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Microbial interactions associated with secondary cucumber fermentation

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Abstract

Aims: To evaluate the interaction between selected yeasts and bacteria and associate their metabolic activity with secondary cucumber fermentation.
Methods and Results: Selected yeast and bacteria, isolated from cucumber secondary fermentations, were inoculated as single and mixed cultures in a cucumber juice model system. Our results confirmed that during storage of fermented cucumbers and in the presence of oxygen, spoilage yeasts are able to grow and utilize the lactic and acetic acids present in the medium, which results in increased brine pH and the chemical reduction in the environment. These conditions favour opportunistic bacteria that continue the degradation of lactic acid. *Lactobacillus buchneri*, *Clostridium bifermentans* and *Enterobacter cloacae* were able to produce acetic, butyric and propionic acids, respectively, when inoculated in the experimental medium at pH 4·6. Yeast and bacteria interactions favoured the survival of *Cl. bifermentans* and *E. cloacae* at the acidic pH typical of fermented cucumbers (3·2), but only *E. cloacae* was able to produce a secondary product.
Conclusions: The methodology used in this study confirmed that a complex microbiota is responsible for the changes observed during fermented cucumber secondary fermentation and that certain microbial interactions may be essential for the production of propionic and butyric acids.
Significance and Impact of the Study: Understanding the dynamics of the development of secondary cucumber fermentation aids in the identification of strategies to prevent its occurrence and economic losses for the pickling industry.

Introduction

Observations reported during the characterization of commercial fermented cucumber spoilage samples concluded that during bulk storage the fermented product underwent a secondary fermentation in which lactic acid was converted into acetic, propionic and in extreme cases into butyric acids (Franco *et al.* 2012). The degradation of lactic acid led to increases in brine pH, which made the product unstable and suitable for the action of undesirable organisms to become established and produced aromatic compounds associated with spoilage of the fermented product. Yeasts, such as *Pichia manshurica* and *Issatchenkia occidentalis*, and bacteria, such as *Lactobacillus buchneri*, *Clostridium bifermentans* and *Enterobacter cloacae*, have been observed and isolated in commercial spoilage and laboratory reproduced secondary fermentation (Franco *et al.* 2012; Johanningsmeier

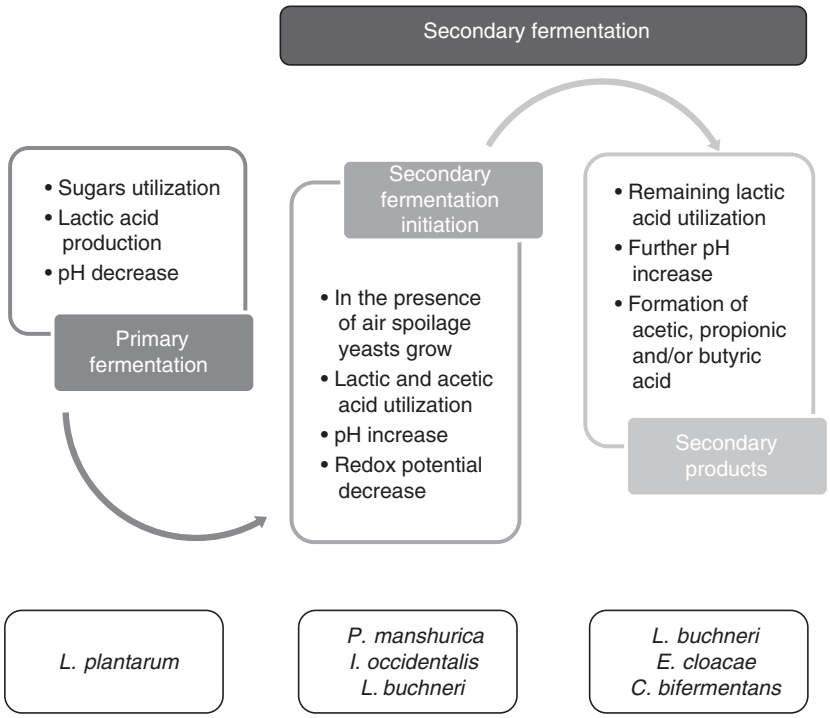

Figure 1 Proposed microbial succession and environmental changes produced during primary fermentation and storage of the fermented cucumbers in $CaCl_2$ brines. Oxidative yeasts, such as *Pichia manshurica* and *Issatchenkia occidentalis*, utilize the organic acids produced during primary fermentation and therefore initiate the secondary fermentation (or spoilage) of cucumber pickles under aerobic conditions. Increases in brine pH and chemical reduction in the environment allow other bacteria, such as *Lactobacillus buchneri, Clostridium bifermentans* and *Enterobacter cloacae*, to grow and continue the utilization of lactic and acetic acids to finally produce propionic and butyric acids (Adapted from Franco and Pérez-Díaz 2012a).

et al. 2012). Studies of secondary fermentations in the model system proposed by Franco and Pérez-Díaz (2012a) lead to the hypothesis that a succession of microbial growth and related changes in the studied environment resulted in the development of secondary cucumber fermentation (Fig. 1). The ability of the yeasts and bacteria isolated from commercial secondary fermentations to utilize lactic and acetic acids contained in fermented cucumber medium was evaluated later (Franco and Pérez-Díaz 2012b).

The spoilage yeasts *P. manshurica* and *I. occidentalis* were able to utilize lactic and acetic acids in the presence of oxygen. Organic acid utilization led to the chemical reduction in the environment and an increase in the medium pH. It seems that these changes could favour the growth of nondesirable bacteria, such as *L. buchneri*, *Cl. bifermentans* and *E. cloacae*, which later were found responsible of converting lactic acid into acetic, butyric and propionic acids, respectively (Franco and Pérez-Díaz 2012b). Acetic acid production was not dependent on pH, while butyric and propionic acids were only detected when the bacteria were inoculated in fermented medium with a pH of 5·0 and above. Production of such organic acids would explain the cheesy and manure-like aroma characteristic of the commercial spoilage of the fermented fruit.

The development of secondary cucumber fermentation has been observed in the commercial process when the fruits are brined with ~5·8% sodium chloride. However, as the pickle industry searches for ways to reduce the NaCl content in the fermentation process, special attention has to be given to the fact that the lack of this preservative in solution will increase the frequency to which the secondary fermentation proceeds. A recent pilot experiment at the commercial scale resulted in stable cucumber fermentations only when potassium sorbate was added to the fermentation tanks during primary fermentation and later bulk storage (McFeeters and Pérez-Díaz 2010).

