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Role of selected oxidative yeasts and bacteria in cucumber secondary fermentation associated with spoilage of the fermented fruit☆

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ABSTRACT

Changes during the spoilage of fermented cucumber pickles have been attributed to the metabolism of different yeasts and bacteria. In this study six organisms isolated from commercial spoiled cucumber pickles were evaluated for their possible role in primary and secondary cucumber fermentations. The ability of the yeasts *Issatchenkia occidentalis* and *Pichia manshurica* to utilize lactic and acetic acids during aerobic metabolism was confirmed and associated with increases in brine pH and the chemical reduction of the fermentation matrix. *Lactobacillus buchneri* and *Pediococcus ethanolidurans* were able to produce lactic acid from sugars, but only *L. buchneri* produced acetic acid at the expense of lactic acid under both aerobic and anaerobic conditions regardless of the initial acidic pH of 3.2 in the medium. The formation of secondary products was associated with the metabolism of *Clostridium bifermentans* and *Enterobacter cloacae*, which metabolic activity was observed at medium pH above 4.5. Individually, the selected spoilage microorganisms were found to be able to produce changes associated with secondary cucumber fermentations. The fact that oxidative yeasts and *L. buchneri* were able to produce chemical changes associated with the initiation of the spoilage process indicates that prevention of the secondary fermentation could be achieved by inhibiting these organisms.

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1. Introduction

Cucumber secondary fermentation or spoilage of fermented cucumber pickles has been described as the result of lactic acid degradation, increase in cover brine solution pH, and the chemical reduction of the matrix which leads to the production of secondary spoilage products, such as propionic and/or butyric acids (Franco and Pérez-Díaz, in press; Franco et al., 2012). Organisms naturally present in the cucumber fruit, such as yeasts and gram positive, and gram negative bacteria, were isolated as possible causative agents of spoilage during bulk storage of fermented cucumber pickles (Franco and Pérez-Díaz, in press; Franco et al., 2012; Johanningsmeier, 2011). Cucumber secondary fermentation associated with spoilage has been reproduced in the laboratory in cucumber pickles fermented with reduced sodium chloride (2%) under anaerobic conditions (Johanningsmeier, 2011; Kim and Breidt, 2007), and under aerobic and anaerobic conditions in which 6% sodium chloride or 1.1% calcium chloride cover brine solutions were used for packing (Franco and Pérez-Díaz, in press). Organisms presenting morphologies similar to those isolated from commercial spoilage samples were observed in spoilage reproduced in the laboratory. Among the isolated microorganisms, the yeasts *Pichia manshurica* and *Issatchenkia occidentalis*; the lactic acid bacteria *Lactobacillus buchneri* and *Pediococcus ethanolidurans*; the gram negative bacteria *Enterobacter cloacae*; and the anaerobe *Clostridium bifermentans* were repeatedly observed in commercial spoilage samples and laboratory experiments.

The laboratory reproduced cucumber spoilage aided in the identification of a succession of events and microorganisms potentially involved in the development of cucumber secondary fermentations. It was observed that increases in brine pH and the chemical reduction of the fermentation developed concomitantly with lactic acid disappearance and were necessary to trigger the production of secondary products, such as propionic and butyric acids (Franco and Pérez-Díaz, in press). The aerobic metabolic activity of the yeasts *P. manshurica* and *I. occidentalis* has been suggested as a key factor for the initial changes to occur (Franco and

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Pérez-Díaz, in press). Different yeast genera have been reported as able to utilize an array of organic acids present in table olive fermentation brines (Ruiz-Cruz and Gonzalez-Cancho, 1969). Specific to the cucumber industry, film forming yeasts were reported as potentially able to utilize lactic acid during bulk storage of the fermented product (Binsted et al., 1962; Etchells and Bell, 1950). *L. buchneri* and *P. ethanolidurans* were frequently observed in the commercial spoilage and laboratory reproduced secondary fermentations (Franco and Pérez-Díaz, in press; Franco et al., 2012; Johanningsmeier, 2011). *L. buchneri* caused lactic acid degradation with production of 1,2 propanediol and acetic acid when incubated in fermented cucumber juice media (4% NaCl and initial pH 3.8) under anaerobiosis (Johanningsmeier et al., 2012), and correlated with increases in propionic acid concentrations during aerobic storage of silage (Ranjit and Kung, 1999). *Pediococcus* populations are commonly established at the end of the primary fermentation (Singh and Ramesh, 2008) and remain stable during storage. The *Pediococcus* isolate (*P. ethanolidurans*) studied here, has been found to survive harsh conditions characterized by high ethanol and low sugar concentrations (Liu et al., 2006), but the ability of the bacterium to utilize other carbon sources, such as organic acids, has not been documented.

Clostridium tertium (Fleming et al., 1989) and a possible *C. bifermentans* B431 (Franco and Pérez-Díaz, in press) have been isolated and are suggested to be responsible for butyric acid production during the spoilage of fermented pickles. *Clostridium* species are ubiquitous to the environment and well known for their capacity to produce butyric acid from different carbon sources. While *C. tertium* was able to utilize lactic acid and produce butyric acid in fermented brines with the initial pH adjusted to 5.0, *C. bifermentans* populations were observed at lower pH values.

The increased brine pH that characterizes spoilage samples might favor the development of other organisms which could be responsible for further deterioration of the fermented product. Enterobacteria, which are naturally present on the cucumber fruit, have been associated with spoilage problems in fermented cucumbers and table olives (Bevilacquia et al., 2009; Etchells et al., 1945). Control of the growth of these organisms is of most importance since they might cause deterioration of the food product. The most important barrier to prevent growth of these organisms is the production of acid and acidic pH generated from the primary fermentation. However, during the spoilage of fermented cucumber pickles, the brine pH might increase to values above 4.5 (Fleming et al., 1989; Franco and Pérez-Díaz, in press). Additionally, enterobacteria spp, such as *E. cloacae*, have been isolated from spontaneous table olive fermentations and reported as potential spoilage factors for the fermented product (Bevilacquia et al., 2009).

