ABSTRACT

FRANCO MELAZZINI, WENDY VERONICA. Effect of Aeration in Calcium Chloride Cucumber Fermentation. (Under the direction of Drs. Ilenys M. Pérez-Díaz and Roger F. McFeeters).

The preservation of cucumber fruits in the form of fermented cucumber pickles is a traditional process used to increase the shelf life of the final product and enhance its flavor. The process, carried out by the natural lactic acid bacteria (LAB) present in the fruits, proceeds at the commercial scale in large fiberglass fermentation tanks on which cover brine solutions, containing sodium chloride (NaCl) concentrations (about 6%), are packed with the fresh fruits. The LAB metabolism results in utilization of sugar and production of lactic acid with the concomitant decrease in the pH of cover brine solutions. These changes in chemistry work synergistically to preserve the product for a prolonged period of time. Recently, the U.S. Environmental Protection Agency (EPA) has challenged the food industry to reduce the NaCl content used in most processed food products. These changes will significantly affect the fermented cucumber industry which relies on the high salt concentration to ensure a proper fermentation process. Several studies to reduce NaCl content for the preservation of fresh cucumbers have been conducted. Recently, calcium chloride ($CaCl_2$) fermentations have been successfully used for the fermentation of the fruits under anaerobic conditions. However, at the commercial scale fermentations currently proceed under the presence of oxygen, introduced to the fermentation tanks by air-purging practices. The presence of oxygen provides an opportunity for selected microorganisms to become established and spoil the fermented cucumbers. Occasionally as reported by commercial pickle producers, the spoilage of fermented cucumbers is characterized by an increase in cover brine pH, reduced lactic acid concentration and, in some cases, strong odors resembling manure and cheese like

aromas. Characterization of this phenomenon has been limited in part due to the scarce availability of commercial spoilage samples and the complex microbiota involved in the process. Laboratory efforts, conducted under anaerobic conditions, concluded that several bacteria are able to utilize lactic acid when the medium pH is above 4.5 and NaCl is reduced to 2%. However, the fermented product has a final pH of 3.3 ± 0.3, suggesting that other microorganisms may be involved in the initiation of the spoilage process. In this study, the opportunity presented by a spoilage outbreak in 2010 resulted in the characterization of a significant number of commercial spoiled samples. The outbreak samples were microbially and chemically compared to samples from commercial standard fermentations. A number of yeasts and bacteria were isolated from this outbreak study. Similar morphologies, observed when the spoilage was reproduced in the laboratory using a $CaCl_2$ model system, were associated with the changes that led to the secondary fermentations. Selected microorganisms were studied for their ability to initiate and propagate the secondary fermentation that led to the spoilage of the fermented product in $CaCl_2$ fermentation in the presence of air. The oxidative yeasts *Issatchenckia occidentalis* and *Pichia manshurica* and the bacterium *Lactobacillus buchneri* were able to utilize lactic acid under these aerobic conditions. Organic acid utilization by the yeasts was faster than the bacterium, and led to chemical reduction of the fermentation environment. Changes in redox potential and the increase in brine pH due to organic acid utilization were determined to be necessary for the establishment of anaerobic and facultative anaerobic microorganisms to continue lactic acid utilization. *Clostridium bifermentans* and *Enterobacter cloacae* were able to convert lactic acid into butyric and propionic acids, respectively, but only when the initial medium pH was above 4.5. The anaerobic/reduced fermentation environment was necessary for the

Clostridium isolate to be metabolically active. The interactions between yeasts and bacteria in the model system enhanced the survival of *C. bifermentans* and increased *L. buchneri* cell populations at low pH (3.8), however did not affect positively the survival of the enterobacteria isolate. Although, it is clear that synergistic activities are necessary to trigger the secondary fermentation, the complete characterization of fermented and spoilage samples is a complex task, and more studies are necessary to identify other possible organisms related to the secondary fermentation. Given that both yeasts and the spoilage LAB, *L. buchneri* are capable of utilizing lactic acid under the acidic conditions prevailing after the lactic acid fermentation, we suggest that commercial pickle producers monitor, besides pH and acidity, the numbers and presence of yeasts and LAB with morphologies similar to *I. occidenlatilis, P. manshurica* and *L. buchneri* in order to better control the onset of the spoilage process.

Effect of Aeration in Calcium Chloride Cucumbers Fermentation

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor in Philosophy

Food Science

Raleigh, North Carolina

2011

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DEDICATION

This dissertation is dedicated to Alfo my strength and support.

"Por dos futuros venturosos"

BIOGRAPHY

Ms. Wendy Franco was born and raised in La Paz (Bolivia). She has been always passionate and intrigued by the microorganisms and their interaction with food matrixes. After earning her Bachelor degree in Food Engineering (2000), Ms. Franco worked as a researcher and a lecturer at the Food Engineering department of Our Lady of La Paz University. Eager to continue her education, in Fall 2006 she was granted with a Fulbright Scholarship and joined the University of Florida to pursue dual Master degrees in Food Science and Agribusiness under the direction of Dr. Amy H. Simonne. Her master research focused in the characterization of pathogens survival on acidified sauces. This wonderful experience impulse her to continue her academic training and in Spring 2009 she joint the North Carolina State University – Food, Bioprocessing and Nutrition Sciences Program as a Ph.D. student under the direction of Dr. Ilenys M. Pérez-Díaz. Her dissertation work has focused on the study and characterization of microbial changes that lead to the spoilage of fermented cucumbers using an air-purged calcium chloride fermentation model system. The results of this research have aid in the better understanding of a problem that for more than 25 years has been an ongoing research topic at the USDA-ARS, SAA Food Science Research Unit located within the Food, Bioprocessing and Nutrition Sciences Department at NC State University. The findings of this work will aid the pickle industry to better control and prevent the onset of the undesirable secondary fermentation that lead to the spoilage of fermented cucumbers. In October 2011, Ms. Franco will join the researchers and faculty staff at the

Regional Center for Studies on Healthy Food (CREAS for its acronym in Spanish) in Viña del Mar, Chile.

ACKNOWLEDGMENTS

I would like to express my heartfelt thanks to my advisor Dr. Ilenys M. Pérez-Díaz who is an amazing mentor and teacher. Her warm personality, professional guidance, and profound knowledge encouraged me to complete this work. Thanks for all the advice, patience and words of encouragement that make this process a very enjoyable one.

I would also like to express my gratitude to Dr. Roger F. McFeeters whose advice and experience were valuable for the understanding of cucumber fermentations. My complete gratitude goes with the members of my committee, Dr. Amy M. Grunden and Dr. Todd R. Klaenhammer for their thoughtful advice, consideration, and support. I am grateful for all their help and encouragement in completing this work.

Thanks to Mt. Olive Pickle Co. and especially to Lisa Moller for having your door always open and for providing some of the materials for this study.

A special thanks to the faculty and staff at the USDA "Pickle lab" and especially to Janet Hayes and Suzanne Johanningsmeier. Thanks for your warm welcome and all the help you gave me while conducting my research. I hope our friendship and collaborative work grows with time.

During my stay at NC State I have found valuable friends. Thanks for all the laughs, cheerfulness and friendship. I will have you all in my heart forever.

A big thanks to my family whom regardless of the time and the distance have always supported and encouraged me to keep going. Mom, Dad, Mau and Patty I love you.

My heart and love to my Alfo. You have taught me to follow my dreams and show passion in my work. Thanks for your patience, support and wise words. This accomplishment is yours too. I love you so much.

Finally, I would like to thanks and give praise to God whose guide made this dream come true.

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Chapter 1 Literature review

Fermentation of cucumber fruits

Along with drying and dehydrating, fermentation is one of the oldest forms of food preservation. For thousands of years, food materials have been fermented as a means of preservation. In its beginnings the fermentation process was related to unknown events, and not until 1857 when Louis Pasteur described the role of microorganisms in the process were these considered as responsible (Dubos 1998). The benefits of the fermentation process include preservation, extension of the product shelf-life, and enhancement of its flavor and nutritional content (Breidt 2006; Breidt and others 2007). Worldwide fermentation is used in the preparation of several food items, such as bread, beer, dairy products (cheese and yogurt among others), and fermented meat and vegetable products, including cucumber pickles (Hutkins 2006).