The commercial fermentation of cucumbers is not a sterile process and it is likely to have a complex microbiota. Furthermore, during current processing practices, the fermentation is carried out in open top fermentation tanks (8000 gallons) that are air-purged to prevent bloater damage to the fruits due to carbon dioxide (CO_2) accumulation (Flemings *et al.* 1975). Tank aeration does not have a defined standard, and in some cases, fermentation tanks are air-purged only during the active lactic acid fermentation, while others are continuously purged during storage (personal communication from several pickle processors).

Therefore, the objectives of this study were to evaluate interactions of selected yeasts and bacteria and its effect on cell growth, organic acids utilization and spoilage products and determine the role of aeration during the spoilage process. In addition, the succession of microorganism proposed in previous research (Franco and Pérez-Díaz 2012a) as responsible for the deterioration process in fermented cucumbers was tested in laboratory-controlled experiments.

Materials and methods

Experimental medium

2A cucumbers were acquired from a local processing company. Juice from the fruit was obtained by processing the cucumbers in a food processor (Juiceman, Jr., Salton Inc., Quebec, Canada). Cucumber juice was centrifuged for 1 h at 10 000 ***g*** (Sorbal RC58; DuPont Instuments, Red Oak, IA, USA). The supernatant was then collected and stored at −20°C before use. Fresh medium, labelled as cucumber juice medium (CJM), was prepared by mixing (50 : 50 – w/w) cucumber juice with cover brine (50 mmol l^{-1} acetic acid and 200 mmol l^{-1} anhydrous $CaCl_2$). The mixture was filter sterilized using a 0·2-μm PES filter membranes (Nalgene; Nalge Nunv International, Rochester, NY, USA). Filter-sterilized CJM was stored at 4°C before use. Fermented cucumber juice medium (FCJM) was obtained by the inoculation of CJM with a mixture of three *L. plantarum* strains LA 445, LA 98 and LA 285 (USDA-ARS Food Science Research Unit, Raleigh, NC, USA; Culture Collection). The mixture was incubated at 30°C for a time that allowed the conversion of sugars into lactic acid. Fermented medium was also filter sterilized (Nalegene; Nalge Nunv International). Ten millilitre aliquots were aseptically transferred to 50- and 15-ml conical tubes (Corning Inc., NY, USA), for aerobic and anaerobic incubation, respectively.

Cultures

The different cultures used during this study were obtained from the culture collection of the U.S. Department of Agriculture-Agricultural Research Service, Food Science Research Unit, located in Raleigh, NC, USA (USDA-ARS, culture collection). Isolates from frozen stock were streaked in appropriate media as described in Table 1. Broth cultures were then harvested at 10 000 ***g*** for 15 min (Centrifuge 5810; Eppendorf, Hamburg, Germany) and washed twice with 0·85% saline solution. The final cell pellet was resuspended with 0·85% saline solution and the density was adjusted by measuring the optical density at 600 nm to achieve a final concentration of approximately 8 log CFU ml^{-1}. For all cultures, serial dilutions were made to achieve the desired inoculation concentrations of 5 log CFU ml^{-1}. All culture media were obtained from Becton, Dickinson & Co. (Sparks, MD, USA).

Evaluation of yeast and bacterial interactions

Bacterial and yeast interactions were evaluated by inoculating filter-sterilized FCJM (prepared as described above) with a mixture of *P. manshurica* and *I. occidentalis* and pure bacterial cultures to a final concentration of 5 log CFU ml^{-1}. Triplicate experimental tubes were incubated at 30°C under aerobic conditions for *L. buchneri*–yeasts and *E. cloacae*–yeasts mixtures, and under anaerobic conditions for *Cl. bifermentans*–yeasts culture.

Table 1 Isolates used in this study

Micro-organism	Culture collection	Source	Culture media
Lactobacillus plantarum	LA 0219	Fermented cucumbers	MRS
L. plantarum	LA 98	Fermented cucumbers	MRS
L. plantarum	LA 445	Fermented cucumbers	MRS
Pichia manshurica	Y88	Spoiled $CaCl_2$fermented brine – Commercial	YMA
Issatchenkia occidentalis	Y89	Spoiled $CaCl_2$-fermented brine – Commercial	YMA
L. buchneri	LA1149	Spoiled $CaCl_2$-fermented brine – Commercial	MRS
Pediococcus ethanolidurans	LA1150	Spoiled $CaCl_2$-fermented brine – Commercial	MRS
Clostridium spp. presumptive *Cl. bifermentans*	B431	Spoiled NaCl-fermented brine – Commercial	DRCA
Enterobacter spp. presumptive *E. cloacae*	B515	Spoiled $CaCl_2$-fermented brine – Laboratory reproduced	VRBG

Cultures were incubated at 30°C for 48 h under aerobic conditions, except for the clostridial culture that was incubated under anaerobic conditions and the enteric culture that was incubated at 37°C.
DRCA, differential reinforced clostridia agar; YMA, yeast and moulds agar; VRBG, violet red bile agar supplemented with 1% glucose.

Evaluation of microbial interactions during secondary cucumber fermentation by successively inoculating pure cultures in cucumber juice medium

To test the ability of the isolates to reproduce the changes characteristic of secondary cucumber fermentation, as determined from commercial (Franco *et al.* 2012) and experimental samples (Franco and Pérez-Díaz 2012a), the isolates were inoculated into fresh CJM following a successive inoculation approach. Two litres (2 l) of filter-sterilized CJM were packed into 2-l glass water-jacketed fermentation vessels of a BioFlo110 Modular Benchtop Fermentor (New Brunswick Scientific Co., Inc., Edison, NJ, USA). The vessels were air-purged at a rate of 2·5 ml min^{-1} controlled by a Matheson PG-1000 (U001) flow meter (Matheson Instruments, Montgomeryville, PA, USA). A constant temperature of 30°C was maintained on the fermentation vessels using a re-circulating chiller (NESLAB Merlin M-75; Thermo Electron Co., Newington, NH, USA). Parameters such as redox

potential (Pt4805-DPAS-SC-K8S/200 redox electrode; Mettler-Toledo, Bedford, MA, USA), dissolved oxygen (dO_2) (Inpro 6830/220 electrode; Mettler-Toledo) and pH (pH electrode model 405-DPAS-SC-K8S/225; Mettler-Toledo) were monitored with time. The measured redox potentials were converted to the redox potential against the standard hydrogen electrode (E_h) by adding 203·4 mV (30°C) according to the manufacturer's instructions. Electrodes for all of these measurements were calibrated, rinsed with 70% ethanol and secured into fittings on the head plate of each fermentation vessel. First, three *L. plantarum* strains LA 445, LA 98 and LA 85 (USDA-ARS, culture collection) were inoculated along with *P. manshurica* and *I. occidentalis* at 5 log CFU ml^{-1}. The inoculated medium was incubated for 1 week to allow for the completion of the primary fermentation. *Lactobacillus buchneri* (5 log CFU ml^{-1}) was inoculated 10 days postpacking, while *Cl. bifermentans* and *E. cloacae* inocula were added (5 log CFU ml^{-1}, each) on day 14.