The objective of this study was to characterize the role of selected spoilage organisms isolated in previous research. The role of each organism during the fermentation process (primary fermentation) was evaluated using fresh cucumber juice media while the potential behavior during bulk storage (secondary fermentation) was assessed in fermented brines. Given the interest of the pickle industry to move toward no NaCl fermentations, the $CaCl_2$ fermentation matrix proposed by Franco et al. (2012) in the study of secondary cucumber fermentations was used as the experimental matrix.

2. Materials and methods

2.1. Experimental media preparation

2A cucumbers were acquired from a local processing company. Juice from the fruit was obtained using a food processor (Juiceman, Jr.). Cucumber juice was spun for 1 h at 10,000 × g (Sorbal RC58, DuPont Instruments, Red Oak, IA, USA). The supernatant was then collected and stored at −20 °C until used. Cucumber juice medium (CJM), was prepared by mixing (50:50) cucumber juice with a cover brine solution such that after equilibration the mixture contained 25 mM acetic acid as vinegar and 100 mM $CaCl_2$. The mixture was filter sterilized using 0.2 μPES filter membranes (Nalgene, Nalge Nunv International, Rochester, NY, USA). Filter sterilized CJM was stored at 4 °C until used. Fermented cucumber juice medium (FCJM) was obtained by inoculation of CJM with a mixture of lactic acid bacteria (LAB) composed of *Lactobacillus plantarum* strains LA 445, LA 98, and LA 285 (United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Food Science Research Unit, Raleigh, NC, USA, Culture Collection). The inoculated CJM was incubated at 30 °C for 7–10 days to allow sugar utilization and lactic acid production by the LAB. The course of the fermentation was monitored by pH measurements and High Performance Liquid Chromatography (HPLC) analysis for sugars and organic acids (McFeeters and Barish, 2003) as described in the chemical analysis section. The fermented CJM was spun (10,000 × g, 15 min, Sorbal) and the supernatant filter-sterilized (Nalgene) after a pH of 3.2 or lower was measured and limited (<1 mM) quantities of sugars were detected.

For aerobic incubation, 50-mL conical tubes were aseptically filled with 10 mL CJM or FCJM providing a sufficient head space for the continuous supply of oxygen between sampling times. Anaerobic experiments were performed in 15-mL conical tubes aseptically filled with 10 mL of either media and incubated inside a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, MI, USA).

2.2. Cultures

The different cultures used during this study were obtained from the culture collection of the USDA-ARS, Food Science Research Unit, located in Raleigh, NC, USA (Table 1). Isolates were streaked from frozen stock cultures in the agar plates indicated in Table 1 and manipulated as described. Broth cultures were harvested at 10,000 × g for 15 min (Centrifuge 5810, Eppendorf, Hamburg, Germany) and washed twice with 0.85% saline solution. The cell pellets were re-suspended in 0.85% saline solution and the density adjusted to an optical density at 600 nm of 0.132 to achieve a final estimated cell number of approximately 8 log CFU/mL. For all cultures, serial dilutions were made to achieve the desired inoculation level of 5 log CFU/mL. All culture media were obtained from Becton, Dickinson and Co. (Sparks, MD, USA).

2.3. Inoculation of CJM and FCJM

Pure cultures were inoculated in both CJM and FCJM to 5 log CFU/mL. *C. bifermentans* and *E. cloacae* pH sensitivity was tested by inoculating the bacteria in FCJM to which the initial pH was adjusted to 4.0, 4.5, and 5.0 using 5 N NaOH solution. Experimental tubes were incubated at 30 °C under both aerobic and anaerobic conditions. Spoilage yeast isolates were cultured alone and with *L. plantarum* LA 445 into CJM to simulate a natural fermentation.

2.4. Redox potential measurements

The ability of yeast isolates to reduce the environment was monitored in 8 oz glass jars containing 200 mL of FCJM. Jars were sealed with metal lug caps with holes inserted into the caps to hold a redox electrode (InLab 501/170, Mettler-Toledo, Bedford, MA, USA) and a rubber septum for taking brine samples using sterile syringes. The proper functioning of the redox electrode probes was

Table 1
Cultures used in this study.

Microorganism	Culture collection	Source	Reference	Culture media	Incubation temperature	Incubation time
Lactobacillus plantarum	LA 219	Fermented cucumbers	FSRU culture collection	MRS	30 °C	48 h
L. plantarum	LA 98	Fermented cucumbers	NCDO (Reading, England) courtesy of Todd Klaenhammer	MRS	30 °C	48 h
L. plantarum	LA 445	Fermented cucumbers	McDonald et al. (1993)	MRS	30 °C	48 h
Pichia manshurica	Y88	Commercial secondary cucumber fermentation with $CaCl_2$ cover brine solution	Franco et al. (2012)	YMA	30 °C	48 h
Issatchenkia occidentalis	Y89	Commercial secondary cucumber fermentation with $CaCl_2$ cover brine solution	Franco et al. (2012)	YMA	30 °C	48 h
Lactobacillus buchneri	LA1149	Commercial secondary cucumber fermentation with $CaCl_2$ cover brine solution	Franco et al. (2012)	MRS	30 °C	48 h
Pediococcus ethanolidurans	LA1150	Commercial secondary cucumber fermentation with $CaCl_2$ cover brine solution	Franco et al. (2012)	MRS	30 °C	48 h
Clostridium bifermentans	B431	Commercial secondary cucumber fermentation with NaCl cover brine solution	Franco et al. (2012)	DRCA	30 °C - Anaerobic	48 h
Enterobacter cloacae	B515	Laboratory reproduced secondary cucumber fermentation with $CaCl_2$ cover brine solution	Franco and Pérez-Díaz (in press)	VRBG	37 °C	24 h

