cover brine at a much lower concentration (100 mM; McFeeters & Pérez-Díaz, 2010; Pérez-Díaz et al., 2015). $CaCl_2$ is already commonly employed in cucumber fermentation as an additive to maintain tissue firmness during fermentation and long-term bulk storage (Franco et al., 2016; McFeeters & Fleming, 1990).

While $CaCl_2$ brine substitution could potentially reduce chloride and consequently sodium deposition from commercial production by 60–80%, there are significant barriers to adoption of NaCl-free fermentation, combined with long-term bulk storage, as a new industry practice. In multiple commercial-scale trials, primary fermentation in experimental tanks packed with 100 mM (1.1%) $CaCl_2$ and 6 mM (0.09%) potassium sorbate cover brine proceeded comparably to fermentations in control tanks in which cucumbers were brined with 1 M NaCl (Pérez-Díaz et al., 2015). However, lactic acid levels declined in the experimental tanks following fermentation, and chemical indicators of spoilage such as increases in acetic acid and formation of propanol, propionic acid, and butyric acid were detected along with a rise in pH. Microbiological analysis of tanks with rising pH values detected lactic acid bacteria and yeasts associated with spoilage.

Preservatives commonly used in pickling may be a means to improve the microbial stability during the bulk storage phase of commercially fermented cucumbers brined with $CaCl_2$. Sodium benzoate (6.9 mM) was previously shown as an effective preservative for stabilizing low or no salt fermentations (Fleming et al., 1996), and potassium sorbate represses respiring yeasts and molds in commercial fermentations conducted in NaCl cover bri (Etchells et al., 1961; Stratford et al., 2020). Potassium sorbate additionally enabled the completion of cucumber fermentations in the absence of NaCl when added to 6.0 mM in open top tanks (Pérez-Díaz et al., 2015). The dissociation constants for both preservatives, potassium sorbate (4.75) and sodium benzoate (4.08), make them effective in fermentations within the pH range where most spoilage associated metabolic activity occurs (Franco et al., 2012). Earlier studies also indicated that preservatives such as benzoic acid, fumaric acid, and allyl-isothiocyanate (AITC) were effective in suppressing the indigenous microbiota of acidified cucumbers when used in adequate concentrations and thereby maintain pH (Pérez-Díaz, 2011; Pérez-Díaz & McFeeters, 2008, 2010). Therefore, this study evaluates the effects of these preservatives to maintain microbial stability throughout bulk storage of cucumbers fermented without NaCl. We hypothesize that the combined effects of selected preservatives may be able to extend the bulk storage life of fermented cucumbers in a $CaCl_2$ cover brine.

2 | MATERIALS AND METHODS

2.1 | Inhibition of microbes during long-term storage (up to 50 days) of laboratory-scale cucumber fermentations brined with $CaCl_2$ and no preservative ($n = 2$) potassium sorbate ($n = 2$) or sodium benzoate ($n = 2$)

All fermentations were subjected to air purging to mimic the conditions in the commercial process. The laboratory-scale cucumber fermentations were conducted in a BioFlo110 Modular Benchtop Fermentor (New Brunswick Scientific, Co., Edison, NJ, USA) as described by Franco & Pérez-Díaz (2012a). Briefly, the fermentation cover brine (1.5 kg) contained enough anhydrous $CaCl_2$ (Brenntag North America, Durham, NC, USA) and acetic acid as vinegar (20%, Fleischmann's Vinegar Company Inc., Cerritos, CA, USA) to equilibrate at 100.0 and 25.0 mM, respectively. The fermentation substrate was size 3A cucumbers diced into ¼″ cubes (1.5 kg). All the fermentations were subjected to air purging at a rate of 5 mL/min controlled by a Matheson PG-1000 (U001) flowmeter (Matheson Instruments, Montgomeryville, PA, USA). Fermentations free of preservatives were used as negative controls ($n = 2$). Experimental treatments included the supplementation of fermentation cover brines with 2.7 ± 0.3 mM (0.05%) potassium sorbate ($n = 2$) (Sigma-Aldrich, St. Louis, MO, USA) or 3.0 mM (0.04%) sodium benzoate ($n = 2$) (Sigma-Aldrich). Fermentation cover brine samples were aseptically collected via sampling tubes for microbiological and chemical analyses performed as described in sections 2.2 and 2.3. Fermentation cover brine samples were spun for 10 min at 15,294 rcf at room temperature (Eppendorf Centrifuge 5810R, Fisher Scientific, CA, USA) to remove cells and particulate, so that clarified supernatants could be used for chemical analyses.

2.2 | Microbiological analysis of fermentation cover brine samples collected from laboratory- and commercial-scale bioreactors

The collection of samples occurred using aseptic techniques. A 0.85% NaCl solution was the diluent for serial dilutions. Colony counts were from full strength and diluted fermentation brine samples derived from spiral plating (Autoplate 4000, Spiral Biotech, Norwood, MA). Colony counts for lactic acid bacteria derived from Lactobacilli deMan Rogosa and Sharpe agar plates (MRS, Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 10 mL/L of a 0.1% cycloheximide solution (OXOID, Ontario, Canada) to inhibit the growth of yeasts. The incubation of MRS plates occurred in a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, MI) at 30°C for 48 h. Colony counts for yeasts came from Yeast and Mold agar plates (YMA, Becton Dickinson and Co.) supplemented with 0.01% chloramphenicol (Sigma-Aldrich) and 0.01% chlortetracycline (Sigma-Aldrich) to inhibit bacterial growth. The aerobic incubation of YMA plates occurred at 30°C for 48 h. The enumeration of colonies from agar plates occurred using a Flash & Go Automated Colony counter (cat. 90006010, IUL Instruments, Barcelona, Spain).

2.3 | Chemical analysis of fermentation cover brine samples collected from laboratory- and commercial-scale bioreactors

Concentrations of organic acids and sugars were measured by HPLC analysis using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) for the separation of components (McFeeters & Barish, 2003). The column temperature was held at 65°C for laboratory fermentation samples and 37°C for commercial fermentation samples. Sample components were eluted with 0.03 N sulfuric acid at a flow rate of 0.6 mL/min. The higher column temperature was used to separate butyric and propionic acids. A Thermo Separations UV6000 diode array detector (Spectra System Thermo Scientific, Waltham, MA, USA) set to collect data at 210 nm was used to quantify malic, lactic, acetic, benzoic, fumaric, sorbic, propionic, and butyric acids. A Waters model 410 refractive index detector (Waters Corp., Millipore Corp., Billerica, MA, USA) connected in series with the diode array detector was used to measure glucose, fructose, ethanol, and propanol. External standardization of the detectors was done using eight concentrations of the standard compounds. Full-strength cover brine supernatants or the 10-fold diluted counterparts were injected in the HPLC system by an autosampler. Samples were diluted when the expected concentration of metabolites were outside of the calibration curve ranging from 0.50 to 100.0 mM for most sugars and acids, except for benzoic acid, fumaric acid, and sorbic acid for which the maximum value of the curve was 10.0 mM. When needed, samples were diluted with the mobile phase.

2.4 | Effects of benzoic and sorbic acids, and zinc sulfate on the viability of the spoilage associated yeasts *Issatchenkia occidentalis, Pichia manshurica*, and *Zygosaccharomyces globiformis* in fermented cucumber juice used as a model system

Fermented cucumber juice medium (FCJM) was made from cucumber fermentations brined with 100 mM anhydrous $CaCl_2$ (Brenntag North America) and 25 mM acetic acid added as vinegar (20%, Fleischmann's Vinegar Company Inc., Cerritos, CA, USA) as described by Franco and Pérez-Díaz (2013). Two independent lots of fermented cucumbers were used to prepare FCJM in parallel. The fermented cucumbers (pH 3.2 ± 0.3) were blended with the fermentation cover brine in equal weights in a sterilized vessel (Waring Commercial Blender 700S, Torrington, CT, USA) and frozen until used. The fermented cucumber slurries were defrosted and spun by centrifugation at 12,880 rcf for 10 min at 21°C (Eppendorf Centrifuge 5810R) to produce the clarified supernatant, FCJM. FCJM was filter sterilized through a 0.22-μm membrane prior to experimentation. Sterile solutions of sodium benzoate and potassium sorbate were filter sterilized and added to FCJM to produce concentrations of 5.0, 10.0, and 15.0 mM. Such concentrations are within the levels currently used in the pickling industry in acidified and fermented products (Pérez-Díaz and McFeeters, 2008). The FCJM pH was at 3.3 ± 0.2 after supplementation remaining below the dissociation constant for both preservatives and maintaining the efficacy as antimicrobials.