Impact of the microbial metabolisms on secondary cucumber fermentation

Size 3B (39–51 mm diameter) cucumbers acquired from a local processor were washed with tap water to remove debris. Fruits that presented visual damage (cuts and bruises) were excluded. Fruits and cover brine (50 : 50 – w/w) were packed into four 50-l plastic containers (pails) such that after equilibration the mixture contained 100 mmol l^{-1} anhydrous $CaCl_2$ and 25 mmol l^{-1} acetic acid added as vinegar (20%). A mixture of three *L. plantarum* strains LA 445, LA 98 and LA 385 (USDA-ARS, culture collection) were inoculated (5 log CFU ml^{-1}) into each experimental container. The containers were covered with a translucent food grade plastic wrap and incubated at room temperature. The vessels were exposed to sunlight to prevent film forming yeast growth for 5 h day^{-1}. The primary fermentation was stopped once the cover brine pH approached 3·2 ± 0·3 and sugars were significantly depleted.

The fermented brine was centrifuged at 10 000 ***g*** for 30 min (Sorvall RC-5B; DuPont Instruments, Wilmington, DE, USA). The supernatant was then filter sterilized using a 0·22-μm bottle top filter apparatus (Nalegene; Nalge Nunv International). Three-litre (3 l) aliquots of the sterile brine were aseptically transferred into nine 3·8-l glass jars. The jars were sealed with lids that were fitted with an inlet and outlet for air purging application and a rubber septum for sample collection. A silicone sealant was applied around the fittings holding the air inlet and outlet to prevent air leakage into the jars. Three jars were air-purged, three nitrogen-purged and three kept under anaerobic conditions by placing them inside an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI, USA). Gas flows for the purged jars were kept constant at 5 ml min^{-1} by a Matheson PG-1000 (U001) flow meter (Matheson Instruments). The fermentation jars were inoculated with a mixed culture composed of the spoilage yeasts, *P. manshurica* Y089 and *I. occidentalis* Y090 (USDA-ARS, culture collection) to 5 log CFU ml^{-1} and incubated at 30 ± 3°C.

The secondary fermentation was stopped once the pH of cover brine was 4·5–5·0. Cover brine was spun at 10 000 ***g*** for 15 min and filter sterilized (Nalgene). Two hundred millilitre (200 ml^{-1}) aliquots of the filter-sterilized brine were then packed into 12- to 8-oz sterile glass jars. Each jar was equipped with a gas inlet and outlet and a rubber septum for sample collection. Half of the jars were air-purged and the other half was placed inside an anaerobic chamber (Coy, Laboratory Products Inc.). Three jars were inoculated with *L. buchneri*, three with *Cl. bifermentans*, three with *E. cloacae* and the remaining three were inoculated with a mixed culture of the three bacteria. Cultures were added to the experimental jars in concentrations about 5 log CFU ml^{-1}.

Chemical and microbiological analysis

Samples at different time points were aseptically collected. 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, USA) for the separation of components (McFeeters and Barish 2003). The column temperature was held at 37°C, and sample components were eluted with 0·03 N sulphuric acid at a flow rate of 0·6 ml min^{-1}. 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 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.

Aseptically collected samples were serially diluted in 0·85% saline solution and spiral plated using an Autoplate 400 (Spiral Biotech, Norwood, MA, USA) on Lactobacilli deMan, Rogosa and Sharpe agar (MRS; Becton Dickinson & Co., Franklin Lakes, NJ, USA) supplemented with 1% cycloheximide (0·1% solution; Oxoid, Hampshire, UK); yeast and moulds agar (YMA; Becton, Dickinson & Co.) supplemented with 0·01% chloramphenicol (Sigma-Aldrich, St Louis, MO, USA) and 0·01% chlortetracycline (Sigma-Aldrich); violet red bile agar supplemented with 1% glucose (VRBG; Becton, Dickinson &

Co.); and differential reinforced clostridia agar (DRCA; Becton, Dickinson & Co.) supplemented with 1% cycloheximide (Oxoid) for the enumeration of presumptive lactic acid bacteria (LAB), yeasts, enterobacteria and *Clostridium* spp., respectively. MRS and YMA plates were incubated at 30°C anaerobically and aerobically, respectively, for 48 h. Purple and pink colonies after 24-h aerobic incubation on VRBG plates were recorded as presumptive enterobacteria. Black colonies after 48 h of anaerobic incubation on DRCA plates were recorded as *Clostridium* spp.

Results

Interaction of bacteria and yeasts in fermented cucumber juice medium

Changes in FCJM medium pH were observed concomitantly with lactic acid utilization and acetic acid production when the spoilage yeasts and *L. buchneri* were co-cultured (Fig. 2a). A total of 41·7 ± 1·5 mmol l^{-1} lactic acid was consumed, and 27·4 ± 1·9 mmol l^{-1} acetic acid was produced, corresponding to a final medium pH of 4·5. *Lactobacillus buchneri* population increased 1 log in the first week of incubation (Fig. 2b). Increase in the bacterium population could be attributed to the utilization of the remaining sugars in the medium (Liu *et al.* 2008). *Lactobacillus buchneri* remained at about 6 log CFU ml^{-1} during the lactic acid utilization stage.