The United States (U.S.A.) produced 2.1 billion pounds of cucumbers from 2004 to 2006. From this production more than 50% of the cucumbers were consumed fresh, while the remaining billion were converted into pickle products (Lucier and Lin 2000). The retail market for cucumber pickles in the U.S.A. is dominated by preserved refrigerated fresh pack pickles such as dill and sweet pickles, followed by sour and half sour pickles, which are fermented and non-pasteurized (Lucier and Lin 2000).

Current practice to ferment cucumber fruits consists of packing the fresh fruits and cover brine solution into open top fermentation tanks (8,000 to 10,000 gallons), to achieve equilibrated concentrations of 5 to 6% sodium chloride (NaCl), 0.1 to 0.4% calcium chloride ($CaCl_2$), and 0.05 to 0.2% acetic acid (Breidt and others 2007). The cucumber fermentation is

possible due to the presence of a number of microorganisms, responsible for the chemical changes with time. Enterobacteria spp. may grow at the beginning of the fermentation, producing carbon dioxide and hydrogen, which may influence the initial development of anaerobic conditions. Numbers of enterobacteria drastically decrease early during the fermentation due to their sensitivity to the acidic environment developed as the fermentation proceeds (Etchells and others 1945). Relatively low numbers of lactic acid bacteria (LAB) are naturally present at the beginning of the fermentation. However, they are able to outcompete the remaining natural microbiota due to their ability to survive in extreme environments characterized by high salt and acid (Breidt 2006; Hutkins 2006). *Lactobacillus plantarum* is the predominant LAB present in fresh cucumbers. This homofermentative organism produces primarily lactic acid via the Embden-Meyerhoff-Parnas pathway (Breidt and others 2007). Other LAB present during the fermentation are *Pediococcus pentosaceous, L. brevis and Leuconostoc mesenteroides* (Singh and Ramesh 2008). These LAB are in general heterofermentative and use the phosphoketolase pathway to produce lactic acid, carbon dioxide, ethanol, and acetic acid (White 2007). Yeasts are also naturally present in fresh cucumbers. These eukaryotic organisms primarily carry out ethanol fermentations, converting glucose to ethanol and carbon dioxide, and have been considered as contributors in flavor and growth factors during lactic acid fermentation in cucumber pickles (Etchells 1941). The initial pH of brined cucumbers is about 6.5, and at the end of the fermentation, there may be up to 2% lactic acid, a final pH of 3.1 to 3.5, and residual or no sugar. Such

conditions work synergistically to prevent microbial survival or growth, thereby effectively preserving the cucumbers (Breidt and others 2007).

Although lactic acid is the major product of the fermentation process, some other by-products are formed. Carbon dioxide (CO_2) is generated from respiration of cucumbers when they are submerged in brine (Potts and Fleming 1979), and by the decarboxylation of malate during fermentation. Some LAB, especially the heterofermentative, have an inducible malolactic enzyme which converts malate to lactate and carbon dioxide (Johanningsmeier and others 2004). The presence of coliforms and yeasts also increases the chances of CO_2 production. Excessive CO_2 can lead to bloater pickles, an undesirable quality problem produced by the formation of gas pockets in the cucumber flesh (Corey and others 1983). To remove carbon dioxide, Flemings and others (1975) recommended including nitrogen-purging routines or, if possible, using anaerobic fermentations. Nowadays, the pickle industry commonly uses air-purging to prevent bloater damage. The change in the practice has been mainly based on costs and in the fact that air displaces CO_2 from the fermentation tanks in a fashion similar to the proposed nitrogen-purging routine. Air-purging is commonly applied during the active lactic acid fermentation (7 to 10 d in summer months and up to a month at colder temperatures during the winter season); however, there are processors that follow a continuous air-purging schedule even during storage of the fermented product (personal communication, unpublished). A gradient of dissolved oxygen that changes, among other factors, with the depth of the tank and the fermentation age was observed in commercial fermentation tanks subjected to air-purging practices (Pérez-Díaz, unpublished).

Potts and Fleming (1979) observed that introduction of air into the fermentation might lead to changes in the microbiota present in the fermenting tanks. Oxygen availability induces the growth and establishment of aerobic microbiota that might alter the characteristics of the fermented product. To limit the growth of aerobic microorganisms, particularity molds and yeasts, potassium sorbate (350 μm/ml) or 0.9% acetic acid can be used (Bell and Etchells 1952; Binsted and others 1962). Excessive growth of aerobic microorganisms which can cause spoilage problems is also controlled by stopping the purging for several hours each day (Breidt, McFeeters and Pérez-Díaz, 2007).

Once fermentation is completed, the fermented cucumbers are stored in the same fermentation tanks for prolonged periods of time. The firmness of the product is maintained by the calcium salts in the brine, while spoilage microorganisms and LAB are controlled by the low pH and the high salt concentration (Buescher and others 1979). After fermentation and storage, the fermented cucumbers are removed from the tanks, desalted in water, packed, and covered in fresh brine cover solution and seasonings.

An important outcome from the fermentation process is the production of large volumes of waste waters that come from fermentation tanks and the washing/desalting step. This waste flow is rich in NaCl and has a high biological oxygen demand (BOD) from the lactic acid and other organic components that diffuse from the cucumbers during the desalting process. A continuing problem for the pickle industry is to meet discharge limits concerning chloride concentrations which are regulated by the U.S. Environmental Protection Agency (EPA). Two hundred and thirty ppm (230 ppm) was the limit established by the regulatory

agency for disposal in fresh waters (Environmental Protection Agency 1987); however, nowadays the limit has been reduced to 140 ppm.

The limitation imposed by the EPA has challenged the pickle industry to find ways to decrease or eliminate the NaCl used during the fermentation process. Currently, processors have adopted the practice of recycling fermentation brines to be used in subsequent fermentations. Prior to recycling, fermentation brines may be processed to remove "softening enzymes", primarily polygalacturonases, which can degrade peptides in the cucumber cell wall and soften the fruits (Buescher and Hamilton 2002). The cleared brines are then mixed (50:50) with fresh brines and used in subsequent fermentations. Although these practices decrease the volume of waste water, a major reduction in waste salt could be achieved, if NaCl is eliminated from the cucumber fermentation process.

Reduced sodium chloride and calcium chloride cucumber fermentations

Different alternatives have been studied to reduce NaCl concentrations during the fermentation of cucumber pickles. Early efforts to ferment cucumbers with reduced NaCl concentrations concluded that the fermentation could be carried out in brines containing 4% NaCl when cucumber fruits are blanched prior to fermentation (Fleming and others 2002). If pasteurization is not included, brines containing 0.2% $CaCl_2$, and 0.2% postassium sorbate could be used to successfully ferment cucumbers in reduced NaCl concentrations (Guillou and others 1992). Recently, McFeeters and Pérez-Díaz (2010) reported that cucumbers can be fermented in cover solutions without NaCl, but containing 100 to 200 mM $CaCl_2$,

provided the fermentation takes place under anaerobic conditions. Replacement of NaCl with $CaCl_2$ in cover brines will benefit the pickle industry by reducing about 80% of the chloride concentration in waste waters. However, the effect this will have upon the application of the air-purging in open containers has not been studied.

Concerns when considering the implementation of $CaCl_2$ fermentations in commercial tank yards arise from the potential changes that could occur during lactic acid fermentation and later storage in open top fermentation tanks. Moreover, the air-purging practices followed by the processors give the fermentation tanks a gradient of dissolved oxygen. The major problem that the industry could face is the spoilage of the product which represents economic loses ranging from $6,000 to $11,000 per affected tank. Spoilage of fermented cucumbers occasionally develops when cucumbers are fermented in NaCl brines, however its incidence may increase in $CaCl_2$ fermentations. Spoiling tanks are commonly reported as having increased brine pH and in some cases malodorous emissions from the fermentation tanks. Industry efforts to prevent spoilage consist of increased monitoring frequency of total acidity and salt concentration on tanks, but when a tank is identified with significant differences compared to the set standards it is too late to prevent the loss.