tested in buffered pH calibration solutions (4 and 7) saturated with quinnhydrone (Acros Organics, NJ, USA). The probes were rinsed thoroughly with 70% ethanol and secured into the fittings on the jar lids. The tips of the electrodes were placed in the center of the jar in the brine solution. A silicone sealant was applied around the fittings holding the redox electrodes to prevent air leakage. Each jar was equipped with an air inlet and outlet to allow for air purging at 5 mL/min and pressure release, respectively. Duplicate jars were used for each experiment and replicated two times. Jars were kept at room temperature (24–27 °C).

Non-inoculated media (CJM and FCJM) were used as control on the respective experiments. Fresh and fermented cucumber juice media inoculated with the *L. plantarum* strains was used as a reference for standard fermentations. Periodically, during the course of the different fermentations, 1 mL aliquots were aseptically sampled. Changes in substrates, products, and microbial populations were followed as described below in the microbiological and chemical analysis sections.

2.5. Microbiological analysis

Collected samples were serially diluted in 0.85% saline solution, and spiral plated (Autoplate 400, Spiral Biotech, Norwood, MA, USA). LAB and *Clostridia* enumeration was done using the deMann Rogosa and Sharpe Lactobacilli agar (MRS, Becton, Dickinson and Co.) and Differential Reinforced Clostridial agar (DRCA, Becton, Dickinson and Co.), respectively. Both media were supplemented with cycloheximide (1% solution, OXOID, England) to inhibit yeast growth. Only black colonies proliferating in the DRCA plates were counted. Plates were incubated anaerobically at 30 °C for 48 h. Yeasts were enumerated using Yeast and Malt agar (YMA, Becton, Dickinson and Co.) supplemented with 0.01% chloramphenicol (Sigma–Aldrich, St. Louis, MO, USA) and 0.01% chlorotetracycline (Sigma–Aldrich) to inhibit bacterial growth. YMA plates were incubated aerobically at 30 °C for 48 h. Enterobacteria were enumerated using Violet Red Bile Glucose agar (VRBG, Becton, Dickinson and Co.), and plates were incubated aerobically at 37 °C for 24 h. Purple and pink colonies after 24 h incubation on VRBG plates were recorded as presumptive enterobacteria.

2.6. Chemical analysis

Sample pH was measured with a Fisher Accumet pH meter, model 825 MP (Pittsburgh, PA, USA). Sample components such as, sugars, organic acids, and ethanol were determined by HPLC on a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) (McFeeters and Barish, 2003). The column was heated to 37 °C and 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, USA) set to collect data at 210 nm was used to detect malic, lactic, acetic, propionic, and butyric acids. A Waters model 410 refractive index detector (Waters Corp., Millipore Corp., Billerica, MA, USA) 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.

2.7. Statistical analyses

Experiments conducted in the fermentation jars were conducted, in duplicate, in two independent runs, while experiments conducted to characterize the single isolates and the yeast–bacteria interactions were conducted in triplicate and repeated twice independently. The results were analyzed using the ANOVA procedure with the Duncan's multiple range test of the Statistical Analysis Systems version 9.0 (Statistical Analysis System, SAS Institute, Cary, NC, USA).

3. Results

3.1. Characterization of the spoilage yeasts

The metabolic activity of the spoilage yeasts incubated in CJM under aerobic and anaerobic conditions is shown in Table 2. Under anaerobic conditions, yeasts were inhibited and no significant changes in cell population, substrate utilization, or medium pH were observed. On the other hand, under aerobic conditions, the yeasts utilized sugars as the cell density increased accompanied by increases in pH from 4.2 to 5.7 and 6.0 for *P. manshurica* and *I. occidentalis*, respectively. The acetic acid contained in the medium was depleted during the course of the incubation. Ethanol was produced only under aerobiosis in similar amounts for both isolates (data not shown). Lactic acid was not detected in samples inoculated with the spoilage yeasts either aerobically or anaerobically. Both oxidative yeasts showed similar growth patterns in CJM (Fig. 1). The highest yeast population (about 8 log CFU/mL) was achieved after 5 days of incubation (Fig. 1). After 20 days samples inoculated with *I. occidentalis* reached a pH value above 5.6 while samples inoculated with *P. manshurica* reached a final pH close to 6.0. As expected, the CJM inoculated with the *L. plantarum* culture alone had high levels of lactic acid (Table 2).

Table 2
Metabolism of selected cucumber secondary fermentation isolates incubated in cucumber juice medium (CJM).

Sample	ΔpH ($pH_f - pH_i$)[c]		Lactic acid (mM)		Acetic acid (mM)	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Control[d]	4.2 ± 0.0	4.2 ± 0.0	N/D	N/D	25.4 ± 0.0	25.5 ± 0.0
Lactobacillus plantarum	−0.9	−1.1	75.4 ± 3.9[a]	67.6 ± 2.2[a]	25.0 ± 0.7	22.2 ± 0.5
Pichia manshurica	+1.5	N/C	N/D[b]	N/D[b]	N/D[b]	5.7 ± 0.02[a,b]
Isstachenckia occidentalis	+1.8	+0.1	N/D[b]	N/D[b]	N/D[b]	1.0 ± 0.3[a,b]
Lactobacillus bunchenri	−0.9	−0.9	59.7 ± 1.1[a,b]	74.0 ± 0.1[a]	35.7 ± 0.4[a,b]	34.6 ± 0.3[a,b]
Pediococcus thanolidurans	−0.7	−1.3	92.6 ± 0.4[a]	77.2 ± 0.3[a]	25.2 ± 0.3	26.1 ± 0.1
Clostridium bifermentans	None	None	N/D	N/D	25.4 ± 0.02	25.5 ± 0.0
Enterobacter cloace	None	None	N/D	N/D	25.4 ± 0.02	25.5 ± 0.0