While the co-culture of *E. cloacae* and the spoilage yeasts did not have any positive effect in the bacterial growth (Fig. 3a), the mixed culture of *Cl. bifermentans* and the spoilage yeasts enhanced the survival of the bacterium at the lower initial pH of the FCJM (3·8). The bacterium increased 1 log in population, while spoilage yeast counts decreased with time presumably due to the

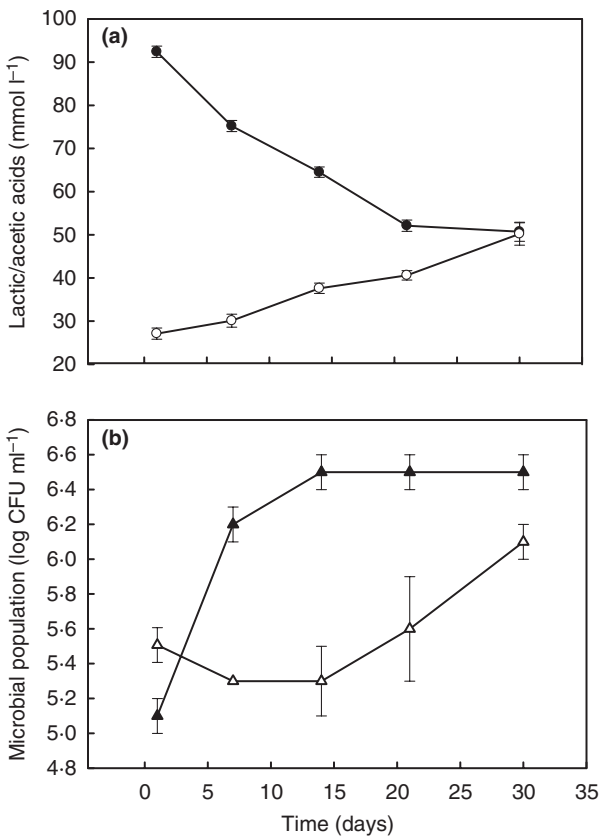

Figure 2 Characteristics of *Lactobacillus buchneri* and spoilage yeasts mixed culture in fermented cucumber juice medium (FCJM) with an initial pH of 3·8. Changes in lactic (●) and acetic (○) acids concentrations are shown in (a), while changes in *L. buchneri* (▲) and spoilage yeasts (△) populations are shown in (b). Values represent the mean ± standard deviation of six replicates incubated under aerobic conditions (30 ± 2°C) for 30 days.

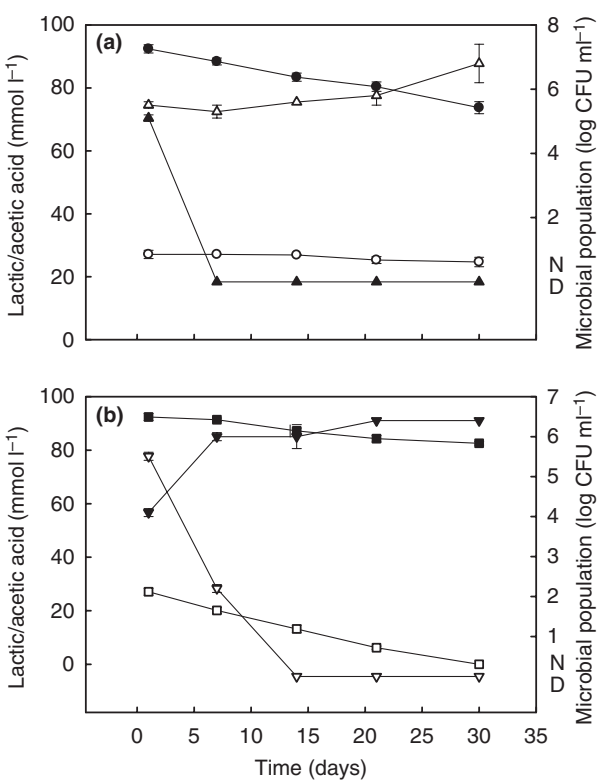

Figure 3 Characteristics of mixed cultures in fermented cucumber juice medium with an initial pH of 3·8. (a) Changes in *Enterobacter cloacae* (▲) and yeast (△) populations and lactic (●) and acetic (○) acids concentrations with time. (b) Changes in *Clostridium bifermentans* (▼) and yeasts (▽) populations and lactic (■) and acetic (□) acids concentrations as a function of time. ND, Cells were not detected (below the detection threshold of 2·6 log CFU ml^{-1}). Values represent the mean ± standard deviation of six replicates incubated for 30 days.

lack of oxygen required to grow (Fig. 3b). However, butyric acid production by *Cl. bifermentans* was not detected in the mixed culture.

Effect of mixed cultures in the development of secondary fermentation in cucumber juice medium

Interactions between the micro-organisms potentially involved in the spoilage were studied by successively inoculating their pure cultures in a fermentation system as described in Table 2. Lactic acid produced during the primary fermentation by *L. plantarum* was consumed by the yeasts, which reached more than 6 log CFU ml^{-1} within the first week of experimentation. This in turn increased the medium pH and induced the chemical reduction in the fermentation matrix (Tables 2 and 3). Propionic acid production was noticed at day 14 at pH 3·7, while butyric acid production was detected on day 30 when the fermentation matrix was further reduced (−110·5 ± 14·8 mV) and the pH was 5·5 ± 0·1 (Table 2).

The community effect favoured the survival of the non-pH tolerant isolates, specifically *E. cloacae* (Table 6, mixed culture experiments). Although *E. cloacae* populations decreased to 0·6 log CFU ml^{-1} after inoculation, probably due to the acid stress, the bacterium population increased to a final density of 5·4 log CFU ml^{-1}. *Clostridium bifermentans* populations increased after its inoculation about 0·6 log CFU ml^{-1}.

Table 2 Changes in pH, redox potential and dissolved oxygen, substrates and products observed as the result of the successive inoculation of selected isolates in cucumber juice medium as described in Fig. 1