Fermented cucumbers spoilage

Occasionally as reported by commercial pickle producers, spoilage of fermented cucumbers is associated with increased brine pH (above 3.6), decreased lactic acid concentration, and in some cases off odors emitted from the fermentation tanks. More detailed characteristics on how the spoilage proceeds and which organisms are involved are

limited in part due to the sporadic occurrence of the event and the inability to predict the steps that lead to the secondary fermentation. In addition, the complex microbiota that is present during and after the lactic acid fermentation makes the isolation and identification of potential causative agents very challenging. The first documented fermented cucumber spoilage was reported by Fleming and others (1989). Cucumbers fermented with low NaCl concentrations (2.3%), spoiled during storage after an apparently normal lactic acid fermentation. The spoilage was characterized by complete depletion of lactic acid and production of butyric and propionic acids. Butyric acid production was attributed to *Clostridium tertium*, isolated from the spoiled brines. However, the authors concluded that the bacterium was not able to initiate the spoilage process since it was only able to convert lactic acid into butyric acid at pH 5 and above.

Later studies corroborated the previous observations that pH and NaCl content are important factors that trigger the spoilage process (Kim and Breidt 2007). Spoilage has been induced at pH above 3.8 and NaCl concentration below 4% under anaerobic conditions. Organisms implicated and isolated from these laboratory spoilage cases were identified as *Propionibacterium, Clostridium* and *Lactobacillus* spp., but no specific organism has been correlated with the initiation of the spoilage process at the conditions prevailing once the primary fermentation is completed (low pH, high acid and NaCl). Recently, a number of LAB have been isolated from commercial and laboratory reproduced spoilage samples (Johanningsmeier and others 2011). Among these bacteria, *L. buchneri* and *L. parafarringis*

have been reported as able to utilize lactic acid contained in fermented brines (Johanningsmeier and McFeeters 2011; Johanningsmeier and others 2011).

Similar to the fermented cucumber spoilage, the decay process termed "zapatera spoilage" has been observed in fermented table olives. Zapatera spoilage has been characterized as the decomposition of organic acids at a time when little or no sugar is present and lactic acid fermentation stops before the pH has decreased below 4.5. Researchers have demonstrated that "zapatera spoilage" in green olives is related to the increase of short chain volatile compounds, mainly propionic and butyric acids, with a concurrent degradation of lactic acid (Montaño and others 1992). During of greek table olives controlled fermentations, isobutyric, propionic and valeric acids were formed after 40 days of storage contributing to the decay of the table olives (Panagou and Tassou 2006). Researchers have hypothesized that bacteria that compete against LAB, such as *clostridia*, propionic acid bacteria, enterobacteria, yeast and fungi, are able to produce volatile compounds, including alcohols, esters, ketones, aldehydes and organic acids, that might be associated with the spoilage of the table olives (Kawatomari and Vaughn 1956; Plastourgos and Vaughn 1957; Fleming and others 1989).

Microflora potentially associated with secondary fermentation in cucumber pickles

When trying to identify potential causative agents responsible for the secondary fermentations in cucumber pickles, it is reasonable to think about the natural microbiota that is present in the cucumber fruit, among them lactic acid bacteria, yeasts, enterobacteria, and

Clostridium spp. If oxygen availability is considered, it is reasonable to postulate that organisms other than those reported under anaerobic conditions may have a role in the spoilage of fermented cucumber pickles. For instance, under aerobic conditions *L. plantarum* is able to grow faster and reach higher cell densities as compared to anaerobic conditions (Bobillo and Marchal 1991). When glucose is present, this bacterium produces mainly lactate. However, once the sugar is exhausted, an oxygen-dependent pathway promotes the formation of acetic acid at the expense of the lactic acid (Murphy and Condon 1984; Bobillo and Marchal 1991). Other microorganisms able to reproduce this 'lactate oxidizing system' are *Pediococcus pentasaceuous, L. casei, Streptococcus faecium* and *S. faecalis* (Thomas and others 1985).

During bulk storage, yeasts may grow at the surface of the brine and oxidize the organic acids produced during primary fermentation (Etchells and Bell a and b1950; Etchells and others 1952). It has been observed in the laboratory that common pickling spoilage yeasts such as *Zygosaccharomyces globiformis* (Bell and Etchells 1952) may grow even in the presence of micromolar concentrations of oxygen (personal communication/unpublished). Additionally, various yeasts have been implicated to spoilage problems in the table olives industry (Vaughan and others 1969; Durán Quintana and others 1979). Under aerobic conditions, species from the genera *Candida*, *Pichia* and *Saccharomyces* have been reported as able to utilize lactic and/or acetic acids (Dakin and Day 1958; Ruiz-Cruz and Gonzalez-Cancho 1969).

Other microorganisms of interest belong to the *Enterobacteriaceae* family. These bacteria, commonly present in fresh produce, are usually inhibited by the acidic conditions and low pH that develop as the primary fermentation proceeds (Etchells and others 1945). However, a recent study reported that *Enterobacter cloacae* might be a vector of contamination in fermented green olives (Bevilacquia and others 2009). A number of anaerobic organisms have been isolated and related with fermented cucumber spoilage (Kim and Breidt 2007). Among those *Clostridia* spp. are of interest due to their ability to sporulate under stress conditions and germinate once environmental conditions are favorable.

Hypotheses and objectives

Hypothesis 1: That aerobic $CaCl_2$ cucumber fermentation can lead to the development of spoilage.

- Objective 1: To characterize the biochemical and microbiological changes that take place during aerobic cucumber $CaCl_2$ fermentations and storage.

Hypothesis 2: That the agents responsible for the spoilage of fermented cucumbers brined with $CaCl_2$ can be isolated and identified from vessels subjected to aeration.

- Objective 2: To isolate and identify microorganisms potentially involved in spoilage of air-purged cucumber fermentations.

Hypothesis 3: That the isolates are capable of inducing the spoilage under aerobic conditions and generate changes similar to those observed in commercial and air-purged spoilages.

- Objective 3: To determine whether potential spoilage isolates are able to utilize lactic acid under aerobic conditions.
- Objective 4: To test the ability to reproduce the changes observed in $CaCl_2$ aerobic fermentations by following a succession of potential spoilage microbes.

Significance

The fermented cucumber industry will be highly benefited by the development of a fermentation process in which NaCl is not utilized. From a waste generation point of view, the chloride concentration in 1.1% calcium chloride is less than 20% of what it would be when cucumbers are fermented with 6% NaCl and 30 mM calcium chloride. $CaCl_2$ fermentations have been proven (in the laboratory) effective under anaerobic environmental conditions, but no studies have evaluated the effects under aerobic conditions. If spoilage proceeds under these aerobic conditions, the study of the organisms present during this process will help to understand the complex microbiota responsible for a process that to date is not well elucidated. The characterization of the role of individual organisms during this process, will contribute to determe the environmental factors that influence to the spoilage process. This information will serve to evaluate measures to prevent the development of spoilage microbes and therefore control the fermentation and storage of cucumbers fermented in $CaCl_2$ brines.

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Chapter 2 Characterization of the cucumber secondary fermentation

Submitted to the Journal of Applied and Environmental Microbiology – August 2011

Abstract

Characteristics of secondary cucumber fermentations that occurred in 40 commercial tanks were investigated during a 2010 outbreak. These secondary fermentations can result in spoilage that causes total loss of the fermented product at an estimated cost of $6,000 to $15,000 per affected tank. Previous research has suggested that such fermentations are the result of microbiological utilization of lactic acid and formation of acetic, butyric and propionic acids. The objectives of this study were to characterize the biochemical and environmental changes associated with secondary cucumber fermentations, characterized by increasing pH and decreasing lactic acid concentrations and to isolate and characterize potential causative microorganisms. Two yeasts, *Pichia manshurica* and *Issatchenkia occidentalis* were identified and most commonly detected concomitantly with lactic acid utilization. In the presence of oxygen, the yeast metabolic activity led to lactic acid degradation, a small decline in the redox potential (E_h, Ag/AgCl, 3M KCl) of the fermentation brines and an increase in pH to levels at which bacteria, other than the lactic acid bacteria responsible for the primary fermentation, can grow and produce acetic, butyric and propionic acids. These yeasts are also capable of utilizing acetic acid. Additionally, three gram positive bacteria, *Lactobacillus buchneri*, *Clostridium bifermentans/sordelli*, and *Pediococcus ethanolidurans* were identified as potentially relevant to different stages of the secondary fermentation. The unique opportunity to study commercial spoilage samples generated a better understanding of the microbiota and environmental conditions associated with secondary cucumber fermentations.