The effect of zinc chloride was also evaluated as it has been recommended for microbial inhibition in fermented olive (Bautista-Gallego et al., 2011). Several zinc compounds, including zinc sulfate, have antifungal activity (Savi et al., 2013). Zinc sulfate was added to individual aliquots of FCJM at concentrations of 0.62, 3.1, and 6.2 mM.

The yeast strains used for this experiment were *I. occidentalis* Y0089, Y0090, Y0093, and Y0094, and *P. manshurica* Y0088, Y0091, Y0092, and Y0098 all of which were isolated from commercial fermentations undergoing spoilage (Franco et al., 2012). Four strains of *Zygosaccaromyces globiformis* were also included in the experiment. The *Z. globiformis* strains Y0068, Y0069, Y0070, and Y0071 were isolated from spoiled sweet cucumbers by Bell and Etchells in 1952. All strains used in this experiment are maintained in the culture collection of the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) Food Science & Market Quality and Handling Research Unit (FSMQHRU) located in Raleigh, NC, USA. Three inocula of a single yeast species were transferred to FCJM individually. The inocula contained four strains of *I. occidentalis* (Y0089, Y0090, Y0093, and Y0094), *P. manshurica* (Y0088, Y0091, Y0092, and Y0098), or *Z. globiformis* (Y0068, Y0069, Y0070, and Y0071).

To produce inocula, the yeast cultures were transferred from frozen stocks into Yeast and Mold (YM) broth (Difco, Becton and Dickinson Company, Sparks, USA) and incubated at 30°C under static aerobic conditions for 48 h. Aliquots of 15 ml of each culture were spun at 4500 *g* for 5 min (Eppendorf Centrifuge 5810R, Fisher Scientific). Pellets were washed with the same volume of sterile 0.85% NaCl solution and spun prior to re-suspension in 0.5 mL of the sterile saline solution. Suspensions of each strain belonging to a single species were mixed in equal volumes to produce the inocula. The yeasts were inoculated into 4.5 mL of FCJM to 6.81 ± 0.16 Log CFU/mL (approximately a 0.1 mL inoculation) and incubated at 30°C under static aerobic conditions. Both the control, native FCJM and the experimental, supplemented FCJM were inoculated at this point. Two independent lots of FCJM were inoculated with an independent inoculum of each species prepared as described above from independent cultures. The yeast colony counts from the remaining cultures in YM broth and the initial time point in FCJM were confirmed by enumeration on YM agar plates. The yeast colony counts in the experimental and control FCJM were also determined on days 4, 7, and 14. Culture dilutions were done with saline solution. An Autoplate 4000 spiral plater was used for the inoculation of the YM agar plates, which were incubated aerobically at 30°C for 48 h. Colonies from agar plates were enumerated using a Flash & Go Automated Colony counter. This technique had a limit of detection of 1.3 Log CFU/mL.

The pH of all yeast cultures in FCJM was measured on days 0, 4, 7, and 14 using an Accumet Research 25 pH meter (Accumet, Fisher Scientific, Waltham, MA, USA) equipped with a Gel-Filled Pencil-Thin pH Combination Electrode (Accumet, Fisher Scientific) and calibrated with certified standards of pH 2.00, 4.00, and 7.00 (Fisher Scientific).

2.5 | Effects of benzoic, sorbic, and fumaric acids on the viability of spoilage-associated lactic acid bacteria of the *Lentilactobacillus* spp. in cucumber juice used as a model system

Four unique strains of *Le. buchneri*, ATCC 4005 (LA0030), LA1147, LA1149, and LA1155, and *Le. parafarraginis* strain LA1153 were used as spoilage LAB indicator strains. A mixture of strains belonging to the *L. plantarum* phylogenetic cluster, including *L. plantarum* LA0098 and LA0089 and *L. pentosus* LA0445, was used as a positive control.

L. pentosus LA0445 is susceptible to 20 mM (0.23%) fumaric acid (Pérez-Díaz, 2011). McFeeters and Pérez-Díaz (2008) demonstrated that lactic acid bacteria intrinsic to cucumber fermentations are susceptible to sodium benzoate. The strain LA0098 is widely known as ATCC14917. LA0089 is known as NCDO82, a probiotic *L. plantarum* strain. All strains were obtained from the USDA-ARS-FSMQHRU culture collection located in Raleigh, NC, USA. Frozen stocks in MRS broth with 10% glycerol were streaked onto MRS agar plates and incubated at 30°C. Incubation of the *L. plantarum* and *L. pentosus* cultures occurred for 48 h under aerobiosis. The cultures of the spoilage LAB, *Le. buchneri* and *Le. parafarraginis*, were incubated anaerobically for 5–7 days.

The cultures were transferred from MRS plates to cucumber juice medium (CJM). The CJM was prepared with juice pressed from size 2B pickling cucumbers (31.75–38.1 mm in length and a diameter of 31–38 mm) using a Juiceman Jr. JM-1C (Salton, Beachwood, OH, USA) automatic juice extractor. The fresh juice was sieved through 5 layers of 100% cotton cheesecloth (grade #90, 44×36 threads/inch, Cartridge Plus, Inc., Riva, MD, USA) to remove large particulate. Deionized water was added to the fresh cucumber juice (CJ) in a 50:50 ratio (w:w), prior to the addition of 100 mM anhydrous $CaCl_2$. The solution was centrifuged in 400 mL increments at 9,500×*g* for 30 min at 4°C (RC-3B Refrigerated Centrifuge, Sorvall Instruments, Thermo-Fisher, Waltham, MA, USA). CJ supernatant was sterile filtered through a 0.2-µm membrane (Nalgene FAST PES Daigger, Vernon Hills, IL, USA). The CJ growth medium (CJM) was aseptically transferred into sterile conical-bottomed tubes in 10-mL aliquots prior to use. The pH of the CJM was not adjusted (pH = 5.8). Each LAB strain was independently cultivated in duplicate in CJM for 4 days at 30°C under anaerobic conditions.

FCJM was used for experimentation to evaluate the effect of preservatives on LAB. The FCJM was prepared as described above. Three preservatives, potassium sorbate, sodium benzoate, and fumaric acid, were used in this experiment. As stated above, the preservatives potassium sorbate and sodium benzoate are commonly used in commercial-scale pickling. A concentration of 20 mM fumaric acid was known to effectively prevent growth of lactobacilli in acidified cucumbers at pH 3.3 (Pérez-Díaz, 2011). One batch of FCJM was partitioned out for the varying levels of preservative additions. Sodium benzoate and potassium sorbate test media contained concentrations of 5-, 10- or 15-mM, and FCJM with no preservative addition was used as a control. Fumaric acid was independently added to test media at 5-, 10-, 15-, and 20-mM concentrations. Media containing fumaric acid was readjusted to 3.3 ± 0.01 with 5N NaOH after addition of fumaric acid. Each sterile-filtered FCJM was aseptically transferred in 10-mL volumes to sterile 15-mL conical-bottom tubes preceding inoculation.

FCJM was individually inoculated with each independent spoilage culture of *Lentilactobacillus* spp. or the *L. plantarum* cluster mixture to 5.6 ± 0.5 Log CFU/mL and incubated anaerobically at 30°C. Duplicate FCJM cultures were prepared with one batch of medium and independently cultivated cells per species. Samples were taken at days 4 and 7 for plating on MRS media using the simplified agar plate method (Jett et al., 1997) with a limit of detection of 1.0 Log CFU/mL. Media pH was measured after 30 days of incubation as an indicator of long-term stability.