Time (days)	Organisms present at the sampling point	pH	Lactic acid (mmol l^{-1})	Acetic acid	Propionic acid	Butyric acid	dO_2 (mg l^{-1})	Redox potential (E_h, mV)
0	LP, PM, IO	4·2 ± 0·04	ND	23·6 ± 0·3	ND	ND	ND	322·0 ± 3·1
7	LP. PM, IO	3·3 ± 0·0	72·6 ± 1·7	14·0 ± 0·2	ND	ND	1·6 ± 0·0	369·5 ± 3·5
14	LP, PM, IO, LB, CB, EC	3·7 ± 0·05	29·8 ± 0·9	1·6 ± 0·03	4·0 ± 0·1	N/D	3·7 ± 0·1	171·5 ± 2·1
21	LP, PM, IO, LB, CB, EC	4·2 ± 0·1	19·0 ± 0·7	0·8 ± 0·1	4·0 ± 0·6	N/D	1·3 ± 0·1	153·0 ± 9·9
30	LP, PM, IO, LB, CB, EC	5·5 ± 0·15	ND	0·5 ± 0·1	4·1 ± 0·2	3·3 ± 0·03	ND	−110·5 ± 14·8
40	LP, PM, IO, LB, CB, EC	6·0 ± 0·01	ND	0·5 ± 0·1	4·6 ± 0·0	3·8 ± 0·02	0·6 ± 0·1	−83·5 ± 3·5
60	LP, PM, IO, LB, CB, EC	6·5 ± 0·01	ND	0·5 ± 0·1	4·0 ± 0·3	3·8 ± 0·02	0·6 ± 0·01	−41·0 ± 2·8
Anaerobic Control*	LP, PM, IO, LB, CB, EC	3·3 ± 0·1	81·5 ± 0·2	28·7 ± 0·1	ND	ND	NA	382·5 ± 1·5

Values represent the mean ± standard deviation of six replicates incubated for 60 days.
LP, *Lactobacillus plantarum*; PM, *Pichia manshurica*; IO, *Issatchenkia occidentalis*; LB, *L. buchneri*; CB, *Clostridium bifermentans*; EC, *Enterobacter cloacae*; ND, not detected; NA, not available.
*Control experiments corresponded to the same experimental design, but excluding air purging. The results showed in the table represent samples collected after 60 days of experimentation.

Table 3 Population changes observed during incubation of the spoilage isolates in cucumber juice medium in a succession approach

Time (days)	Organisms present at the sampling point*	*L. plantarum** (Log CFU ml^{-1})	Yeasts*	*L. buchneri*†	*E. clocae*	*Cl. bifermentans*
0	LP, PM, IO	5·0 ± 0·05	5·5 ± 0·1	–	–	–
7	LP. PM, IO	6·9 ± 1·1	7·3 ± 0·7	–	–	–
14	LP, PM, IO, LB, CB, EC	6·9 ± 0·0	6·2 ± 0·0	5·9 ± 0·0	5·1 ± 0·3	4·9 ± 0·3
21	LP, PM, IO, LB, CB, EC	5·6 ± 0·0	6·2 ± 0·1	5·7 ± 0·1	4·5 ± 0·7	5·1 ± 0·2
30	LP, PM, IO, LB, CB, EC	4·4 ± 0·1	6·3 ± 0·1	5·6 ± 0·1	5·4 ± 0·0	5·5 ± 0·3
Anaerobic Control†	LP, PM, IO, LB, CB, EC	1·2 ± 0·1	ND	1·5 ± 0·1	ND	ND

Values represent the mean ± standard deviation of six replicates incubated for 30 days.
LP, *Lactobacillus plantarum*; PM, *Pichia manshurica*; IO, *Issatchenkia occidentalis*; LB, *L. buchneri*; CB, *Clostridium bifermentans*; EC, *Enterobacter cloacae*; ND, not detected; NA, not available.
*Organisms were successively inoculated as explained in Materials and Methods section following the succession approach proposed in Fig. 1.
†Control experiments corresponded to the same experimental design, but excluding air purging. The results showed in the table represent samples collected after 30 days of experimentation.

Impact of the microbial metabolism on the secondary cucumber fermentation

Although a secondary fermentation was observed when the selected isolates were successively inoculated in CJM (described in the previous section), the changes observed could not be attributed to a specific micro-organism, the overall community effect or specific metabolites. Therefore, in this section, a similar successive inoculation approach was followed with the introduction of a filtration step between the inoculations of the micro-organisms. Following the approach described in Fig. 1, once the expected change was observed, the experimental medium was spun to remove the micro-organisms responsible for the change, and the filter-sterilized medium was inoculated with the corresponding micro-organism for further experimentation.

As expected, a primary fermentation was observed when the cucumber fruits were inoculated with the *L. plantarum* culture (Table 4). As the bacteria utilized sugars, brine pH decreased, while lactic acid concentration and LAB populations increased. Seven days post-packing brine pH was 3·1 ± 0·1 corresponding with 90·3 ± 4·3 mmol l^{-1} of lactic acid. The population of natural yeasts increased 1 log during the progression of the primary fermentation. No film forming yeasts were observed in any of the experimental containers. As the environment became more acidic, enterobacterial population, naturally present at the beginning of the experimentation, disappeared (Table 4).

The oxidative metabolism of the spoilage yeasts was observed only when the experimental jars containing filter-sterilized medium were air-purged (Table 5). About 30 mmol l^{-1} of lactic acid was utilized during aerobic yeast metabolism, which in turn increased the medium pH to 4·5. Acetic acid, originally in concentrations about 25 mmol l^{-1}, was decreased to a final concentration of 17·6 ± 0·1 mmol l^{-1} by day 14 (Table 6). No significant changes in pH or organic acids were observed in nitrogen-purged jars or in those kept under anaerobic conditions.

Table 4 Changes in substrates, products and microbial populations during laboratory primary fermentation of 2A cucumber fruits in cover brines containing 100 mmol l^{-1} $CaCl_2$ and 25 mmol l^{-1} acetic acid after equilibration

Variable	Initial conditions	After primary fermentation
pH	4·2 ± 0·2	3·1 ± 0·1
Glucose (mmol l^{-1})	12·9 ± 1·6	1·3 ± 0·2
Fructose (mmol l^{-1})	18·0 ± 2·0	5·2 ± 0·3
Lactic acid (mmol l^{-1})	ND	90·3 ± 4·3
Acetic acid (mmol l^{-1})	25·4 ± 0·3	23·4 ± 3·2
LAB population (log CFU ml^{-1})	5·3 ± 0·1	8·3 ± 0·4
Yeast population (log CFU ml^{-1})	3·0 ± 0·0	4·1 ± 0·2
Enterobacterial population (log CFU ml^{-1})	3·3 ± 0·1	ND
Clostridia population (log CFU ml^{-1})	ND	ND

Values represent the mean ± standard deviation of six replicates incubated for 7 days.
ND, Not detected; LAB, lactic acid bacteria.