Introduction

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

Early efforts to understand secondary cucumber fermentations concluded that the lactic acid produced during the primary fermentation by lactic acid bacteria is consumed, which consequently induces an increase in pH (Fleming and others 1989). It was also noticed that acetic, propionic and butyric acids are produced (Fleming and others 1989). More recent studies of the secondary fermentation of cucumbers under anaerobic conditions concluded that lactic acid can be utilized by *Lactobacillus buchneri* and/or *Lactobacillus parafarraginis*, which are naturally present in the fermented cucumber brines and fruits (Johanningsmeier and McFeeters 2011; Johanningsmeier and others 2011). It is suspected the increasing pH leads to the growth of other microorganisms capable of converting lactic acid into acetic, propionic and/or butyric acids (Fleming and others 1989; Kim and Breidt 2007).

Modern practices for the commercial fermentation of cucumbers in bulk include the use of open top tanks and an air-purging routine to prevent cucumber bloating. Gas-purging practices to control bloater damage due to carbon dioxide accumulation in the fermentation

tanks were first introduced with the use of nitrogen purging routines (Flemings and others 1975). Nitrogen was later replaced with air-purging primarily due to a lower cost without sacrificing the efficiency in preventing bloater damage (Potts and Fleming 1979). However, the presence of oxygen in commercial fermentation tanks creates an opportunity for oxidative yeasts to grow during or after the primary fermentation (Potts and Fleming 1979). The presence of oxidative yeasts in open top fermentation tanks containing fermented cucumbers located across the United States was demonstrated by Etchells and co-workers in the 1950s (Etchells and Bell 1950.; Etchells and Bell 1950; Etchells and others 1952; Etchells and others 1953). Shortly after these reports were made, the ability of a selected number of yeasts present in foods to assimilate acetic and lactic acids was discovered (Dakin and Day 1958; Ruiz-Cruz and Gonzalez-Cancho 1969). More recent studies suggest that yeasts such as *Candida utilis* express two mediated transport systems for lactic acid, one of which appears to be able to transport other monocarboxylic acids, such as acetic acid (Cássio and Leão 1993). Since lactic and acetic acids are critical to the preservation of fermented cucumbers during bulk storage, the potential role of yeasts capable of utilizing the organic acids present in fermented cucumbers was evaluated.

Investigations of the development of secondary cucumber fermentations have been limited by the inability to predict the event at the commercial scale and by its sporadic occurrence in a small number of fermentation tanks. In the 2010 cucumber brining season, a considerable number of commercial tanks spoiled due to secondary fermentations, which resulted in loss of lactic acid, increased pH and the discard of the fermented fruits. This

outbreak provided a unique opportunity to investigate the development of secondary fermentations in commercial fermentation tanks. The observations made from these investigations are reported here. The objectives of this research were to characterize the biochemical changes that accompanied secondary cucumber fermentations, characterized by increasing pH and decreasing lactic acid concentrations, and to initiate the isolation and characterization of potential causative microorganisms, including oxidative yeasts.

Materials and Methods

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

addition to pH, the detection of manure-like and cheesy aromas and the presence of bubbles at the surface of the tanks also indicate tanks may be in the process of spoiling.

For this study, 40 spoiling and 20 standard fermentation tanks were monitored on the same day. Dissolved oxygen (dO_2) measurements were made 2 inches below the wooden cover boards with an Oxi330i portable set from WTW Measurements Systems Inc. (Fort Myers, FL) and adjusted for the estimated salt levels in the tanks (6%). A portable AP61 Accumet pH meter equipped with an AP50a electrode (Fisher Scientific, Pittsburgh, PA) was used to collect pH measurements from the cucumber tanks. Redox potential was measured using a submergible redox electrode model PHEH-65-10 (Omega Engineering, Inc., Bridgeport, NJ) connected to a portable conductivity tds/pH meter (Omega Engineering, Inc.). The appropriate functioning of the redox potential probe was verified by measuring the redox potential of both pH 7.0 and 4.0 calibration buffers with added quinhydrone to saturation levels. The metal tip of the probe was polished using an alumina powder mixture (1.0 micron; Precision Surfaces International, Houston, TX), as needed. The redox probe was rinsed thoroughly with 70% ethanol to sanitize between tank measurements. Millivolt measurements were converted to E_h following the manufacturer's instructions ($E_h = E_{meas} +$ 207 mV, at 25°C). Samples for chemical and microbiological analyses were aseptically collected from below the wooden cover boards from three distant spots at the same depth (9 inches) in the tank.

Additional commercial spoilage brine samples (samples 1- 4) were provided by processors in 2009 and 2010. These samples were incubated at 30°C statically for 15 days to

follow the utilization of lactic acid and acetic acid remaining once they were received. Sample #4, in which lactic acid was completely depleted when received, was spiked with 50 mM lactic acid and then further incubated for 10 days. An AccuFet solid-state pH combination electrode (Fisher Scientific) and Accumet AR25 pH meter (Fisher Scientific) were used to measure the pH of these samples during the incubation period.

Chemical analysis. The concentrations of organic acids and sugars were measured by HPLC analysis using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, CA) for the separation of components (McFeeters and Barish 2003). The column temperature was held at 37°C and samples components were eluted with 0.03N sulfuric acid at a flow rate of 0.6 mL/ min. A Thermo Separations UV6000 diode array detector (Spectra System Thermo Scientific, Waltham, MA) set to collect data at 210 nm was used to quantify malic, lactic, acetic, propionic, and butyric acids. A Waters model 410 refractive index detector (Waters Corp., Millipore Corp., Billerica, MA) connected in series with the diode array detector was used to measure glucose, fructose, and ethanol. External standardization of the detectors was done using four concentrations of the standard compounds.

Microbiological analysis. Brine samples were serially diluted in 0.85% saline solution, and spiral plated using an Autoplate 400 (Spiral Biotech, Norwood, MA). Lactic acid bacteria enumeration was done using Lactobacilli deMan Rogosa and Sharpe agar (MRS, Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 1% cycloheximide (0.1% solution, OXOID, New England). MRS plates were incubated anaerobically using a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, MI) at 30°C for 48 h.

Yeasts were enumerated using yeast and molds agar (YMA, Becton Dickinson and Co.) supplemented with 0.01% chloramphenicol (Sigma-Aldrich, St. Louis, MO) and 0.01% chlortetracycline (Sigma-Aldrich). YMA plates were incubated aerobically at 30°C for 48 h.

Induction of secondary cucumber fermentations in the laboratory using commercial spoilage samples as the source of spoilage microorganisms. The experimental medium (EM) used for the reproduction of the secondary fermentation was prepared from 2B cucumbers acquired from a local processing company. The fruits were washed, packed into 1 gallon sized glass jars at a 55:45 pack-out ratio (weight cucumbers to volume brine) with brine to equilibrate to 4% NaCl, and inoculated with *Lactobacillus plantarum* LA0219 starter culture (USDA-ARS Food Science Research Unit, Culture Collection, Raleigh, NC) at 6 log CFU/g. Fermentation progress was monitored by pH and HPLC analysis of acids and sugars. Upon completion of the lactic acid fermentation, fermented cucumbers were blended into slurry and stored at -10°C. To prepare media for inoculation with spoilage microorganisms, fermented cucumber slurry was thawed, pressed through cheesecloth to remove large particulates, and spun at 23,400 x g for 15 min (Sorbal RC 5B, DuPont Instruments, Wilmington, DE). NaCl concentration was adjusted to 6% to be representative of NaCl levels used commercially, and pH was raised to 3.8 with 6N NaOH to allow for more rapid development of secondary fermentation (Kim and Breidt 2007; Johanningsmeier and others 2011). The resulting EM was filter - sterilized with a 0.2 µm bottle top filter apparatus (Nalgene FAST PES, 0.2 µm pore size, 90-mm-diameter membrane, Daigger, Vernon Hills, IL). Twelve mL aliquots of the filter sterilized medium were aseptically transferred to either

50 or 15 mL conical centrifuge tubes for aerobic and anaerobic incubation at 25°C, respectively. Incubation under anaerobic conditions was done using a Coy anaerobic chamber (Coy Laboratory Products, Inc.).