2.6 | Evaluation of the long-term microbial stability of commercial cucumber fermentations brined with 100 mM $CaCl_2$ and stabilized with preservatives post fermentation

Commercial-scale cucumber fermentations were brined with 100 mM anhydrous $CaCl_2$, 6 mM potassium sorbate (Brenntag North America), and a starter culture of *L. pentosus* LA0445 to 5 Log CFU/mL. Commercial-scale fermentations were performed as described by Pérez-Díaz et al. (2015) in fiberglass open top tanks of 12,500 L. The starter culture was prepared as described by Pérez-Díaz and McFeeters (2011). Fresh cucumbers of various sizes, pieces and nubs, including those with physical damage and off-shoots of fresh-pack production operations, were packed in the fermentation tanks. These fermentations are used for relish production, and are the most susceptible to microbial spoilage during long-term storage. Air purging was applied at an estimated rate of 7 ± 5 L/min for 2 to 3 h twice a day for the first 7 days and on day 9, 11, and 13. Preservatives were added to the fermentation vessels after 14 days of packing. Duplicate tanks were supplemented with no preservatives (negative controls), 10.0 mM fumaric acid (Brenntag North America), 10.0 mM fumaric acid and 2 mM AITC (Sigma-Aldrich), 10.0 mM fumaric acid and 12.0 mM sodium benzoate (Brenntag North America), or 12.0 mM sodium benzoate. AITC was added at this stage as it was known to aid in preventing growth of spoilage yeasts in acidified cucumbers (Pérez-Díaz & McFeeters, 2010). Two positive control tanks that represented typical commercial production methods were brined with 1.06 M NaCl, 1.0 mM potassium sorbate, and 40.0 mM anhydrous $CaCl_2$ and were not inoculated with starter culture. Acetic acid was not added to the control or the experimental tanks to increase microbiological stability by eliminating an energy source for spoilage-associated microbes (Franco & Pérez-Díaz, 2012b). Fermentation cover brines were circulated, and samples were collected periodically for

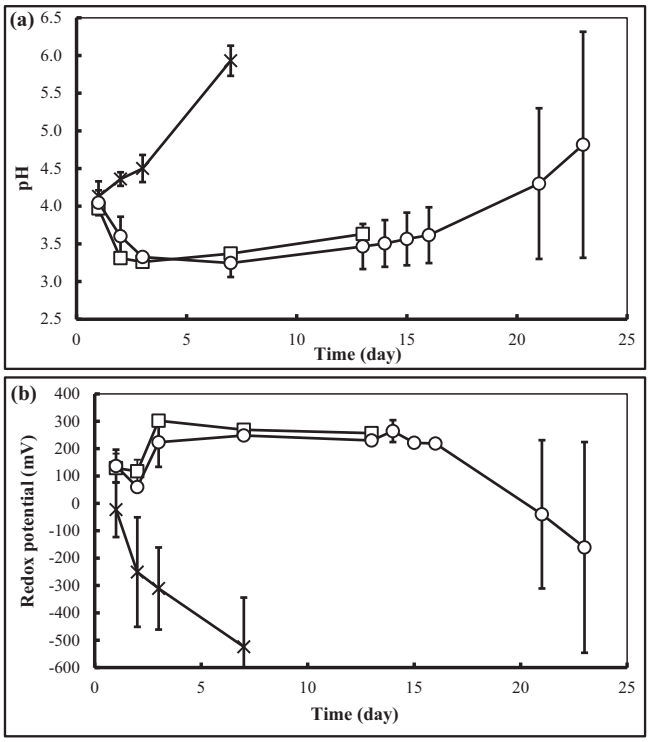

FIGURE 1 pH (a) and redox potential (b) trends from laboratory-scale fermentations brined with 100 mM $CaCl_2$ and 25 mM acetic acid. Cucumber fermentations were supplemented with no preservative (×, n = 2), 3 mM sodium benzoate (□, n = 2), or 3 mM potassium sorbate (○, n = 2). Error bars indicate standard error of the mean from independent duplicates

341 days using aseptic techniques for microbiological and chemical analyses, which were performed as described above.

3 | RESULTS AND DISCUSSION

3.1 | Effect of sub-lethal concentrations (3.0 mM) of sodium benzoate and potassium sorbate on the microbial stability of cucumber fermentations brined with $CaCl_2$ during long-term storage

In the absence of preservatives, the pH increased within the first few days of fermentation (Figure 1), abundant yeast and mold growth was observed on the surface of the fermentation brines, and the contents turned malodorous within the first week of fermentation (data not shown). Such preservative-free low-salt fermentations, containing no NaCl and 100 mM $CaCl_2$ ($n = 2$), produced ethanol at concentrations greater than 100 mM in the first 3 weeks of incubation, which indicated yeast contamination. The observation made from such low-salt fermentations that remained non-supplemented resembled those described by Franco et al. (2012) and Guillou et al. (1992) for spoilage-associated secondary fermentation. These fermentations were stopped after a week of incubation given the observed microbial instability, increases in pH above the threshold for safety (>4.6), and the development of malodors.

Supplementation of low-salt fermentations with 3 mM potassium sorbate or sodium benzoate enabled a decline in the cover brine pH (Figure 1a), maintained an oxidized system (Figure 1b), and delayed secondary fermentation (Figure 2a,b). The supplementation of fermentations with preservatives reduced ethanol production to less than 10 or 70 mM, as with potassium sorbate ($n = 2$) and sodium benzoate ($n = 2$), respectively. While sugars were converted to lactic acid (<70.0 mM; $n = 4$; Figure 2a) and acetic acid (<15.0 mM; $n = 4$; Figure 2b) in all treatments, a steady decline in acid concentration was observed in all fermentations as a function of time during long-term incubation (after 10 days) (Figure 2). The data indicate that the lactobacilli reached the peak of the population and entered death phase within the first 5 days of the fermentations (Figure 3a), which corresponds to peak production of lactic acid and acetic acid. The disappearance of the acids coincides with the growth and/or presence of abundant yeast populations and the revival of the population of lactobacilli (Figure 3b).

The ability of the spoilage-associated yeasts *I. occidentalis* and *P. manshurica* to utilize lactic and acetic acids in cucumber juice medium and fermented cucumber juice under aerobiosis was previously documented by Franco and Pérez-Díaz (2012b). The presence of yeasts in fermentations at pH 3.3 was found to enhance the survival of bacteria capable of producing propionic and butyric acids such as *Enterobacter cloacae* and *Clostridium bifermentans* (Franco & Pérez-Díaz, 2013). Thus, it is likely that the native populations of yeast in the fermentations degraded the acids, which resulted in a substantial increase in pH and the chemical reduction of the medium (Figure 1) and/or potentiated the growth of spoilage lactobacilli. The pH of the fermentations remained below that expected for fresh cucumbers, (6.05 ± 0.15, Lu et al., 2002) suggesting that the changes observed for such parameter was strictly related to the disappearance of the acids rather than the production of a base. An increment in pH to 4.6 was observed in the potassium sorbate treatment (Figure 1).

The initial increment in colony counts for presumptive lactobacilli was reverted around the time in which lactic and acetic acids started to disappear and the yeast populations were at maximum densities (Figures 2–4). A revival of the lactobacilli population was observed in most instances after acid utilization initiated (Figures 2–4). Colony morphologies observed from MRS agar plates during the revival of the population were like those associated