Values represent the mean ± standard deviation of six replicates after 20 days under aerobic conditions (30 ± 2 °C) and 12 months under anaerobiosis (25 ± 2 °C).
N/C: No significant change observed.
N/D: Non detected.
[a] Indicates a significant difference compared to the not-inoculated samples ($\alpha = 0.05$).
[b] Indicates a significant difference compared to samples inoculated with *L. plantarum* ($\alpha = 0.05$).
[c] pH_i: Initial pH, pH_f: final pH. (+) indicates an increase in media pH, and (−) indicates a decrease in media pH.
[d] Control samples represent non-inoculated medium containing 14 mM Glucose, 15 mM fructose, 25 mM acetic acid, and 100 mM $CaCl_2$. No significant changes in pH were observed in control samples during the course of the experimentation. The values in the table represent the metabolites and pH measurement by the culmination of experiment.

Both yeasts showed a similar behavior when co-cultured with *L. plantarum* in CJM (Table 3). As expected, the LAB rapidly utilized the sugars in the CJM and produced lactic acid with the concomitant decrease in medium pH regardless of the availability of oxygen. However, under aerobic conditions, and after sugars were depleted, lactic and acetic acid concentrations declined with time in the presence of the oxidative yeasts. Organic acid utilization led to increases in medium pH and yeast populations to 7 log CFU/mL. Under anaerobiosis, lactic acid production was associated with increases in LAB populations. The fermented medium remained stable once sugars were depleted, such that after a year the pH was 3.2 and more than 80 mM lactic acid remained. In anaerobic experiments yeasts were not detected after 30 days of incubation.

Yeast metabolic activity in FCJM purged with air resulted in organic acid utilization with the concomitant increase in brine pH (Fig. 2, Panels A and C). The spoilage yeasts were able to deplete more than half of the initial lactic acid in about 3–4 days. The brine pH increased as lactic acid was utilized and reached values above 4.6 (critical safety limit in fermented products) after 6 days of incubation. At this pH about 75% of lactic acid was depleted (Fig. 2, Panel A). Yeast populations were close to 7 log CFU/mL

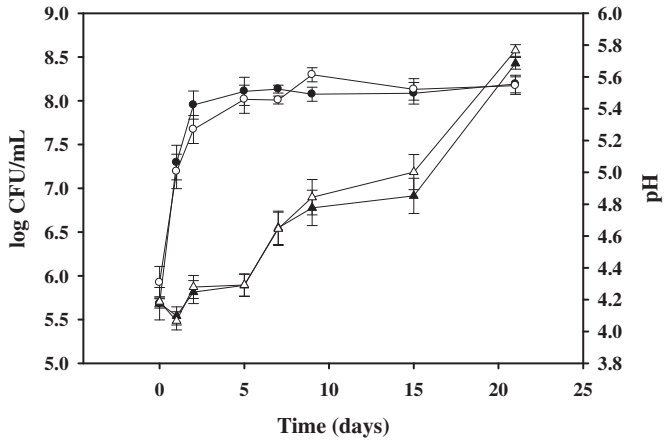

Fig. 1. Changes in cucumber juice medium pH and spoilage yeasts population under aerobic conditions. Changes in pH (△,▲) and in cell numbers (●,○) for *Issatchenkia occidentalis* (△,●), and *Pichia manshurica* (▲,○) grown in cucumber juice media (CJM) at 30 °C, under aerobic and static conditions. Values represent the mean and standard deviation of six replicates.

during active lactic acid utilization. In a similar fashion, the yeast isolates were able to utilize acetic acid present in the fermented medium (Fig. 2, Panel B). About 98% of the acetic acid originally present in FCJM was utilized in 4 days. Similar to the experiments conducted in CJM, the metabolic activity of the yeast isolates was inhibited under anaerobiosis in FCJM, and no changes in either lactic or acetic acids were observed. Furthermore, the experimental tubes remained stable for more than 12 months of anaerobic incubation at room temperature. Yeasts were not detected after a month of experimentation (data not shown).

The utilization of organic acids, specifically lactic and acetic acids by oxidative yeasts detected in the FCJ medium resulted in the

Table 3
Changes in organic acids, pH, and cell population observed in cucumber juice medium (CJM) inoculated with *Pichia manshurica* and *Lactobacillus plantarum*.