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

Isolation and Identification of Microorganisms. A variety of cucumber fermentation spoilage samples were used for the isolation and identification of potential causative microorganisms from MRS, YMA, and differential reinforced clostridial agar (DRCA, Becton Dickinson and Co) (Tables 2 and 5). Different colony morphologies visually observed on the agar plates were selected for this purpose. Two representative colonies from

each morphology type were picked and streaked in the respective culture medium. Isolated colonies were analyzed microscopically (OPTIPHOT-2, Nikon, Tokyo, Japan) and by Gram staining or by a 10% KOH-glycerol fixation for bacteria and yeasts, respectively. To determine the presence of *Clostridium* spp. in sample #4, which had the highest initial pH upon delivery to the laboratory, 25 mL of the spoiled brine was aseptically transferred to 50 mL centrifuge tubes in quadruplicate. Two tubes were incubated at 80°C for 30 min to induce clostridial spore germination, while the other two tubes were not heated. An aliquot of 100 µl from each tube was spread plated on DRCA and plates were incubated anaerobically at 30°C for 48 h. Black colonies on DRCA plates were tentatively identified as *Clostridium* species and isolated for further analysis. Frozen stocks of all isolates were prepared in MRS, YM broth, or Differential reinforced clostridial medium (DRCM, Becton Dickinson and Co.), as appropriate, containing 15% glycerol (Fischer Scientific).

The bacterial and yeast isolates were identified using partial *16S* and/or *26S rRNA* gene sequencing, respectively. Bacterial chromosomal DNA was obtained using DNeasy genomic extraction and purification kits (DNeasy, Qiagen, Valencia, CA) while yeast chromosomal DNA was obtained using the MasterPure™ Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). The PCR mix contained 2X master mix (Biorad), chromosomal DNA and forward and reverse primers, which were 27F (5-AGAGTTTGATCCTGGCTCAG-3') (Barrangou and others 2002) and 1492r (5'-GGTTACCTTGTTACGACTT-3') (Wilson and others 1990) for the bacterial isolates, and NL-1 (5'-GCCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-

GGTCCGTGTTTCAAGACGG) for yeasts (Kurtzman and Robnett 1997). All primers were obtained from Integrated DNA Technologies (Coralville, IA). PCR products were purified using the Qiagen PCR purification kit and sequenced by Eton Bioscience Inc. (Raleigh, NC). The sequences obtained were subjected to the basic local alignment search tool (BLAST) (Altschul and others 1990) in the GenBank (Benson and others 2000) database to determine the identity of the isolates.

Inhibition of Lactic Acid Disappearance by Yeasts Isolated from the Spoilage Samples. Fresh cucumber juice was prepared from 2A pickling cucumbers (25.4 mm-31.8 mm diameter) obtained from a local processor using an automatic juice extractor (JM400 Juiceman Jr., Black and Decker, Towson, MD). Particles were removed from the fresh juice using cheese cloth and subsequently centrifugation for 1 h at 10,000 x g using a Sorvall RC-5B centrifuge (Dupont Instruments). The supernatant was mixed (50:50) with cover brine solution such that, after equilibration, the mixture had 100 mM $CaCl_2$ and 25 mM acetic acid. Half of the cucumber and cover brine mixture was supplemented with 100 ppm allyl isothiocyanate (AITC, Aldrich, St. Louis, MO) and the other half was left without the preservative to serve as a control. The mixtures were individually filter-sterilized using a 0.22 µm bottle top filter apparatus (Nalgene, Vernon Hills, IL).

Two liter aliquots of each of the filter-sterilized media containing either 0 or 100 ppm AITC were aseptically transferred into two sterile 3.8 L glass jars. The jars were sealed with lids that were fitted with a redox electrode (InLab 501/170, Mettler-Toledo, Bedford, MA), an inlet and outlet for air purging application and a rubber septum for sample collection. The

appropriate functionality of the redox electrodes was confirmed using pH 7.0 and 4.0 electrode calibration solutions saturated with quinnhydrone. The redox electrodes 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 electrode and the air inlets to prevent air leakage into the jars. Jars were held at room temperature (24 ± 3°C). Air purging was applied at a flow rate of 5 mL/min controlled by a Matheson PG-1000 (U001) flow meter (Matheson Instruments, Montgomeryville, PA). Fermentation vessels were inoculated with a mixture of three *L. plantarum* strains (LA 445, LA 98, and LA 89, USDA-ARS Culture Collection, North Carolina State University, Raleigh, NC), and a mixture of the two yeast isolates presenting morphologies MY1 and MY2 (Table 5, USDA-ARS Culture collection) to 5 log CFU/mL. Brine samples were aseptically collected using 21G-6" sterile metal needles and syringes every 24 h for microbiological and chemical analyses as described above. This process was repeated with 2 lots of fresh cucumber juice. Data presented here represents the means of two independent replicates.

Statistical Analysis. Log microbial count plates and measured concentrations for organic acids, and sugars 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).

Results

Commercial spoilage samples. The outbreak of spoiling cucumber fermentations in commercial tanks reported in August 2010 was characterized by atypical fermentations odors, but not manure or cheese-like aromas. About 80% of the spoiling tanks contained a mixture of whole cucumbers of variable sizes and cucumber slices and/or spears. Fifty eight percent of the spoiling tanks exhibited evidence of gas formation and most of them were packed in July 2010. Sodium chloride (NaCl) concentrations were re-adjusted in 63% of the tanks to the targeted 25 salometer (6%). Concentration of the salt in fermenting cucumber brines before adjustment ranged from 17 to 21 salometer (4 to 5%). Lactic acid concentrations were 45 to 85 mM lower than the mean concentration in normal fermentations, 115.5 ± 24.5 mM. The opposite was observed for acetic acid concentrations, which were on average 2-fold higher than the 25 mM observed in standard fermentations (Table 2.1 and Fig. 2.1). The differences in lactic and acetic acid concentrations were indirectly detected by the increased pH of the brines in spoiling fermentation tanks as shown in Fig. 2.1. The environmental conditions in spoiling tanks were characterized by significantly higher dissolved oxygen (dO_2) and decreased redox potential (E_h) measurements as compared to standard tanks (Table 2.1). Moreover, the tank in which both propionic and butyric acids were detected showed the most reduced environment as denoted by negative E_h readings (Table 2.1). Fifty percent of the spoiling fermentation tank samples had detectable levels of propionic acid (4 to 10 mM). In contrast, only two samples had detectable levels of butyric acid with concentrations ranging between 6 to 12 mM, respectively (Table 2.1). The

presence of butyric acid was only detected in spoiling fermentations with pH > 4.0. However, the presence of propionic acid was noted in spoiling fermentations with pH lower (3.7 ± 0.2) than those observed from spoilage samples in which neither propionic or butyric acids were produced (3.9 ± 0.2) (Table 2.2).

Counts of lactic acid bacteria from samples of spoiled fermented cucumbers were, on average, 4 logs higher than counts from samples of standard cucumber fermentations (Table 2.1). Two distinct colony morphologies recognized on MRS plates inoculated with the spoilage samples were designated as MB1 and MB2, and are described on Table 2.4. These two bacterial morphologies were more abundant in spoilage samples that did not contain propionic and/or butyric acids ($P < 0.05$).

While significant differences in total yeasts and molds counts were not observed between standard and spoiled fermentations, it was possible to identify two morphologies (MY1 and MY2, Table 2.5) that predominated in the spoilage samples. The MY1 morphology was detected in all YMA plates inoculated with spoiled fermentation samples, but in only 9 standard fermentation samples. Numbers of MY1 colonies were higher on plates inoculated with the spoiled fermentation samples by at least 2 logs of CFU/mL ($P < 0.05$). Similarities between the MY2 morphology and other morphologies observed in the YMA plates made it difficult to accurately and objectively establish counts for the samples. The morphology designated as MY3 (Table 2.5) was exclusively detected in standard fermentation samples.