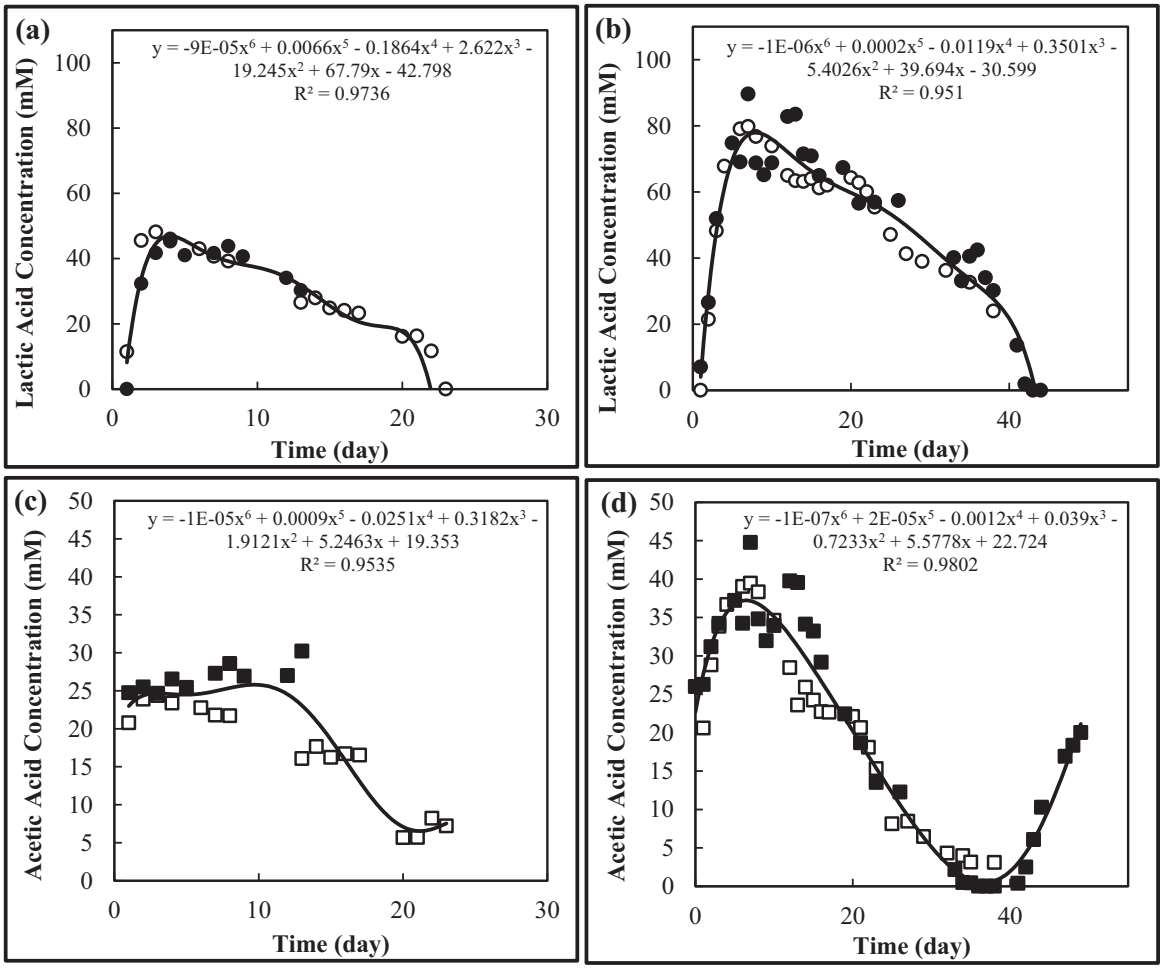

FIGURE 2 Lactic (○, ●) and acetic (□, ■) acid concentrations measured in low-salt cucumber fermentations supplemented with 3 mM sodium benzoate (a), (c) or 3 mM potassium sorbate (b), (d) in 3kg bioreactors subjected to air purging. Independent duplicates are represented in each panel with open and closed symbols. The average trend is represented by the solid black line in each panel

with the spoilage bacterium, *Le. buchneri* by Franco et al. (2012) and Daughtry et al. (2018). *Le. buchneri* is known to convert lactic acid into 1, 2-propanediol and acetic acid at a 1–0.5 M ratio each and traces of ethanol at acidic pH via the 1, 2-propanediol oxidoreductase in the absence of other electron acceptors (Stefanie et al., 2001). However, 1, 2-propanediol does not accumulate during secondary fermentation of cucumber due to rapid conversion to propionic acid and propanol by other microbes that are naturally present, such as *Lentilactobacillus rapi* (Johanningsmeier et al., 2012; Johanningsmeier & McFeeters, 2013). Formation of propionic acid was observed in one out of two air-purged laboratory-scale fermentations free of preservatives (controls) to 6.9 mM after 14 days of incubation. In control bioreactors with no preservatives, spoilage began within a few days after glucose and fructose reached undetectable levels (day 10), as evidenced by elevated pH (Figure 1) and a decrease in redox potential (Figure 1). Nominal lactic acid production (<37.0 mM) was measured in control fermentations and acetic acid disappeared in 8–12 days (data not shown).

In most treatments, butyric acid was detected at concentrations of 7.0–17.0 mM as levels of lactic and acetic acid decreased toward the end point of the fermentations (Figure 4). Butyric acid did not appear in cucumber fermentations treated with potassium sorbate until the concentration of the preservative decreased below the limit of detection (Figure 4). Butyric acid production, between 6.0 and 12.0 mM, occasionally occurs in commercial-scale fermentations at pH above 4.0 (Franco et al., 2012).

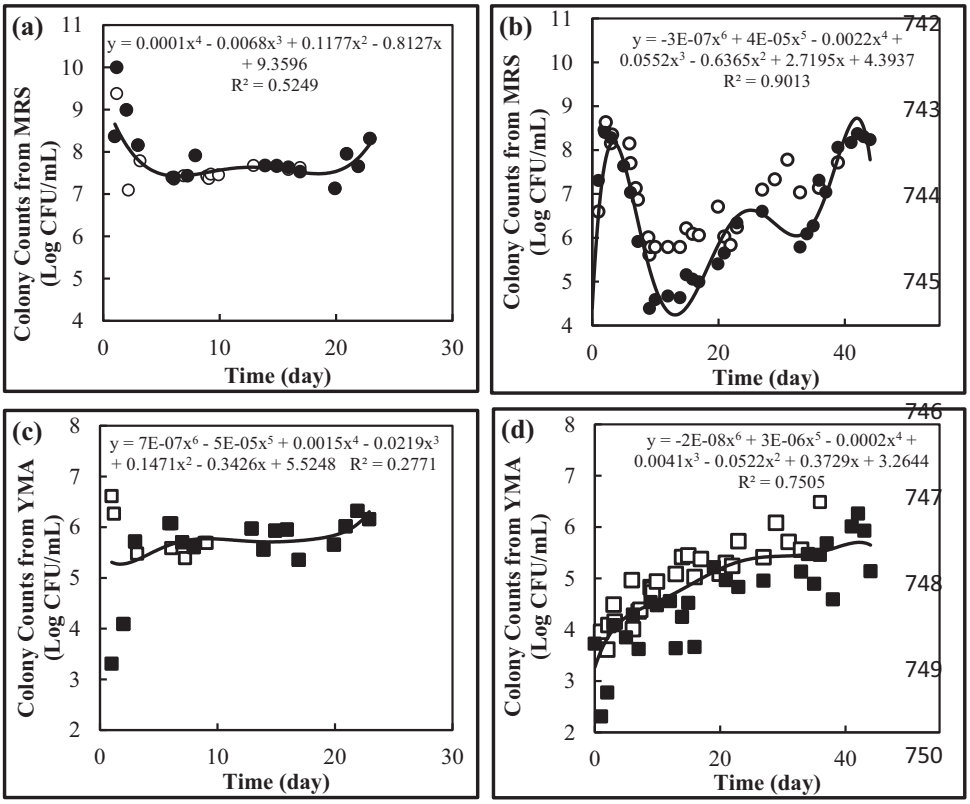

FIGURE 3 Colony counts for presumptive lactobacilli from MRS agar (○, ●) and yeasts from YMA agar (□, ■) in low-salt cucumber fermentations supplemented with 3 mM sodium benzoate (a), (c), or 3 mM potassium sorbate (b), (d) in 3 kg bioreactors subjected to air purging. Independent duplicates are represented in each panel with open and closed symbols. The average trend is represented by a solid black line

3.2 | Effects of potassium sorbate, sodium benzoate, and zinc sulfate on the viability of spoilage-associated yeasts such as *Issatchenkia occidentalis, Pichia manshurica*, and *Zygosaccharomyces globiformis*

Data from a previous spoilage event in commercial cucumber fermentations brined with NaCl indicate that colonization by *I. occidentalis* and *P. manshurica* has a substantial role in triggering microbial instability (Franco et al., 2012). Yeasts are also present during spoilage of fermented cucumbers brined with $CaCl_2$ (Pérez-Díaz et al., 2015). Concentrations of 6.0 mM potassium sorbate or sodium benzoate enabled the completion of fermentation in the absence of NaCl in commercial-scale trials but did not prevent colonization by yeasts during long-term storage (Pérez-Díaz et al., 2015). Sorbic acid was fully degraded after 21 days of fermentation in such commercial trials. Such sorbic acid degradation in cucumber fermentations was consistent with that documented by others (Alderton & Lewis, 1958; Costilow et al., 1957, Etchells et al., 1961).