Time (days)	Lactic acid (mM)	Acetic acid (mM)	pH	Log CFU/mL in YMA	Log CFU/mL in MRS
Aerobic and static incubation at 30 °C					
Pichia manshurica					
0	N/D	25.3 ± 0.0	4.1 ± 0.0	5.0 ± 0.1	5.3 ± 0.1
3	62.4 ± 0.1	25.2 ± 0.0	3.3 ± 0.0	6.6 ± 0.0	7.5 ± 0.0
5	42.9 ± 0.0	25.2 ± 0.1	3.5 ± 0.0	7.0 ± 0.0	7.8 ± 0.1
7	35.9 ± 0.1	24.9 ± 0.0	4.3 ± 0.1	7.4 ± 0.3	7.5 ± 0.2
9	17.6 ± 0.0	17.9 ± 0.0	7.3 ± 0.0	7.4 ± 0.0	7.1 ± 0.0
12	0.4 ± 0.1	1.9 ± 0.0	7.3 ± 0.2	7.8 ± 0.0	6.9 ± 0.1
15	N/D	2.5 ± 0.0	7.4 ± 0.3	7.7 ± 0.1	6.0 ± 0.3
20	N/D	0.3 ± 0.2	7.6 ± 0.1	7.5 ± 0.0	5.7 ± 0.01
Issatchenkia occidentalis					
0	N/D	25.3 ± 0.0	4.1 ± 0.0	5.0 ± 0.1	5.3 ± 0.1
3	61.3 ± 0.0	25.1 ± 0.0	3.4 ± 0.0	6.1 ± 0.0	7.3 ± 0.1
5	42.9 ± 0.1	24.7 ± 0.0	3.7 ± 0.0	6.9 ± 0.0	7.5 ± 0.1
7	35.5 ± 0.0	24.3 ± 0.0	4.2 ± 0.2	7.1 ± 0.1	7.6 ± 0.2
9	16.7 ± 0.1	19.3 ± 0.1	6.9 ± 0.1	7.2 ± 0.1	7.3 ± 0.2
12	1.1 ± 0.1	3.9 ± 0.1	7.0 ± 0.1	7.5 ± 0.0	7.0 ± 0.0
15	N/D	1.5 ± 0.1	7.2 ± 0.1	7.8 ± 0.2	6.5 ± 0.1
20	N/D	0.5 ± 0.0	7.3 ± 0.1	7.3 ± 0.2	5.8 ± 0.0
Anaerobic and static incubation at 30 °C					
Pichia manshurica					
0	N/D	25.3 ± 0.0	4.13 ± 0.0	5.0 ± 0.1	5.3 ± 0.1
30	77.1 ± 1.2	25.3 ± 0.1	3.2 ± 0.0	N/D	4.8 ± 0.1
365	87.3 ± 2.5	22.3 ± 2.9	3.2 ± 0.0	N/D	N/D
Issatchenkia occidentalis					
0	N/D	25.3 ± 0.0	4.13 ± 0.0	5.0 ± 0.1	5.3 ± 0.1
30	78.1 ± 1.5	25.3 ± 0.2	3.2 ± 0.0	N/D	5.1 ± 0.0
365	89.4 ± 2.1	24.3 ± 2.0	3.2 ± 0.0	N/D	N/D

N/D Non detected.
Values represent the mean ± standard deviation of four replicates.

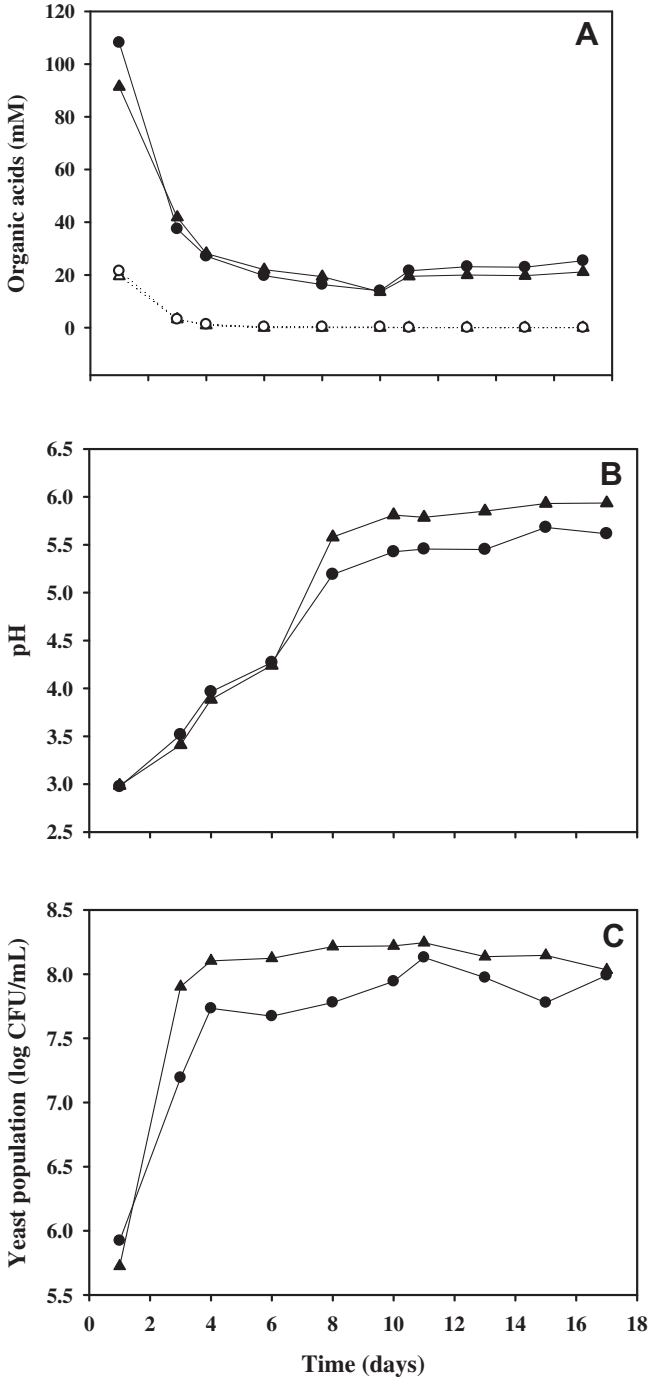

Fig. 2. Changes in organic acids, pH, and yeast populations during the mixed culture incubation of *Pichia manshurica* and *Issatchenkia occidentalis* in fermented cucumber juice medium under aerobic conditions. Changes in lactic acid (closed symbols) and acetic acids (open symbols) (Panel A); pH (Panel B); and yeasts population (Panel C) of the spoilage yeasts *I. occidentalis* (triangles) and *P. manshurica* (circles) inoculated in fermented cucumber juice medium (FCJM) and incubated at 30 °C under aerobic and static conditions. Values represent the mean of two samples for two independent experiment replicates.

reduction of the matrix, as shown by the decreases in redox potential (E_h) (Fig. 3). After two days of incubation the E_h decreased from 220 mV to ~100 mV. The decreasing trend continued thereafter and the culture reached the most reduced value at day 5 (−110 mV). This value remained stable for another 5 days when an increase to final E_h value of 10 mV was measured.