Table 5 Changes in organic acids, pH, redox potential and the yeast population observed during secondary fermentations under air-purged and nitrogen-purged and anaerobic conditions

Variable	Initial conditions (day 7)	Characteristics by the 10th day of fermentation		
		Air-purged	Nitrogen-purged	Anaerobic
pH	3·1 ± 0·0	4·5 ± 0·0	3·1 ± 0·0	3·1 ± 0·0
Redox potential (E_h, mV)	234·5 ± 3·5	−100·4 ± 1·2	240·1 ± 3·2	NA
Lactic acid (mmol l^{-1})	89·7 ± 1·2	60·6 ± 0·9	89·1 ± 1·2	89·1 ± 1·2
Acetic acid (mmol l^{-1})	23·8 ± 1·3	17·6 ± 0·1	23·1 ± 1·9	23·2 ± 1·1
Yeast population (log CFU ml^{-1})	5·3 ± 0·1	7·1 ± 0·2	3·1 ± 0·1	3·2 ± 0·2

Values represent the mean ± standard deviation of six replicates incubated for 10 days.
NA, Not available.

The inoculation of the bacterial isolates into the filter-sterilized brine led to further lactic acid degradation with the concomitant production of the spoilage products (Table 6). The metabolic activity of *L. buchneri* was similar under both aerobic and anaerobic conditions. The bacterium as previously reported was able to convert lactic acid into acetic acid with the concomitant increase in brine pH (Franco and Pérez-Díaz 2012b; Johanningsmeier *et al.* 2012). *Clostridium bifermentans* was able to utilize lactic acid when incubated under anaerobic conditions (Franco and Pérez-Díaz 2012b). As lactic acid was degraded by the bacterium, the medium pH increased and butyric acid was formed (Table 6). The enteric isolated behave similarly under both aerobic and anaerobic conditions (Table 6). About 40 mmol l^{-1} lactic acid was consumed by the bacterium. The concentration of acetic acid increased by 14 mmol l^{-1}, and similar amount of propionic acid was detected. The disappearance of lactic acid and the formation of secondary products resulted in an increase in medium pH by 2 units.

In the mixed bacterial culture (*L. buchneri*, *Cl. bifermentans* and *E. cloacae*) experiments, lactic acid utiliza-

Table 6 Lactic and acetic acid utilization by *Lactobacillus buchneri*, *Clostridium bifermentans* and *Enterobacter cloacae* under controlled laboratory conditions

Variable	Initial conditions (day 12)	Characteristics after 30 days of fermentation	
		Air-purged	Anaerobic
L. buchneri			
pH	4·6 ± 0·1	5·0 ± 0·0	5·0 ± 0·0
Lactic acid (mmol l^{-1})	59·6 ± 1·8	49·1 ± 0·2	47·3 ± 0·3
Acetic acid (mmol l^{-1})	15·5 ± 1·6	22·3 ± 0·1	21·7 ± 0·1
Propionic acid (mmol l^{-1})	ND	ND	ND
Population (log CFU ml^{-1})	5·1 ± 0·0	5·6 ± 0·4	5·2 ± 0·1
Cl. bifermentans			
pH	4·6 ± 0·1	4·7 ± 0·1	5·9 ± 0·2
Lactic acid (mmol l^{-1})	60·1 ± 1·2	55·9 ± 0·1	25·2 ± 0·1
Acetic acid (mmol l^{-1})	15·1 ± 1·3	18·1 ± 0·5	10·5 ± 0·2
Butyric acid (mmol l^{-1})	ND	3·1 ± 0·5	38·1 ± 0·1
Propionic acid (mmol l^{-1})	ND	ND	ND
Population (log CFU ml^{-1})	4·9 ± 0·0	ND	5·3 ± 0·0
E. cloacae			
pH	4·6 ± 0·1	6·9 ± 0·1	7·0 ± 0·1
Lactic acid (mmol l^{-1})	60·1 ± 1·2	21·2 ± 0·6	23·1 ± 0·4
Acetic acid (mmol l^{-1})	17·1 ± 1·3	26·5 ± 0·0	27·1 ± 0·1
Propionic acid (mmol l^{-1})	ND	14·0 ± 0·1	15·1 ± 0·2
Butyric acid (mmol l^{-1})	ND	ND	ND
Population (log CFU ml^{-1})	5·2 ± 0·0	7·1 ± 0·1	6·9 ± 0·3
Mixed culture: *L. buchneri, Cl. bifermentans* and *E. cloacae*			
pH	4·6 ± 0·1	5·9 ± 0·3	5·7 ± 0·0
Lactic acid (mmol l^{-1})	60·2 ± 0·1	25·7 ± 0·4	27·1 ± 0·3
Acetic acid (mmol l^{-1})	17·1 ± 1·2	28·3 ± 0·3	23·1 ± 0·5
Butyric acid (mmol l^{-1})	ND	4·5 ± 1·2	10·1 ± 0·1
Propionic acid (mmol l^{-1})	ND	17·1 ± 0·2	15·3 ± 0·2
LAB population (log CFU ml^{-1})	5·1 ± 0·0	6·5 ± 0·0	6·4 ± 0·0
Enterobacterial population (log CFU ml^{-1})	5·3 ± 0·1	5·3 ± 0·0	5·4 ± 0·0
Clostridia populations (log CFU ml^{-1})	4·9 ± 0·0	3·0 ± 0·0	5·7 ± 0·0

Values represent the mean ± standard deviation of six replicates incubated for 30 days.
ND, Not detected; LAB, lactic acid bacteria.

tion and increase in medium pH were similar under air-purged and anaerobic conditions (Table 6). About 80% of lactic acid was consumed by the spoilage isolates, which in turn increased the medium pH to 5·9 and 5·7 under air-purged and anaerobic conditions, respectively. As lactic acid was degraded, acetic, propionic and butyric acids were detected in the medium. Although small amounts of butyric acid were detected under air-purged conditions, the production of the fatty acid was enhanced under anaerobic environments. The greatest increase in cell population was detected for *L. buchneri*, which increased 1·4 and 1·3 logs under air-purged and anaerobic conditions, respectively. *Clostridium bifermentans*, on the other hand, decreased about 2 logs when incubated under air-purged conditions, but did not disappear after 30 days of incubation. Under anaerobic conditions, the bacterium increased 0·8 logs. Significantly, higher *Cl. bifermentans* populations were associated with larger production of butyric acid under anaerobic conditions. Interestingly, *E. cloacae* populations remained stable at 5·3 log CFU ml^{-1} under both conditions, suggesting that in mixed culture, the bacterium is outcompeted by other organisms and it might be using a survival (no growth) mechanism.