Z. globiformis presents a significant risk as a spoilage microbe considering the tolerance to low pH, high salt and sugar, and to several common preservatives, including potassium sorbate concentrations at less than 6.0 mM (Costilow et al., 1955; Martorell et al., 2007). While *Zygosaccharomyces* has not been implicated in the spoilage of cucumbers fermented for hamburger dill chips, it was isolated from spoiled sweet pickles (Bell & Etchells, 1952). Previous research identified cinnamaldehyde, AITC, potassium sorbate, and sodium benzoate as effective preservatives against spoilage caused by *Zygosaccharmyces* spp. (Pérez-Díaz & McFeeters, 2010, 2011). The suppressive effect of the four preservatives against *Zygosaccharomyces* was tested in FCJM at an acidic pH (3.3 ± 0.2).

In this study, sodium benzoate and potassium sorbate were both effective in suppressing growth of *I. occidentalis* and *P. manshurica* mixtures at concentrations of 10.0 and 15.0 mM (Figure 5a–d). Growth of both yeast mixtures continued at 5.0 mM concentration with both preservatives, which suggests that the effective concentration of both preservatives is between 5.0 and 10.0 mM. Similarly, growth of *Z. globiformis* was suppressed by potassium sorbate at 10.0 mM, but sodium benzoate was ineffective at all the concentrations tested (Figure 5e–f). These observations are in line with results obtained by Costilow et al.

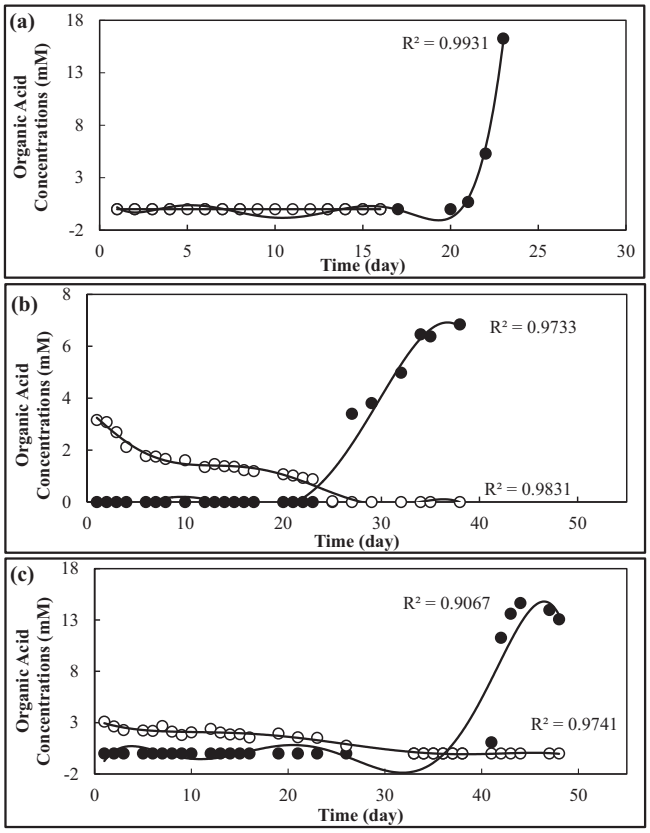

FIGURE 4 Measurement of butyric acid (●) and potassium sorbate (○) levels in cucumber fermentations brined with 100.0 mM $CaCl_2$, 25.0 mM acetic acid, and 3.0 mM sodium benzoate (a) or 3.0 mM potassium sorbate (b), (c). A single fermentation is represented in each panel as the metabolites were not formed in all fermentation vessels

(1955, 1957) and Etchells et al. (1961). Given that the yeasts were inoculated to the maximum attainable cell density (6.8 ± 0.2 Log CFU/mL) to observe die off, no changes in pH were measured in the supplemented and non-supplemented FCJM as a function of time (data not shown). Contrary to the case of fermented olives, the suppressive effect of zinc sulfate against the three oxidative yeasts species used for experimentation was minimal (data not shown).

The observation that a minimum of 10.0 mM potassium sorbate or sodium benzoate is needed to suppress the growth of spoilage-associated yeasts explains the development of spoilage in the laboratory-scale cucumber fermentations described above and provides target concentrations for the stabilization of commercial NaCl-free cucumber fermentations.

3.3 | Inhibition of *Le. buchneri* and *Le. parafarraginis* in FCJM by preservatives

Several strains of *Le. buchneri* and one of *Le. parafarraginis* were previously isolated from commercial- and laboratory-scale cucumber fermentations that spoiled due to secondary fermentations with rising pH (Franco et al., 2012; Johanningsmeier et al., 2012). Both species catabolize lactic acid in fermented cucumbers after the primary fermentation proceeded with a corresponding increase in pH under aerobiosis and anaerobiosis (Johanningsmeier & McFeeters, 2013). Consistent with early studies (Costilow et al., 1955; McFeeters & Pérez-Díaz, 2008), potassium sorbate was non-inhibitory to the lactobacilli tested in a saturated solution (data not shown). All six strains of spoilage lactobacilli were able to grow (~ 1 Log CFU/mL) in FCJM in the absence of preservatives, whereas the lowest level (5.0 mM) of fumaric acid reduced cell densities by at least 0.5 Log CFU/mL (Figure 6). Supplementation of FCJM with 5.0 mM sodium benzoate was insufficient to reduce the cell density of *Le. buchneri* LA1147 and LA1155 (Figure 7). Addition of 10.0 mM sodium benzoate or fumaric acid to FCJM prevented pH rises induced by the *Le. buchneri* strains and *Le. parafarraginis* (Figure 8). Bactericidal effects were observed with increasing concentrations (Figures 6 and 7), but strains varied in their resistance to each of the preservatives ($p < 0.05$). All strains of *Le. buchneri* and *Le. parafarraginis* were more resistant to preservatives than the *L. plantarum* cluster, showing that the preservatives would need to be added after primary fermentation proceeds. While some *Le. buchneri* strains were inactivated at low concentrations of fumaric acid, this preservative was less effective against *Le. parafarraginis* strain LA1153 than sodium benzoate (Figures 7 and 8). Among the *Le. buchneri* strains, LA1155 was the most resistant to both preservatives (Figures 7 and 8). Nonetheless, 15.0 mM sodium benzoate or 20.0 mM fumaric acid induced a 2 to 5 Log CFU/mL reduction in the *Le. buchneri* and *Le. parafarraginis* cell densities. These concentrations are consistent with those that were effective for stabilizing NaCl-free acidified cucumbers for long-term bulk storage (Pérez-Díaz & McFeeters, 2008, 2010). Furthermore, 10.0 mM of either preservative was successful in maintaining pH stability of FCJM in a 30-day challenge with the spoilage lactobacilli (Figure 8). Therefore, either fumaric acid or sodium benzoate could be incorporated in salt-free cucumber fermentations after fermentation is complete to prevent secondary fermentation by spoilage-associated strains of *Le. buchneri* and *Le. parafarraginis*.