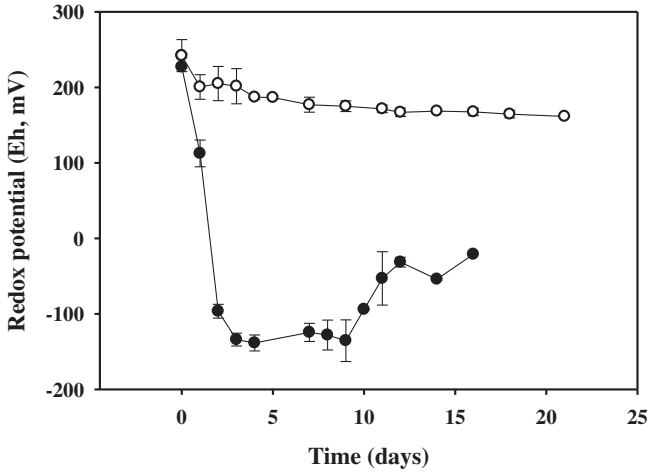

Fig. 3. Redox potential changes during aerobic incubation of the spoilage yeasts *Pichia manshurica* and *Issatchenkia occidentalis* mixed culture in fermented cucumber juice medium. Effect in redox potential by spoilage yeasts *Issatchenckia occidentalis* and *Pichia manshurica* mix culture incubated in fermented cucumber juice media (FCJM, pH 3.2) under air-purged (●) and anaerobic (○) conditions. Values represent the mean ± standard deviation of two independent trials.

3.2. Characterization of spoilage lactic acid bacteria

Both isolated LAB (*L. buchneri* and *P. ethanolidurans*) showed similar behavior when incubated in CJM under both aerobic and anaerobic conditions. The sugars contained in the medium were rapidly utilized and pH decreased (Table 2). *P. ethanolidurans* culture had a final pH of 3.1 ± 0.01 and 2.9 ± 0.01 under aerobic and anaerobic incubation, respectively. The final pH for *L. buchneri* was 3.1 ± 0.01 under both aerobic and anaerobic conditions. In the presence of oxygen, *P. ethanolidurans* produced 22 ± 0.7 mM more lactic acid than *L. buchneri*. During anaerobic incubation both spoilage LAB produced less lactic acid than under aerobic conditions, and about 10 mM acetic acid was also produced by each organism. Propionic and butyric acids were not detected for any isolate under either condition.

When the spoilage LAB were inoculated into FCJM and incubated under aerobic conditions, both utilized the remaining sugars in the brine which resulted in small increases in lactic acid and decreases in pH (data not shown). *P. ethanolidurans* produced 1.5 mM of lactic acid. Only 0.6 mM of lactic acid was produced by *L. buchneri*. Once sugars were depleted, *L. buchneri* was able to metabolize the lactic acid in the fermented medium (Table 4). Higher lactic acid utilization by the bacterium was observed under anaerobic conditions (about 36 mM) than under aerobic (about 10 mM) environments (Table 4). The bacterium, however, did not produce propionic or butyric acids, and the changes observed during incubation were slight increases in brine pH and production of acetic acid (Table 4). *L. buchneri* population increased by 0.5 log CFU/mL during lactic acid utilization (data not shown). Similar changes were observed under anaerobic conditions. *P. ethanolidurans* was not able to utilize the lactic acid present in the fermented medium (Table 4).

3.3. Characterization of C. bifermentans

Growth and metabolic activity of *C. bifermentans* was only observed when it was inoculated in the medium with a starting pH above 4.5. In the fresh CJM, with an initial pH 4.2, the bacterium was not able to utilize the sugar and no changes in pH were observed (Table 2). Furthermore, no growth was detected and cells died off.

Table 4
Metabolism of *Lactobacillus buchneri* and *Pediococcus ethanolidurans* incubated in fermented cucumber juice media (FCJM) after 20 and 360 days under aerobic and anaerobic incubation, respectively.

Sample	ΔpH		Lactic acid (mM)		Acetic acid (mM)		Ethanol (mM)	
	$(pH_f - pH_i)$[a]							
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Control[b]	3.2 ± 0.0	3.2 ± 0.0	84.9 ± 0.3^{A}	85.1 ± 0.2^{A}	26.9 ± 2.4^{A}	26.9 ± 1.6^{A}	N/D	2.3 ± 1.5^{A}
Lactobacillus buchneri	+0.2	+0.4	74.5 ± 0.1^{B}	48.7 ± 0.2^{B}	32.0 ± 1.3^{B}	46.1 ± 0.1^{B}	N/D	10.8 ± 0.0^{B}
Pediococcus ethanolidurans	−0.2	−0.1	86.4 ± 0.2^{A}	86.5 ± 1.2^{A}	27.2 ± 0.6^{A}	28.0 ± 0.0^{B}	N/D	2.9 ± 0.0^{A}

Values represent the mean ± standard deviation of six replicates after 20 days of incubation under aerobic conditions (30 ± 2 °C) and 12 months under anaerobic conditions (25 ± 2 °C). Different capital letters in columns represent significantly different values determined by the Duncan's multiple range test with a significance level of 0.05%.
N/D Non detected.
[a] pHi: Initial pH, pHf: Final pH. Positive values represent an increase in medium pH, and negative values indicate a decrease in medium pH.
[b] Control samples represent non-inoculated medium. The values presented in the table correspond to measurements collected when the experiments were concluded. Decreases and increase in metabolites and pH for the culture experiments were calculated taking as base these control values.