Discussion

A variety of micro-organisms have been observed and isolated from secondary cucumber fermentations. The selected yeasts and bacteria evaluated in this study were proven to have a role during the onset and development of this secondary fermentation. Individual micro-organisms were shown to contribute to different changes in the matrix of study, and together the bacterial and yeast interactions resulted in the controlled reproduction of the spoilage process with results comparable to the commercial secondary cucumber fermentation.

Interestingly, the interaction of bacteria and yeasts in FCJM affected differently each studied bacterium; and this metabolic responses were evidence of the complex microbiota and chemical reactions involved during spoilage or secondary cucumber fermentations.

Different *Lactobacillus* spp. have been reported as able to utilize lactic acid, among them *L. buchneri* LMG6892 has been reported as able to utilize lactic acid in experimental medium and silage and produce acetic acid and 1,2 propanediol, under anaerobic conditions (Oude-Elferink *et al.* 2001). The conversion of lactic acid into another organic acid of lower pKa and an alcohol seems to help the bacterium cope with the environmental stress, but does not support growth (Oude-Elferink *et al.* 2001).

In our experiments, the lack of *L. buchneri* growth concomitantly with lactic acid disappearance from the medium suggests that the utilization of the organic acid plays a role in the survival stress response. Different stress response systems that allow LAB to withstand harsh conditions have been reported; and even though the molecular bases of adaptive response are species specific, it seems that *L. buchneri* stress response is related to a starvation state more than an acid stress response (Van de Guche *et al.* 2002). As observed in this study, the bacterium is able to utilize sugars present in the fermented medium and increase cell population at the initial pH of 3·8 in co-culture with the yeasts, and at lower pH in pure culture (Johanningsmeier 2011; Franco and Pérez-Díaz

2012b). However, once the carbohydrates are depleted, the bacterium growth ceased and in turn lactic acid utilization initiates. Glucose starvation in LAB induces an increase resistance to many stress conditions, including oxidative and osmotic stress (Van de Guche *et al.* 2002), and therefore, it might contribute to longer survival of the bacterium in the environment encountered in commercial cucumber fermentations.

Interestingly, the changes observed in the *L. buchneri* and yeast mixed culture were similar to those reported from commercial spoilage samples (Franco *et al.* 2012), in which decreases in lactic acid were inversely associated with brine pH and acetic acid concentration. In this study, and as expected, about 45% lactic acid disappeared, presumably due to the metabolic activity of both the bacterium and the yeasts. Similarly, to the *L. buchneri* and yeasts mixed culture observations, lactic acid in commercial spoilage samples was not fully depleted (Franco *et al.* 2012). Pure cultures of *L. buchneri* have also been determined to utilize up to 80% of the lactic acid present in the culture medium but will not fully deplete the organic acid (Johanningsmeier *et al.* 2012), while the yeasts alone may fully utilize the lactic acid present in culture media (Franco *et al.* 2012).

The onset of lactic acid utilization by pure cultures of *L. buchneri* in FCJM takes about 10 days and the bacterium decreases significantly in population after 20 days of incubation (Franco and Pérez-Díaz 2012b). Figure 2 shows that lactic acid utilization in a mixed culture of *L. buchneri* and spoilage yeasts initiates after 1 day of inoculation, even though a drop in the yeast counts is initially detected. Together, these observations suggest that the co-culture of *L. buchneri* and yeasts improves the ability of the bacterium to utilize lactic acid by either inducing a stress response system that includes lactic acid utilization and/or by providing nutrient factors that allow for longer survival. At the commercial scale, a mixture of organisms is present, and therefore, longer survival chances for *L. buchneri* will traduce in higher chances to induce secondary fermentation.

Co-culture of yeasts and the clostridial isolate favoured the survival of the bacterium under acidic pH (3·8). In pure cultures, the acidic condition of FCJM inhibited the ability of *Cl. bifermentans* to remain metabolically active, and after 2 days of incubation, the bacterium was not detected in the medium (Franco and Pérez-Díaz 2012b). However, the *Cl. bifermentans* population in the mixed culture increased one log and remained at that level during the 30 days of incubation, but no butyric acid production was observed. Production of butyric acid by *Clostridia* and other anaerobic organisms has been reported as dependent on the presence of other metabolites, such as acetic acid (Bhat and Barker 1947; Bourriard *et al.* 2005). In our experiments, the acetic acid present in the fermented medium was quickly utilized (Fig. 3), which might inhibit the formation of butyric acid by the clostridial isolate.

The overall community effect was observed when the selected isolates were inoculated in CJM following a successive inoculation approach (Tables 2 and 3). In contrast to what was previously reported by Franco and Pérez-Díaz (2012a), propionic acid was produced before butyric acid (Table 3). It seems that production of propionic acid is not dependent on the chemical reduction in the fermentation environment and it could be produced at a lower pH as compared to butyric acid. Similar observations were reported during the characterization of commercial spoilage samples (Franco *et al.* 2012). About 50% of the studied commercial samples had detectable levels of propionic acid, while only two of 42 samples contained butyric acid levels and presented at pH of 4·2 and 4·7. Moreover, negative E_h values were only observed in samples in which butyric acid was detected, while the other spoiled samples showed positive E_h values. Based on the characterization of commercial samples and laboratory experiments, it seems that the chemical reduction in the fermentation matrix is strongly related to high yeast populations, which is a scenario with a lower probability to occur at the commercial scale.

Following the same successive inoculation approach, but including a cleaning step before the different inoculations, it was possible to reproduce the secondary fermentation and relate each selected isolate with different changes in the experimental medium (Tables 4, 5 and 6). Our results confirmed that after the primary fermentation is completed, the presence of oxygen favours the development of yeasts and its oxidative metabolism is trigged (Table 5) (Franco and Pérez-Díaz 2012a,b). A transporter for monocarboxylic acids has been reported for the yeast *Candida utilis* (Cássio and Leão 1993), and although it is unknown if the spoilage yeasts studied here have the same transport system for the assimilation of organic acids, the two isolates were able to degrade lactic and acetic acids under air-purged conditions in pure cultures (Franco and Pérez-Díaz 2012b).