Additional spoilage samples obtained in 2009 and 2010 included an experimental tank packed with fresh cucumbers and a cover brine solution containing no sodium chloride and 100 mM calcium chloride (sample #1); commercial tanks in which recycled NaCl brines were used for packing fresh cucumbers (samples #3 and #4); and a commercial organic tank in which recycled NaCl cover brine solution was used for packing (sample #2). The first indication of spoilage was brine pH significantly higher than the targeted post-fermentation pH of 3.3 ± 0.3 (Breidt and others 2007; Code of Federal Regulations 2010). Samples #1 and #4 were also characterized by off odors resembling manure and cheese. Samples #2 and #3 developed off odors after incubation in the laboratory. Such odors suggested the development of secondary fermentations and the formation of products associated with spoilage such as butyric and propionic acids, which was confirmed by HPLC analysis (Table 2.2). In all samples, lactic and acetic acid concentrations were below the standard post-fermentation values (115.5 ± 24.5 mM and 24.7 ± 4.2 mM, respectively) or not detected as in the case of sample #4 (Table 2.2).

Yeasts and bacterial morphologies detected in spoilage samples collected from the 2010 outbreak were also observed in the four samples unrelated to that outbreak. The MY1 morphology was present in all spoilage samples (#1 thru #4), and the MY2 morphology was detected in samples #1, #3 and #4 (Table 2.2). While the MB1 morphology was present in all spoilage samples; MB2 was exclusively detected from samples #3 and #4 (Table 2.2). Presumptive *Clostridia* spp., black colonies in DRCA plates, were observed from both non-heated and heat treated aliquots of sample #4. As expected, the number of black colonies in

heat treated samples was significantly higher (2 logs, data not shown) than those from non-heated ones. The presumptive *Clostridium* specie isolated from an aliquot of sample #4 was designated as MC1 (Table 2.5).

Variability of acetic acid concentrations in the spoilage samples might be due to the presence of different organisms. Samples in which acetic acid concentration was above the standard 25 mM (Table 2.1) were associated with the presence of the LAB isolate MB1 as the most abundant organism. While decreased acetic acid concentrations in samples incubated for an additional period (Table 2.2) were associated with the presence of yeasts counts of about 5 log CFU/mL.

Induction of a Secondary Cucumber Fermentation in the Laboratory. The changes observed during aerobic and anaerobic incubation of fermented cucumber media inoculated with aliquots from sample #4 are shown in Tables 2.4 and 2.5. The pH of samples incubated under aerobic and anaerobic conditions increased to 8.3 ± 0.1 and 4.3 ± 0.1 at 0.10 and 0.005 pH units/day, respectively, and proceeded together with the reduction in lactic acid concentrations. Lactic acid utilization concomitant with yeast growth was inhibited by anaerobic conditions. Conversely, total yeast counts from samples incubated under aerobic conditions reached about 7 log CFU/mL during active lactic acid utilization. Both MY1 and MY2 morphologies were recognized in YMA plates (Table 2.3). Total yeast counts decreased with time from samples incubated under anaerobic conditions, and only MY1 was detected in YMA plates towards the end of the incubation period (Table 2.3).

Total LAB counts increased with time under both incubation conditions, reaching populations above 7 log CFU/mL (Table 2.4). At first, total LAB population increased with utilization of remaining sugars, which in turn decreased the media pH. These changes were more evident in samples incubated under anaerobic conditions in which pH decreased to 3.2 after 32 days of incubation (Table 2.4). The spoilage LAB, MB2, was not detected at the beginning of the experiment, but it was observed once the media pH was above 4.0 under anaerobiosis and above 6.8 under aerobic conditions. MB2 was the only LAB able to persist in samples incubated under aerobic conditions after 32 days; while both morphologies MB1 and MB2 were detected after 3.5 months of incubation under anaerobic conditions. Acetic and propionic acids were detected from samples incubated under aerobic and anaerobic conditions to similar total amounts by the end of the incubation periods, and their formation was independent of yeast growth. Acetic and propionic acids formed at a rate of 0.55 and 0.53 mM/day under aerobiosis as compared to a more linear rate of 0.24 and 0.14 mM/day under anaerobic conditions. Interestingly, under aerobic conditions, which allowed oxidative yeasts to proliferate, 2.1 ± 0.2 mM acetic acid were utilized between days 0 and 8. The presence of propionic acid coincided with the appearance of the spoilage LAB MB2 (Table 2.4).

Isolation and identification of potential causative agents. A description of the morphological characteristics and identification of the isolates based on the *16S* or the *26S rRNA* sequencing data is shown in Table 2.5. Three yeasts (MY1, MY2, and MY3) were isolated and identified. MY1 and MY2 were the most abundant colony morphologies present

in spoilage samples. MY1 colonies are white, crateiform with wrinkles and radial striations, umbonate in elevation, with undulate margins, and about 1 mm in diameter after 48 h aerobic incubation on YMA plates at 30°C. The colony can be clearly distinguished from others due to its "volcano-like" morphology. The cells occur in buds and have an ellipsoidal shape with ascopores (Table 2.5). MY1 isolates were identified as *Pichia manshurica*. MY2 colonies are white-beige, irregular on shape, with umbonate elevation, and lobate margin after 48 h aerobic incubation on YMA plates at 30°C. The colonies have a "fuzzy" surface with diameters from 1 to 1.5 mm. Under the microscope, cells are ogivall with round ascopores (Table 2.5). MY2 was identified as *Issatchenkia occidentalis*. MY3, observed and isolated only from standard fermentation samples, are little white-beige shiny colonies with spherical shape, a slight elevation, and entire margin after 72 h of aerobic incubation on YMA plates at 30°C. Under the microscope, the cells are round to ellipsoidal with ascopores at the center of the cell. MY3 was identified as *Candida cf. etchellsii*.

The LAB isolate MB1, identified as *Lactobacillus buchneri*, is a Gram-positive, non-spore forming, spherical bacilli. Colonies on MRS plates after 48 h of incubation look filamentous and flat, with a diameter of approximately 1 mm. The shape of the colony resembled a "star". The MB2 isolate, identified as *Pediococcus ethanolidurans*, is a Gram-positive, non-spore forming coccus. Cells, under the microscope, occurred in chains. After 48 h of incubation on MRS agar, colonies look punctiform, convex, with entire margin. The colony size is about 1/10 of a *L. plantarum* colony. The presumptive *Clostridium* isolated from sample #4 (MC1) was identified as *Clostridium bifermentans/sordelli*. The colony was

irregular, umbonate in elevation, and with lobate margin. After 48 h of anaerobic incubation the colony grew embedded in the agar and develops a translucent filament that surrounds the colony. Under the microscope the cell was a Gram-positive, spore-forming rod.

Inhibition of lactic acid disappearance caused by *Pichia manshurica* and *Issatchenkia occidentalis*. As expected, the inoculated *L. plantarum* strains dominated the primary fermentation in the fresh cucumber juice medium. Fermentation proceeded normally in the presence of 0 or 100 ppm AITC within the first week as evidenced by decreases in the medium pH concomitant with increases in lactic acid concentrations and LAB populations (Table 2.5 and Fig. 2.3, Panel A). However, the rate of lactic acid production and the acidification of the medium were delayed by 100 ppm AITC. Supplementation of the fresh cucumber juice medium with 100 ppm AITC prevented the disappearance of lactic acid after 20 days of incubation. Growth of *Pichia manshurica* (MY1) and *Issatchenkia occidentalis* (MY2) was evident in the jars not supplemented with AITC. Growth correlated with lactic and acetic acid disappearance (Table 2.6 and Fig. 2.3, Panel B). Significant changes in pH and redox potential were also detected in the jars not supplemented with AITC (Table 2.6 and Fig. 2.3, Panel C). Yeast populations in the medium containing AITC decreased gradually by 3 logs as the incubation proceeded (Fig. 2.3, Panel B). The fermentation environment remained oxidized in the presence of AITC, as indicated by a positive E_h measurement (Fig. 2.3, Panel C) and the final lactic and acetic acids concentrations (100 mM and 24 mM, respectively) were within concentrations expected for standard cucumber fermentations (Breidt and others 2007).