C. bifermentans also died off in FCJM with an initial pH of 3.2 (Table 5). In medium adjusted to pH 4.5, the bacterium was able to utilize remaining sugars and produce lactic acid (Table 5), but growth was inhibited by the decreasing brine pH (data not shown), and either lactic acid utilization or butyric acid production were detected. Lactic and acetic acids utilization was observed by the bacterium in FCJM in which the initial pH was adjusted to 5.0, as result of the metabolic activity, butyric acid was produced and the medium pH increased. About 50% of the lactic acid and 58% acetic acid were consumed and 25.1 ± 0.4 mM of butyric acid was produced in 14 days. The final pH increased to 5.9 ± 0.1 (Table 5).

3.4. Characterization of *E. cloacae*

The enterobacter isolate was not able to survive in CJM (Table 2) with an initial pH of 4.2 and no changes in substrates were observed. Similarly, the bacterium was not able to metabolize lactic acid contained in the FCJM with initial pH levels of 3.2, 4.0 or 4.5. Only when the medium pH was adjusted to 5.0 was the bacterium able to utilize lactic acid and, in turn, produce propionic and acetic acids and increase the pH of the medium (Table 6).

4. Discussion

The spoilage of fermented cucumber pickles occurs when microbiota present during bulk storage is able to utilize lactic acid and, in turn, make changes in the environment that lead to the final deterioration of the product. In order to make these changes, the microorganisms have to endure extreme conditions characterized by low pH and the presence of organic acids. Both *L. buchneri* and the spoilage yeasts (*P. manshurica* and *I. occidentalis*) studied here were able to utilize lactic acid at the acidic conditions characteristic of the fermented product once the primary fermentation was completed. Therefore, the isolates are able to initiate the secondary fermentation that will lead to spoilage of the product. However, the metabolic activity of the yeasts is more rapid than *L. buchneri* indicating that under aerobiosis yeasts are probably the microorganisms responsible for raising the pH to 4.5 and above if present in the fermentation matrix. Yeasts have been previously reported as being able to transport (Cássio and Leão, 1993) and utilize organic acids as carbon sources (Ruiz-Cruz and Gonzalez-Cancho, 1969). In this study, the yeasts *I. occidentalis* and *P. manshurica* utilized lactic and acetic acids from fermented brines under aerobic conditions. This metabolic activity led to the chemical reduction in the fermentation matrix. In previous studies we have reported that the aerobic spoilage of fermented cucumbers is the result of growth of a series of microorganisms (Franco and Pérez-Díaz, in press). Our results suggest that the aerobic utilization of lactic and acetic acids by the spoilage yeasts is responsible for the first step in the secondary fermentations described in the aforementioned study.

Similar to the behavior reported under anoxic conditions in silage (Oude-Elferink et al., 2001) and fermented cucumber brines (Johanningsmeier et al., 2012), *L. buchneri* was able to utilize lactic acid in the fermented medium under aerobic conditions in $CaCl_2$ brines. The catabolic products from this activity were acetic acid and ethanol. On the other hand, *P. ethanolidurans*, which has also been observed and isolated from different spoilage samples (Franco et al., 2012; Johanningsmeier, 2011), was not able to utilize lactic acid from the fermented medium. Although a wide variety of LAB have been isolated from spoilage samples (Franco et al., 2012; Johanningsmeier, 2011), only *L. buchneri*, and probably *Lactobacillus parafarraginis*, has been shown capable of initiating lactic acid utilization in the low pH and high acid conditions that prevail once a normal/standard primary fermentation is completed under both aerobic and anaerobic conditions (Johanningsmeier et al., 2012).

The fact that *C. bifermentans* and *E. cloacae* were not able to utilize lactic acid at lower brine pH (3.2) shows that these bacteria require other organisms to remove acid and raise the pH of the fermented cucumbers before they can produce butyric and propionic acids (Franco and Pérez-Díaz, in press). Butyric acid production by *C. tertium* has been previously reported in fermented brines with an initial pH of 5.0 incubated under anaerobic

Table 5
Lactic acid utilization and products formation by *Clostridium bifermentans* in fermented cucumber juice medium (FCJM) adjusted to different initial pH.

Medium	Characteristics after 14 days anaerobic incubation						
	Final pH[b]	Glucose (mM)	Fructose (mM)	Lactic acid (mM)	Acetic acid (mM)	Butyric acid (mM)	Ethanol (mM)
Control[a]	3.2 ± 0.1^{A}	1.1 ± 0.1^{A}	1.2 ± 0.1^{A}	100.2 ± 1.3^{A}	25.4 ± 0.1^{A}	N/D^{A}	N/D^{A}
pH 3.2	3.2 ± 0.1^{A}	1.1 ± 0.1^{A}	1.2 ± 0.1^{A}	100.2 ± 1.3^{A}	25.4 ± 0.1^{A}	N/D^{A}	N/D^{A}
pH 4.0	4.0 ± 0.1^{B}	1.1 ± 0.1^{A}	1.2 ± 0.1^{A}	100.2 ± 1.3^{A}	25.4 ± 0.1^{A}	N/D^{A}	N/D^{A}
pH 4.5	4.0 ± 0.1^{B}	N/D^{B}	N/D^{B}	102.8 ± 0.5^{A}	37.9 ± 0.3^{B}	N/D^{A}	1.5 ± 0.3^{B}
pH 5.0	5.9 ± 0.1^{B}	N/D^{B}	N/D^{B}	50.3 ± 0.6^{B}	10.5 ± 0.1^{B}	25.1 ± 0.4^{B}	1.2 ± 0.3^{B}

Values represent the mean ± standard deviation of three replicates after 14 days of incubation under anaerobic conditions (25 ± 2 °C). Different capital letters in columns represent significantly different values determined by the Duncan's multiple range test with a significance level of 0.05%.
N/D: Not detected.
[a] Control: non-inoculated samples subjected to the same experimental conditions.
[b] After 14 days of incubation under anaerobic static conditions.