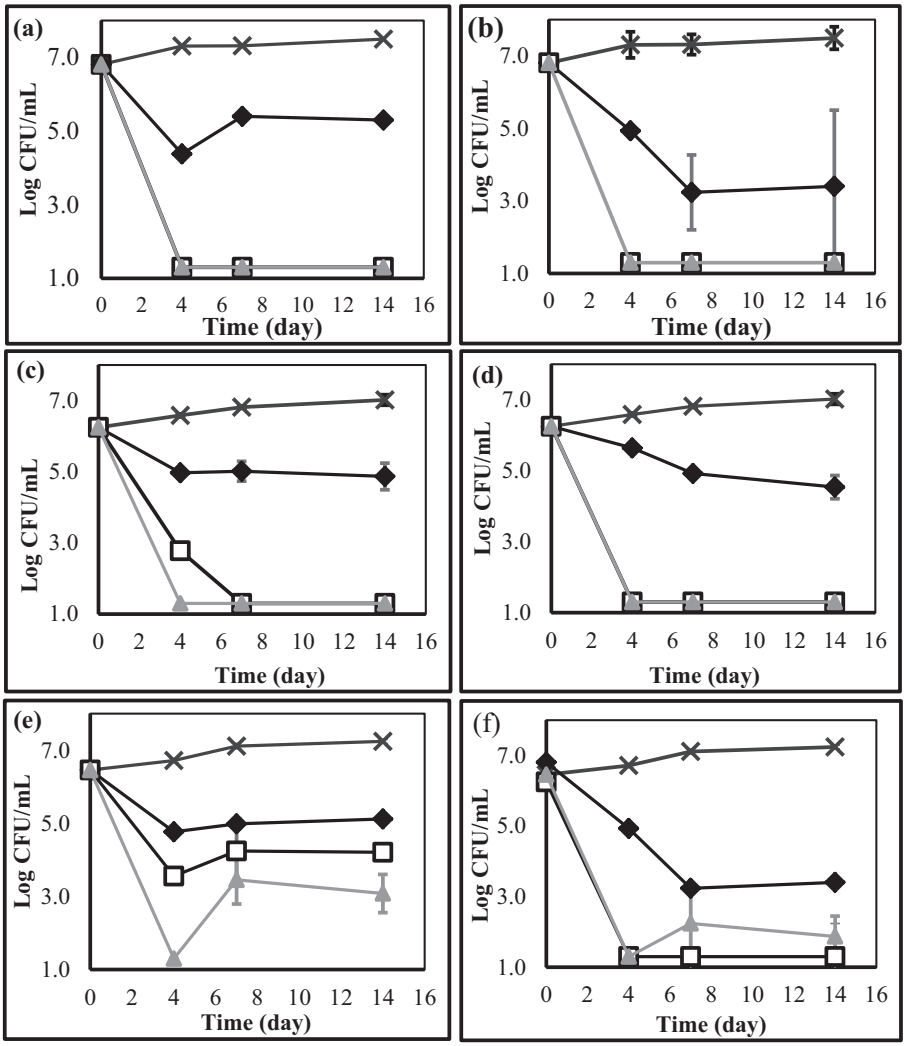

FIGURE 5 Growth of three oxidative yeasts, *Issatchenkia occidentalis* (a) and (b), *Pichia manshurica* (c) and (d), and *Zygosaccharomyces globiformis* (e) and (f) in fermented cucumber medium supplemented with 0 (×), 5.0 (◆), 10.0 (□), or 15.0 (▲) mM sodium benzoate (a), (c), and (e) or potassium sorbate (b), (d), and (f). Error bars represent the standard error of the mean from independent duplicates

3.4 | Stabilization of commercial cucumber fermentations brined with 100 mM $CaCl_2$ by supplementing with preservatives prior to long-term bulk storage

In contrast to experiments conducted at laboratory scale, acetic acid was not added in commercial-scale fermentation cover brines given the emerging evidence of its utilization by spoilage yeasts in this study and that published by Franco et al. (2012). It was the expectation that microbial stability of the NaCl-free fermentation would be enhanced in the absence of acetic acid, an energy source for spoilage microbes. Potassium sorbate (6 mM) was added in all commercial fermentations to enable the complete utilization of sugars by the fermentative lactobacilli as described by Pérez-Díaz et al. (2015) and in line with the results obtained in the two experiments described above. Anticipating that sorbic acid would disappear from the fermentation tanks as a function of time, secondary preservatives were added after the fermentations were completed (14 days) and prior to the development of spoilage. It was hypothesized that the elimination of acetic acid in the cover brine and the addition of preservatives following primary fermentation would improve microbial stability of the fermented cucumbers during long-term storage. In this trial, sodium benzoate, AITC, and fumaric acid were added in commercial fermentations given the observed effectiveness in inhibiting spoilage associated microbes in this study and in enabling microbial stability of acidified cucumbers (McFeeters & Pérez-Díaz, 2008; Pérez-Díaz & McFeeters, 2010).

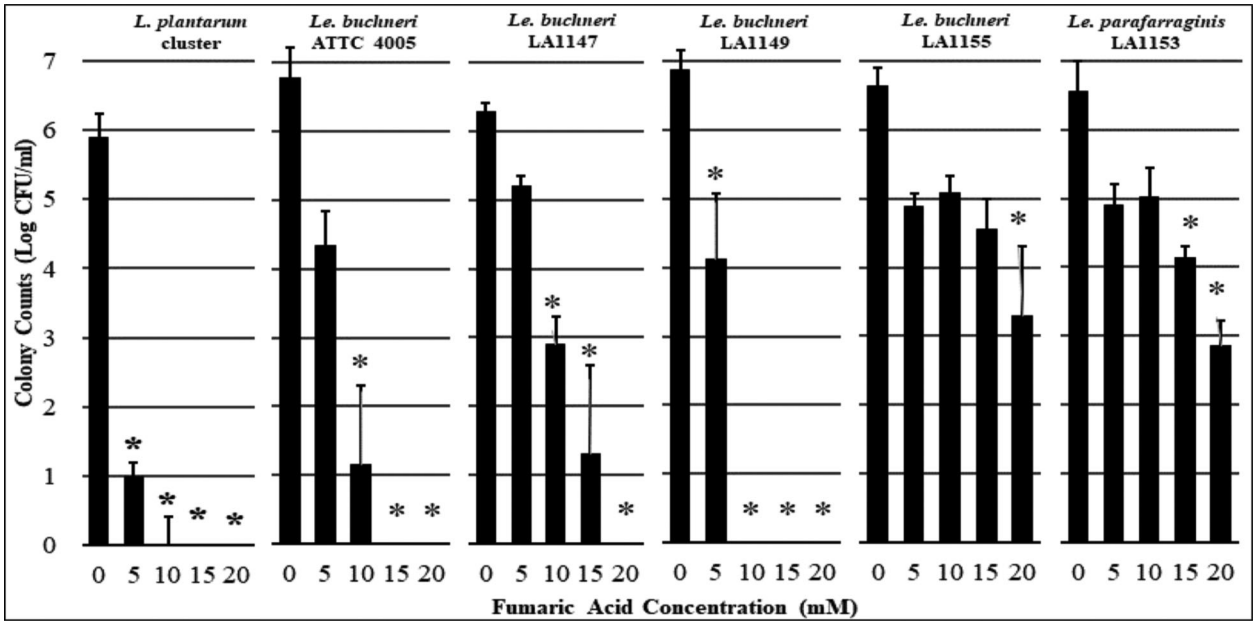

FIGURE 6 Inhibition of the *Lactiplantibacillus plantarum* cluster, the *Lentiplantibacillus buchneri* type strain (ATCC4005), and several strains of spoilage-associated LAB at varying levels of fumaric acid added to fermented cucumber juice medium (100.0 mM $CaCl_2$, pH 3.3, 30°C). Counts are taken 4 days after inoculation to 5.6 ± 0.5 Log CFU/mL. Error bars represent the standard error of the mean for individual treatments, conducted with independent duplicates. The limit of detection was 1.0 Log CFU/mL. Asterisks denote levels of fumaric acid that resulted in a significant reduction in microbial counts ($p < 0.05$) as determined by a Dunnett's multiple comparison of means test with the control set as the treatment without any preservative (0 mM) for each strain