Discussion

The characterization of secondary fermentations that lead to spoilage of fermented cucumbers has been limited due to the sporadic occurrence of the event at the commercial scale and the inability to predict the process. The unique opportunity presented during the 2010 spoilage outbreak allowed for the characterization of commercial brine samples at different stages of spoilage. Based on these observations it was possible to observe that lactic acid degradation is concomitant not only with decreases in brine pH, but also with changes in environmental conditions inside the fermentation tanks, such as reduced environments and increased dissolved oxygen concentration, as compared with standard fermentations. Potential causative agents isolated from commercial brines suggest that oxidative yeasts can be related with aerobic initiation of the secondary fermentations. Additionally, bacterial isolates seem to be associated with different stages of the secondary fermentation as well. Secondary fermentation of cucumbers, which is partially described by the data presented here, is a complex event demanding further studies to determine the identity of all the possible causative agents and their respective contributions. Furthermore, it is still necessary to identify, test and develop tools for the early detection of the development of a cucumber secondary fermentation and strategies to prevent its occurrence. It is the intent of this research to identify some of the factors that contribute to the full development of the secondary cucumber fermentation, establishing a baseline that should help in gaining a better understanding of this event.

The 2010 secondary fermentation outbreak revealed that the type of yeasts present in the fermentation tanks were more relevant than the specific total yeast counts. Oxidative yeasts presenting morphologies similar to those characteristic of *Pichia manshurica* and *Issatchenkia occidentalis* were fully or partially responsible for the disappearance of the lactic acid and the increase in brine pH. The study of this outbreak also confirmed that selected lactic acid bacteria, different from those that carry out the primary fermentation, are capable of proliferating during the secondary fermentation. These spoilage lactic acid bacteria grew concomitantly with the spoilage yeasts. In particular, the ability of the lactic acid bacterium, *L. buchneri* to convert lactic acid to acetic acid and 1,2-propanediol in a fermented cucumber juice medium was recently demonstrated by Johanningsmeier and others (Johanningsmeier and others 2011). Subsequent to such conversion, 1,2 propanediol can be converted into propionic acid by organisms such as *Lactobacillus rapi*, which is also present in the fermented cucumber environment (Johanningsmeier and others 2011; Johanningsmeier 2011). The observation that two microorganisms may be involved in the formation of propionic acid is in agreement with the fact that the numbers of bacterial colonies presenting the MB1 morphology were more abundant in spoilage samples that did not contain propionic and/or butyric acids, suggesting that this bacterium is not directly involved in the formation of such acids.

Variability in acetic acid concentration in the studied spoilage samples might be due to the presence of different organisms in independent commercial fermenting tanks. Samples in which acetic acid concentration were above the standard 25 mM (Table 2.1) were

correlated with the presence of the LAB isolate MB1 as the most predominant organism, which is in agreement with the observation reported by Johanningsmeier and others (2011). Decreased acetic acid concentrations in samples subjected to an extended incubation after their delivery to our laboratory (Table 2.2) were associated with the presence of yeast counts of about 5 log CFU/mL. The ability of the yeast isolates, *Pichia manshurica* (MY1) and *Issatchenkia occidentalis* (MY2) to utilize lactic and acetic acids was evident after inoculating these yeasts in a fresh cucumber juice model system (Table 2.6 and Fig. 2.3). Conversely, the inhibition of yeast metabolic activity when the medium was supplemented with 100 ppm allylisothiocyanate (AITC) allowed the fermented product to remain stable, thus, corroborating that the isolated yeasts are a contributing factor during the spoilage of the fermented product.

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

Once a higher pH develops, due to the microbial lactic acid utilization, a variety of putrefactive microorganisms capable of producing fetid odors, loss in color and softening of

the cucumber tissue may occur (Binsted and others 1962). For instance, during the secondary fermentation reproduced in the laboratory, colony morphologies consistent with *L. buchneri* and *Pediococcus ethanolidurans* became established once media pH was above 4.0 under anaerobic conditions and 6.8 under aerobiosis (Tables 2.3 and 2.4). Butyric acid was observed in commercial samples that had the most reduced (E_h 263 and -139 mV) and higher pH (>4.2) (Table 2.1) environments suggesting that the organisms related with the production of this short fatty acid might be anaerobic or microaerophilic. While *Clostridium bifermentans* was isolated from spoilage sample #4, butyric acid production was not noted in the spoilage reproduced in the laboratory using this sample as inoculum. *Clostridium* spp. have been reported as capable of producing butyric acid at pH values above 4.6 and are known to be sensitive to oxygen (Turton and others 1983; Monteville and others 1985; Fleming and others 1989; Kim and Breidt 2007), thus it could have been possible that the natural clostridial population was disturbed during sample transit to our laboratory and/or during the experimental manipulations.

Redox potential measurements have been proposed as a more accurate tool to monitor fermentation tanks and possibly detect the initiation of a secondary fermentation prior to evident changes in pH and organic acids (Olsen and others *Submitted*). Fermentation tanks in which spoilage was detected by increased pH and reduced lactic acid concentration had also reduced redox potential (E_h) values as compared with standard fermentations (Fig. 2.1 and Fig. 2.2). However, E_h trends in the commercial spoilage tanks suggest that significant

changes in redox potential measurements were not detectable before pH increased to pH 4.0 or above (Fig. 2.1, Panel C), after a significant amount of lactic acid had been utilized.

Although yeast populations from spoiling and standard fermentation tanks were not significantly different, the type of yeast morphologies present in spoiling samples was significantly different from those observed in standard fermentation brines. Of particular interest were the observations that yeast colonies presenting morphologies similar to that of *Candida cf. etchellsii* were exclusively observed, isolated, and predominantly present in standard fermentation samples; and that dissolved oxygen in spoiling cucumber fermentation tanks was significantly higher than in standard fermentation tanks (Fig. 2.2). The potential role of *C. etchellsii* as an oxygen "chelator", and/or as an out competitor or killer yeast (Llorente and others 1997; Regodón and others 1997; Schmitt and Breinig 2002; Gulbiniene and others 2004; Hernández and others 2008), remains to be studied. However, the fact that higher dO_2 measurements characterized the spoiling fermentation tanks may suggest that beneficial oxidative organism(s) may be absent from fermentations prone to undergo a secondary cucumber fermentation.

Although, lower NaCl concentrations were reported as important for the development of spoilage in fermented cucumber pickles (Kim and Breidt 2007), data gathered from commercial spoilage samples indicate that fermentations carried out at 0 to 6% NaCl and 0.2 to 1.1% $CaCl_2$ are susceptible to spoilage. Certain spoilage yeasts and LAB are known for being halotolerant (Deák 2008), which combined with the ability to utilize organic acids as a

source of carbon, gives these organisms a unique competitive advantage in an environment characterized for high NaCl and lactic acid concentrations.

The isolation and identification of yeasts as potential initiators of the secondary fermentation under aerobic conditions is important to the pickle industry. The yeast population naturally present on cucumber fruits is generally controlled once the primary fermentation is completed due to the lack of readily available energy sources (Etchells and others 1953). The exception to this situation is the film-forming yeasts which can be inhibited by the spraying of a concentrated potassium sorbate solution on the surface of the tanks in cloudy weather or by including the preservative in the formulation of the cover brine solutions used for the fermentations (Etchells and others 1961). However, our findings showed that certain yeasts are capable of producing important changes during storage of the fermented product such as the disappearance of lactic acid and increased pH. Certain practices currently followed in the pickle industry might favor the development of these organisms on the fresh fruit prior to the fermentation process. Incubation of the fresh fruits at warmer ambient temperatures (72 to 85ºF) for extended periods of time during transport and/or arrival to the processing plant during the peak of the cucumber tanking season, along with less than ideal sanitation and handling practices, could increase the populations of the microbiota present in the fresh fruit including the yeast populations. Total yeast and mold counts detected in fresh cucumber samples collected from the tank yard analyzed during the 2010 season were 2 log CFU/mL higher than the standard counts of 2.82 ± 0.95 CFU/mL (Pérez-Díaz *In preparation*). Based on our observations it seems that tanks containing pieces

and cuts of the fresh fruits are more susceptible to yeast spoilage than tanks containing the whole fruits. Yeasts, which are generally larger in size as compared to bacteria, are not able to penetrate the cucumber skins (Daeschel and others 1985), consequently, the brined cucumber pieces represent a matrix with more readily available nutrients than the whole fruits.