Table 6
Lactic acid utilization and products formation of *Enterobacter cloacae* inoculated in fermented cucumber juice medium (FCJM) adjusted to different pH.

FCJM media	Final pH[a]	Δ Glucose (mM)[b]	Δ Fructose (mM)[b]	Δ Lactic acid (mM)[b]	Δ Acetic acid (mM)[b]	Δ Propionic acid (mM)[b]
Control[c]	3.2 ± 0.2^{A}	0.9 ± 0.1^{A}	1.0 ± 0.1^{A}	84.8 ± 0.5^{A}	26.4 ± 0.2^{A}	N/D[A]
pH 3.2	3.2 ± 0.2^{A}	0.9 ± 0.1^{A}	1.0 ± 0.1^{A}	None[A]	None[A]	N/D[A]
pH 4.0	4.0 ± 0.2^{B}	0.9 ± 0.1^{A}	1.0 ± 0.1^{A}	None[A]	None[A]	N/D[A]
pH 4.5	4.5 ± 0.1^{B}	0.9 ± 0.1^{A}	1.0 ± 0.1^{A}	None[A]	None[A]	N/D[A]
pH 5.0	6.9 ± 0.1^{B}	N/D[B]	N/D[B]	-39.3 ± 1.2^{B}	$+9.3 \pm 0.7^{B}$	$+14.0 \pm 0.5^{B}$
pH 6.0	7.0 ± 0.1^{B}	N/D[B]	N/D[B]	-51.7 ± 0.7^{C}	$+17.6 \pm 1.2^{C}$	$+19.1 \pm 0.4^{B}$

Values represent the mean ± standard deviation of three replicates after 20 days of incubation under aerobic conditions (30 ± 2 °C). Different capital letters in columns represent significantly different values determined by the Duncan's multiple range test with a significance level of 0.05%.
N/D Non detected.
[a] After 20 days of incubation under aerobic static conditions.
[b] Positive sign (+): production of the compound. Negative sign (−): Utilization of the compound as compared to control samples.
[c] Control: non-inoculated samples subjected to the same experimental conditions.

conditions (Fleming et al., 1989). The same result was observed for *C. bifermentans* studied here. Though enterobacteria are inhibited by the environmental changes produced during the primary fermentation of the cucumber fruits (Etchells et al., 1945), once pH increases sufficiently the bacterium may grow (Franco and Pérez-Díaz, in press). The metabolic activity of these organisms has been previously reported as being related to gas production and softening of fermented vegetable tissue (Bevilacquia et al., 2009; Etchells et al., 1945). In this study, we found that *E. cloacae* is also able to produce propionic acid while utilizing lactic acid as a carbon source when the brine pH is between 5.0 and 6.0.

The changes in the fermentation matrix produced by the metabolic activity of the spoilage yeasts, might favor the establishment of other spoilage bacteria such as *Clostridium* and *Enterobactereceae*. Increased brine pH (above 4.5) and reduced conditions are necessary for *C. bifermentans* and *E. cloacae* to utilize lactic and acetic acids contained in fermented brines and produce butyric and propionic acids. Due to the potential for *Clostridium botulinum* spores to germinate and grow in brines with a pH above 4.6, tanks must be destroyed as they reach such pH value.

Given the nature of cucumber fermentations and the variability on cucumber fruits used for the fermentation process, it is reasonable to assume that other microorganisms may also grow during the later stages of the spoilage process. For example, *propionibacteria* have been previously isolated (Breidt, F., not published) from fermented cucumber spoilage samples. These organisms are able to utilize lactic acid at higher pH values (5 and above) and produce propionic acid as the major metabolic product. Recently Johanningsmeier et al. (2012) reported that *Lactobacillus rapi* can utilize 1,2 propanediol, an intermediate product formed by *L. buchneri* from lactic acid, to produce propionic acid. The complete characterization of fermented and spoilage samples is a complex task and more studies are necessary to identify other possible organisms that may contribute to secondary spoilage fermentations. Given that both yeasts and *L. buchneri* are able of utilizing lactic acid under the acidic conditions that are present after the lactic acid fermentation, it would be useful to monitor the presence of yeasts and LAB with characteristics similar to *I. occidentalis*, *P. manshurica* and *L. buchneri* in addition to pH and acidity in order to better prevent the onset of the spoilage process at the commercial scale.

5. Conclusion

Specific spoilage organisms isolated from commercial secondary cucumber fermentation samples were shown to take part in the process leading to the spoilage of the fermented product. The yeasts *P. manshurica* and *I. occidentalis*, and the LAB *L. buchneri* were able to utilize lactic acid at low pH characteristic of fermented cucumber pickles. The changes produced by these organisms included increase in pH. Additionally, the metabolic activity of the yeasts contributed to the chemical reduction of the fermentation matrix. These changes were necessary for the onset of further lactic acid utilization and the production of propionic and butyric acids by bacteria such as *E. cloacae* and *C. bifermentans*, respectively. Based on the results presented here, we suggest the monitoring of characteristics similar to the yeast isolates and *L. buchneri* to prevent the initiation of the spoilage at the commercial scale.

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