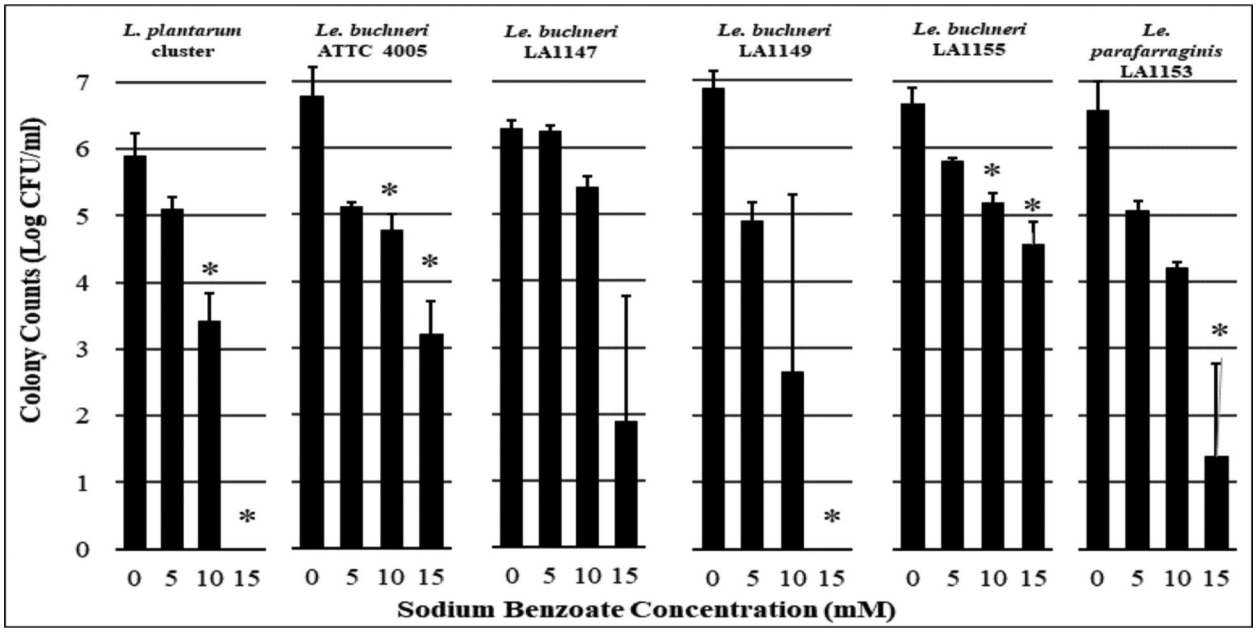

FIGURE 7 Inhibition of the *Lantiplantibacillus plantarum* cluster, the *Lentiplantibacillus buchneri* type strain (ATCC4005), and several strains of spoilage-associated lactobacilli at varying levels of sodium benzoate added to fermented cucumber juice medium (100 mM $CaCl_2$, pH 3.3, 30°C). Counts were taken 4 days after inoculation to 5.6 ± 0.5 Log CFU/mL. Error bars represent the standard error of the mean for individual treatments, conducted with independent duplicates. The limit of detection was 1.0 Log CFU/mL. Asterisks denote levels of sodium benzoate that resulted in a significant reduction in microbial counts ($p < 0.05$) as determined by a Dunnett's multiple comparison of means test with the control set as the treatment without any preservative (0 mM) for each strain

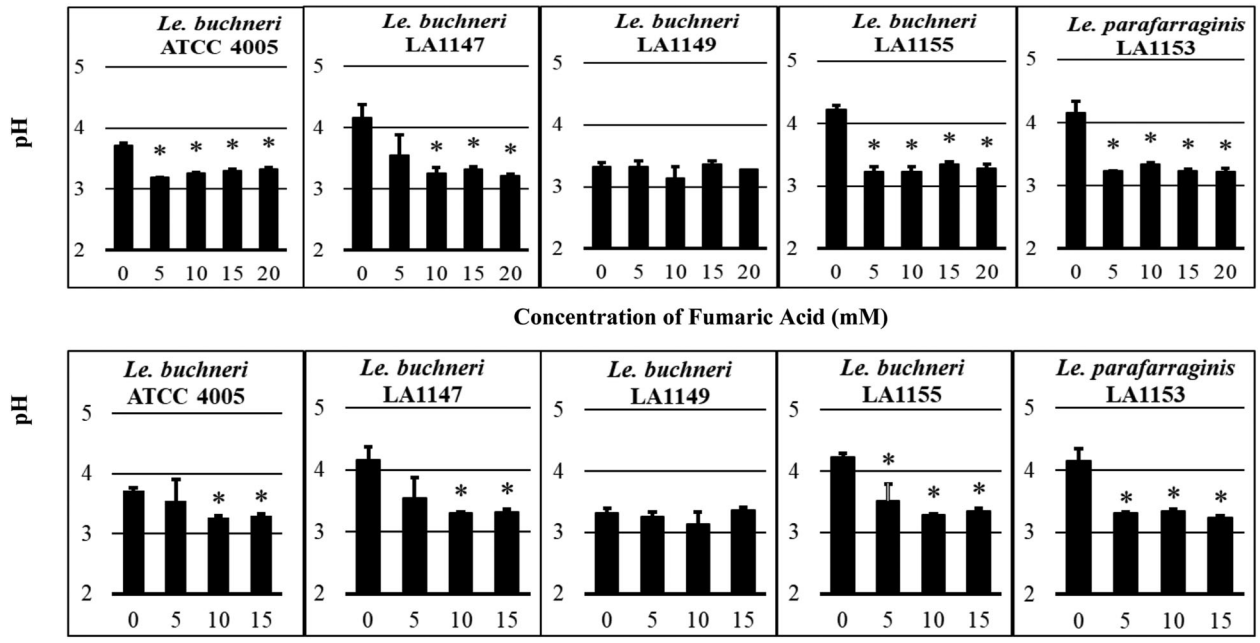

FIGURE 8 pH of fermented cucumber media (100 mM $CaCl_2$, pH 3.3) supplemented with sodium benzoate (top row) or fumaric acid (bottom row), inoculated with *Le. buchneri* or *Le. parafarraginis* spoilage cultures in duplicate and incubated at 30°C for 30 days. Asterisks denote levels of preservative that maintained a significantly lower pH ($p < 0.05$) compared with the control treatment without any preservative (0 mM) for each strain

Primary fermentation of cucumbers in NaCl and $CaCl_2$ brines proceeded as expected at the commercial scale, with the majority (~85%) of lactic acid production occurring in the first 28 (NaCl) or 14 days ($CaCl_2$), producing an average pH of 3.2 ± 0.1 and 3.3 ± 0.1 in NaCl- and $CaCl_2$-brined cucumbers, respectively. NaCl-brined cucumbers maintained a pH below 3.3 ± 0.1 for the remainder of the 341 days of bulk storage, with high levels of lactic acid (≥ 80 mM) (Figure 9a,b). Over the course of the trial, fermented cucumbers in $CaCl_2$ brine without preservatives underwent spoilage, with pH increasing to 4.2 ± 0.3, lactic acid trending toward undetectable levels and acetic acid levels continually increasing (Figure 9c). However, multiple preservative treatments were able to maintain fermented cucumbers in storage in $CaCl_2$ brine without indications of microbial spoilage. All preservative treatments were able to maintain a pH below 3.5 following the completion of fermentation, with two treatments, 10.0 mM fumaric acid with 0.0 or 2.0 mM AITC, maintaining a pH below 3.3 and levels of lactic and acetic acids comparable to NaCl brines throughout the 341 days trial (Figure 9). A horseradish note remained present in tanks treated with 2 mM AITC after months of storage in open top tanks. Typical chemical indicators of spoilage such as propionic acid and butyric acid were not detected in any cucumber fermentations brined with preservatives. Thus, the supplementation of low-salt fermentations with preservatives effectively stabilized microbial activity of stored pickles.

While preservative treatments maintained stable pH levels during the trial, yeasts were detected in all treatments that did not include sodium benzoate as a preservative, including the control NaCl brines. In some treatments, the spoilage yeasts *I. occidentalis* and *P. manshurica* were identified by morphology, but among these treatments, spoilage was only detected in the preservative-free $CaCl_2$-fermented cucumbers (Figure 10a). The colony counts for yeasts and molds remained below 4.0 Log CFU/mL, substantially lower than the maximum attainable cell density for such population in cucumber fermentations (6.0 Log CFU/mL) and the levels observed in laboratory scale bioreactors (Figure 3b). It is presumed that the growth of yeasts and molds was limited by the absence of acetic acid in the cover brines relative to that observed in laboratory-scale fermentations. It remains to be elucidated what levels of such population are needed to trigger spoilage in cucumber fermentations.