The formation of secondary products was attributed to the spoilage bacteria, which were able to produce butyric, propionic and acetic acids (Table 6). Butyric acid production has been associated with clostridial metabolism, and although this bacterium is an obligate anaerobe, under air-purged conditions, small amounts of lactic acid were degraded concomitant with butyric and acetic acid production. In commercial spoilage samples, butyric acid, usually associated with the presence of *Clostridium* spp., was detected in fermentation tanks with reduced environments (negative E_h values) but also small amounts were

detected in oxidized environments (Franco *et al.* 2012). Regardless of redox potential, the short fatty acid was present only when brine pH was above 4·0. It has been reported that certain *Clostridium* spp. are able to metabolize oxygen present in growth medium using a NADH-/NADPH-dependent mechanism (Kawasaki *et al.* 1998). This mechanism along with a set of oxygen scavenger enzymes allows the bacterium to eliminate oxygen in the medium and continue with cell growth. Using this strategy, *Clostridium acetobutylicum* is capable to survive and produce acetic acid (O'Brien and Morris 1971). In the experimental design used in this study, an air flow was constantly supplied into the fermentation jars, and therefore, it is possible that some *Cl. bifermentans* cells were able to utilize the oxygen as an electron acceptor to derive energy, but because oxygen supply was kept constant, the bacterium was not able to keep up with the oxygen uptake and eventually died off.

Similar metabolic activity was observed for *E. cloacae* when incubated in the fermented medium under air-purged and anaerobic conditions. The bacterium was able to utilize lactic acid and produce propionic and acetic acids, with the concomitant increase in medium pH and cell population (Table 6). *Enterobacter cloacae* strains isolated from spontaneous fermentations of Italian table olives were reported as able to grow in nutrient medium supplemented with citric, ascorbic and lactic acid (Bevilacquia *et al.* 2009). Growth and metabolic activity was not inhibited when the medium was supplemented with lactic acid to a final pH of 4·5, but lower growth rates were reported as compared to growth in citric acid- or ascorbic acid-supplemented medium (Bevilacquia *et al.* 2009). Enterobacteria, naturally present in the cucumber fruits, are usually inhibited during the primary fermentation of cucumber fruits as the pH decreases and lactic acid concentration increases (Etchells *et al.* 1945). However, *E. cloacae* populations have been isolated from air-purged fermentations once the medium pH is above 4·5 (Franco and Pérez-Díaz 2012a), suggesting that the proper environmental conditions will encourage the growth of the bacterium. It is possible that under acidic and glucose starvation conditions characteristic of fermented cucumber environments, a viable but nonculturable state (VBNC) is trigged in the bacterium. Cells in the VBNC state typically demonstrate very low levels of metabolic activity, but on resuscitation are again culturable. During the characterization of commercial fermented cucumber spoilage samples, enterobacteria cells were cultured in VRBG agar plates after the incubation of samples aliquots in enterobacteria enrichment broth to allow resuscitation.

The changes observed in the mixed culture experiments were similar to the chemical characteristics reported for commercial spoilage samples (Franco *et al.* 2012). In commercial samples, lactic acid concentrations decreased, but rarely depleted, while the acetic acid concentration increased. Samples in which butyric and propionic acids were detected presented a brine pH above 4·0.

The role of microbial communities in different environments has been studied individually for bacteria and fungi. However, recent studies have suggested that the interaction between these organisms form physic and metabolic interdependent properties distinct from their single components (Tarkka *et al.* 2009). The same was observed in our results, in which the abilities to survive and produce specific metabolites were observed only when the spoilage yeasts and bacteria were grown in mixed cultures.

The interaction between fungi and bacteria can be observed in different mechanisms, among them via the modulation of the physiochemical environment is a relevant one. A common effect is the alteration of the pH, because some micro-organisms (e.g. streptococci, lactobacilli and *Candida*) can occupy environments under a broad range of pH conditions (O'May *et al.* 2005). Changes in pH can affect microbial growth of acid-sensitive organisms, as demonstrated in wine (Alexandre *et al.* 2004) and cheese production (Fox *et al.* 1990). For example, on cheese surfaces, yeasts lactate metabolism and the production of alkali metabolites such as ammonia cause de-acidification that favours the growth of less-acidic tolerant bacterial strains that are essential for cheese ripening, including those that are responsible for propionic acid production (Corsetti *et al.* 2001). Similarly, during the secondary cucumber fermentation, the role of the spoilage yeasts is crucial for organic acids utilization and pH increase, which later allows the establishment of other opportunistic bacteria to produce the secondary fermentation products (propionic and acetic acid). In addition, the environmental pH can also influence other microbial processes, such as the rate synthesis of secondary products (Frey-Klett *et al.* 2011). This activity was observed in our experiments in which *L. buchneri* growth and rate of lactic acid degradation was greater when the bacterium was inoculated in mixed cultures with the spoilage yeast.

Our experiments corroborated that the presence of oxygen is necessary to trigger the rapid onset of the spoilage process by the action of oxidative yeasts (Franco and Pérez-Díaz 2012a). Air purging is a common practice used in the pickle industry to prevent cucumber bloating (Flemings *et al.* 1975). Observations made from standard commercial fermentation tanks suggest that tanks present a gradient of dO_2 dependent on the fermentation stage among other factors (unpublished). Oxygen availability is not uniform during the initiation of primary fermentation and equilibration of the cucumbers and cover brine

solution in the tank. As the fermentation age increases, dO_2 levels may vary between 0·5 and 6·0 mg l^{-1}, depending on environmental temperature and microbial activity among other factors. During the winter season, the lower temperatures induce a higher oxygen intake rate by the tanks. Availability of oxygen during fermentation and/or bulk storage might favour the establishment of the oxidative-spoilage yeasts.

The results reported here corroborate the knowledge that a community of microbes is responsible for the secondary fermentation observed in fermented cucumber spoilage. Although similar changes were observed in the commercial and laboratory scale following the succession of selected spoilage isolates, the matrix in which cucumbers are fermented and stored at the commercial scale is more complex, suggesting that other micro-organisms might also be involved in the spoilage. For instance, propionic acid production in fermented cucumber medium was reported as the catabolic activity of *Lactobacillus rapi* (Johanningsmeier 2011; Johanningsmeier *et al.* 2012). It is clear that synergistic activities are necessary to trigger the posttanking fermentations. In that sense, and based on the metabolic activity of the selected isolates described here, the presence of spoilage yeasts and oxygen in the fermentation tanks is a risk factor for the initiation of the spoilage process. Oxidative yeasts have been reported as able to transport and utilize a wide range of organic acids (Dakin and Day 1958; Ruiz-Cruz and Gonzalez-Cancho 1969; Cássio and Leão 1993; Piper *et al.* 2001). Therefore, strategies to prevent the development of these yeasts should be encouraged in the fermented cucumber industry.

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