Sodium benzoate and fumaric acid appeared to effectively control the growth of lactobacilli in fermentation tanks, reducing the respective colony counts to below detectable levels (2.4 Log CFU/mL) several weeks before such populations were reduced to the same level in NaCl fermentation brines (Figure 10b). *Le. buchneri, Le. parafarraginis*, and the yeasts *I. occidentalis* and *P. manshurica* were putatively identified by morphology in $CaCl_2$ brines without preservatives, which later underwent spoilage

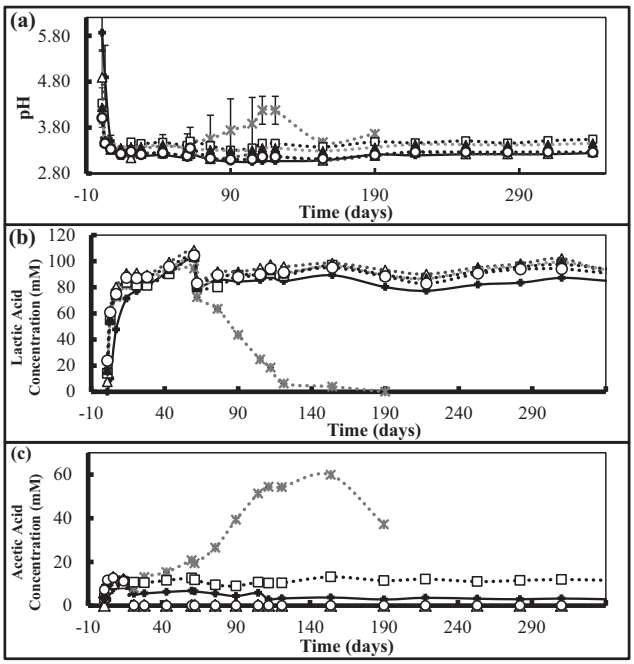

FIGURE 9 Measurement of pH (a), lactic acid (b), and acetic acid (C) in commercial cucumber fermentation cover brines over the course of 341 days of bulk storage. Error bars represent the standard error of the mean from independent duplicates. Commercial scale fermentations brined with NaCl (+) were not supplemented with preservatives during storage. Fermentations brined with $CaCl_2$ (dotted lines) were not supplemented (×) or supplemented with 12 mM sodium benzoate (□), 10 mM fumaric acid (Δ), 10 mM fumaric acid and 12 mM sodium benzoate (▲), or 10 mM fumaric acid and 2 mM allyl-isothiocyanate (○) after 14 days of fermentation

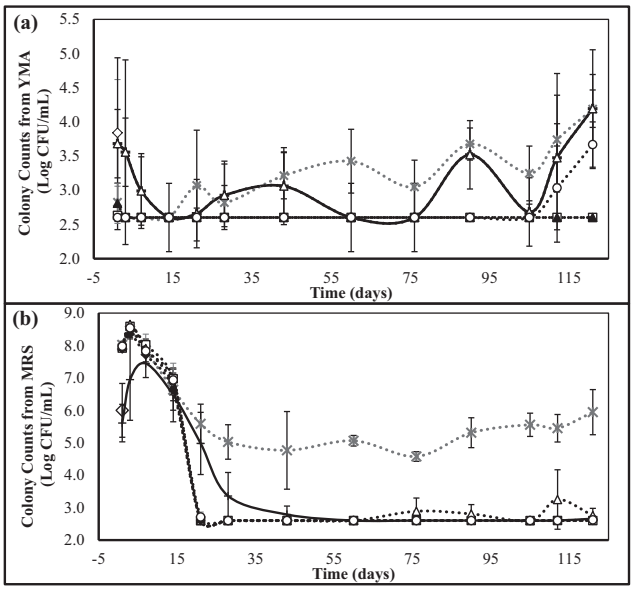

FIGURE 10 Colony counts for yeasts and molds (a) and presumptive lactobacilli (b) in fermented cucumber treatments over the course of a 120-day commercial scale trial. Standard error of the mean from independent duplicates is shown. Fermentations brined with NaCl (+) were free of preservatives. Fermentations brined with $CaCl_2$ (…) were not supplemented (×) or supplemented with 12.0 mM sodium benzoate (□), 10.0 mM fumaric acid (Δ), 10.0 mM fumaric acid and 12.0 mM sodium benzoate (▲), or 10.0 mM fumaric acid and 2.0 mM allyl-isothiocyanate (○)

characterized by the disappearance of lactic acid, formation of propionic acid, and rising pH. An indirect contribution of low yeasts levels to the effectiveness of the preservatives in preventing the metabolic activity of the spoilage lactobacilli cannot be excluded from the data collected.

While sodium benzoate treatments were the most effective in prohibiting growth of oxidative yeasts in fermented and stored cucumbers, some treatments lacking sodium benzoate were ultimately as effective in suppressing spoilage over the course of the trial. Interestingly, in fumaric acid treatments, yeast populations were comparable with control $CaCl_2$ treatments with no preservatives but maintained a pH below 3.3 following primary fermentation and did not develop undesirable lactobacilli populations. Therefore, it is concluded that fumaric acid was effective at suppressing growth of undesirable lactobacilli in the presence of low yeast counts and the absence of acetic acid. Considering the downstream environmental effects of sodium benzoate (WHO, 2019), these data suggest that a benzoate-free preservative option may be available for NaCl-free-cucumber fermentations.

It is relevant to mention that a concurrent study of the bacterial populations present in the commercial cucumber fermentations brined with $CaCl_2$ and no preservatives and the NaCl controls using non-culture-based methods revealed the presence of *Limosilactobacillus panis*, *Le. buchneri*, *Levilactobacillus namurensis*, *Lactobacillus acetotolerans*, *Acetobacter peroxydans*, *Acetobacter aceti*, and *Acetobacter pasteurianus* during initiation of spoilage at pH below 3.4 (Medina et al., 2016). The *Acetobactereaceae* prevailed within the bacterial population in the two tanks brined with $CaCl_2$, regardless of the fermentation age, reaching relative abundance values between 71.6% and 99.9%. The lactobacilli were detected to a relative abundance of 25% in 1 sample collected on day 76 of one of the fermentations brined with $CaCl_2$. While there is evidence implicating some of the lactobacilli in lactic acid degradation and acetic acid, propionic acid, and propanol formation (Johanningsmeier et al., 2012; Johanningsmeier & McFeeters, 2013), more research is needed to understand the role of the *Acetobacter* spp. in spoilage of fermented cucumbers at pH below 3.4. *Acetobacter* spp. are known for colonizing lactic acid fermented foods given their ability to catabolize lactic acid to acetic acid and some can

tolerate pH as low as 3.6 (Aries et al., 1982; De Ley et al., 1984; Gossele et al., 1984).

While a sensory evaluation was not conducted specifically on the products of the research presented here, an extensive evaluation of the impact of salt reduction on the sensory attributes of fermented cucumbers was documented by Wilson et al. (2015). Such sensory evaluation indicates that fermentation of cucumber in calcium chloride brine insignificantly alters consumer liking. The same study identified texture quality as an attribute to be optimized for low-salt cucumber fermentations brined with $CaCl_2$. The amounts of preservatives remaining in the finished fermented cucumber products are nominal due to the dilution that occurs during post-fermentation desalting. Products packed with the cucumbers fermented as part of the research presented here were successfully commercialized without consumer complaints (personal communications with processors).

4 | CONCLUSION

This study demonstrates that post-fermentation supplementation with 12 mM (1.73%) sodium benzoate, 10 mM (0.12%) fumaric acid, a combination of the two, or 10 mM fumaric acid with 2 mM AITC prevents microbial activity during long-term bulk storage of cucumbers fermented with 100 mM $CaCl_2$, 6 mM potassium sorbate, and no acetic acid. The data available to date suggest that it would be counterproductive to apply either air or nitrogen purging in cucumber fermentations as both induce rising pH spoilage led by aerobes, facultative anaerobes, and/or anaerobes. Development of standards for the precise regulation of oxygen levels and spoilage microbe colony counts in low-salt fermentations can assist processors in preventing spoilage in the absence of preservatives. Future research is to elucidate if purging and preservative-free cucumber fermentation is viable.

AUTHOR CONTRIBUTIONS

Ilenys M. Pérez-Díaz: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing. **Eduardo Medina**: Formal analysis; Investigation; Methodology, Validation; Writing – original draft; Writing – review & editing. **Clinton A. Page**: Data curation; Formal analysis; Writing – original draft; Writing – review & editing. **Suzanne D. Johanningsmeier**: Conceptualization; Formal analysis, Methodology; Supervision; Visualization; Writing – original draft; Writing – review & editing. **Katheryne V. Daughtry**: Investigation; Methodology; Validation; Writing – original draft; Writing – review & editing. **Lisa Moeller**: Investigation; Methodology; Resources; Supervision

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CONFLICT OF INTEREST

No conflict of interest is declared.

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