Steps that should reduce the incidence of secondary cucumber fermentation leading to spoilage in a commercial environment include better process controls including the reduction of the holding times for fresh cucumbers prior to tanking, elimination of a washing step for cucumber pieces after cutting, as well as the contact of such pieces with the processing surfaces prior to tanking, and the utilization of processing equipment with a sanitary design. Acidification of the fermented cucumbers with vinegar should not be used to prevent development of an incipient spoilage because acetic acid is utilized by the spoilage associated with the oxidative yeasts as an energy source and could encourage their proliferation. The oxygen availability in the tanks should also be reduced by covering the tanks with food grade plastic sheets forced down with cover brine solution on top of them and by minimizing air purging, as the lack of air would limit the metabolic activity of the spoilage associated with oxidizing yeasts. Additionally, spoiled brines should be isolated to prevent the enrichment of the yeasts in future fermentations. If spoiled brines will be recycled, it is recommended that the cover brine solutions are treated to reduce yeast populations. The use of plant extracts, such as AITC evaluated in this study might be an option to control yeast populations before fermentation (Mochida and others 1988; Pérez-

Díaz and McFeeters 2008; Pérez-Díaz and McFeeters 2010). However, as observed in this study, AITC seems to affect not only yeasts, but also *L. plantarum* as indicated by the lower LAB populations and lactic acid production compared with brines not supplemented with the AITC (Fig. 2.3, Panel A and Table 2.6). Another alternative yet to be studied consists of using clay to reduce yeast populations in a fashion similar to that suggested for the removal of softening enzymes (Marshman and Marshall 1981; Buescher and Hamilton 2002).

Conclusions

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

Acknowledgments

The authors would like to thank Mr. Roger W. Winstead and Mr. Marc Hall with the University Communications Department at North Carolina State University (Raleigh, NC)

for their assistance with the colony photographs shown in Table 5 and Ms. Sandra Parker with the USDA-Agriculture Research Service, Food Science Research Unit for excellent secretarial assistance.

Table 2.1. Chemical and microbiological characteristics of commercial spoiling cucumber fermentation tanks sampled in the year 2010

Variables	Type of Fermentations Characterized				
	Standard Cucumber Fermentations	Spoiling Cucumber Fermentations			
				6-12 mM Butyric Acid	
	No Propionic or Butyric Acid	No Propionic or Butyric Acid	4-12 mM Propionic Acid; No Butyric Acid	No Propionic Acid	8 mM Propionic Acid
Number of Samples Monitored	20	19	19	1	1
Chemical properties					
pH	3.3 ± 0.1^{a}	3.9 ± 0.2^{b}	3.7 ± 0.2^{c}	4.2^{d}	4.4^{e}
dO_2 (mg/L)	0.3 ± 0.1^{a}	1.7 ± 0.3^{b}	1.8 ± 1.1^{c}	0.9^{d}	2.2^{e}
E_h (mV)	351.2 ± 9.5^{a}	$277.0 \pm 9.5^{b\backslash a}$	331.04 ± 7.3^{a}	263.0^{a}	-139.0^{b}
Lactic Acid (mM)	115.5 ± 24.5^{a}	69.5 ± 25.4^{b}	58.9 ± 14.9^{b}	33.9^{c}	50.1^{d}
Acetic Acid (mM)	24.7 ± 4.2^{a}	45.3 ± 14.2^{b}	42.8 ± 13.3^{b}	48.3^{b}	53.1^{c}

Table 2.1 Continued

Microbial Counts (log CFU/mL)					
MRS	0.2 ± 0.0^{a}	4.7 ± 0.1^{b}	3.6 ± 0.0^{c}	5.6 ± 0.0^{c}	NA
YMA	5.7 ± 0.2^{a}	5.0 ± 0.0^{b}	4.8 ± 0.0^{b}	5.4 ± 0.0^{a}	NA
Microbial Counts Based on Characteristic Morphologies (log CFU/mL)					
MB1	ND^{a}	4.4 ± 0.0^{b}	3.2 ± 0.0^{c}	5.2 ± 0.0^{d}	NA
MB2	ND^{a}	3.7 ± 0.1^{b}	2.5 ± 0.1^{c}	4.9 ± 0.1^{d}	NA
MY1	1.4 ± 0.0^{a}	3.6 ± 0.0^{b}	3.9 ± 0.1^{b}	4.1 ± 0.0^{c}	NA
MY3	5.4 ± 1.3^{a}	N/D^{b}	N/D^{b}	N/D^{b}	NA

NA: Not available. By the time samples for microbial characterization were collected the fermentation tank was already disposed of.

ND: Not detected

Means with the same letter in a row are not significantly different ($P<0.05$)

Table 2.2.Chemical and microbiological characteristics of the commercial spoilage samples delivered to the laboratory and not related to the 2010 spoilage outbreak

	Commercial Spoilage Samples Delivered to Our Laboratory (Years 2009-2010)							
	Expected Values	**Sample #1**	**Sample #2**		**Sample #3**		**Sample #4[§]**	
Variables	**Standard Fermentation (n=21)**	**T_i[*]**	**T_i**	**T_f[†]**	**T_i**	**T_f**	**T_i**	**T_f**
pH	3.1-3.5	5.1	3.6 ± 0.1	6.9 ± 0.1	3.6 ± 0.1	7.2 ± 0.04	4.7 ± 0.2	5.0 ± 0.1
Measured Concentrations (mM)								
Lactic Acid	70-100	ND	65.2 ± 1.1	18.7 ± 1.1	63.9 ± 1.3	1.2 ± 1.2	ND	13.1 ± 1.1
Acetic Acid	25	8.3	27.3 ± 0.8	1.9 ± 0.5	29.6 ± 0.8	9.6 ± 0.5	38.8 ± 0.1	0.6 ± 0.3
Propionic Acid	ND[¥]	ND	3.1 ± 0.1	0.2 ± 0.1	4.1 ± 0.1	23.3 ± 0.1	46.4 ± 0.3	58.7 ± 0.3
Butyric acid	ND	11.0	ND	ND	ND	ND	44.0 ± 0.1	61.6 ± 0.3

Table 2.2 Continued

Microbial Counts (log CFU/mL)								
MRS	0.2 ± 0.02	6.2 ± 0.3	5.3 ± 0.1	3.8 ± 0.1	6.1 ± 0.1	4.6 ± 0.1	5.3 ± 0.1	6.8 ± 0.1
YMA	5.7 ± 0.2	5.3 ± 0.4	5.5 ± 0.1	5.5 ± 0.1	2.4 ± 0.1	6.5 ± 0.1	4.2 ± 0.1	4.8 ± 0.1
Estimated Microbial Counts Based on Characteristic Morphologies (log CFU/mL)								
MB1	ND	NA[£]	0.1 ± 0.1	1.1 ± 0.1	ND	1.4 ± 0.1	1.6 ± 0.1	3.6 ± 0.1
MB2	ND	NA	ND	1.3 ± 0.1	ND	ND	2.7 ± 0.1	3.7 ± 0.1
MY1	1.4 ± 0.02	NA	5.5 ± 0.1	5.5 ± 0.1	3.6 ± 0.1	6.5 ± 0.1	3.8 ± 0.1	3.2 ± 0.1

[*]T_i designates observations obtained from the commercial spoilage samples upon delivery.

[†]T_f designates observations obtained from the commercial spoilage samples after incubation at 30°C for 15 days under aerobic and static conditions.

[§]Sample #4 was spiked with 50 mL lactic acid before incubation. After adding lactic acid sample pH was 3.9.

[£]NA: Data is not available

[¥]ND: Not detected

Table 2.3 Trends in pH, lactic acid utilization and yeast counts during secondary cucumber fermentation reproduced in the laboratory using commercial spoilage #4 as inoculum.

Incubated under aerobic conditions for 1 month.					
Time (days)	**pH**	**Microbial counts (log CFU/mL)**			**Lactic acid (mM)**
		Total YMA Counts	**MY1 Counts from YMA**	**MY2 Counts from YMA**	
0	3.8 ± 0.1	2.8 ± 0.0	ND*	ND	110.7 ± 0.1
4	4.1 ± 0.1	6.7 ± 0.1	2.2 ± 0.1	ND	90.7 ± 3.4
8	6.8 ± 0.1	7.4 ± 0.2	1.5 ± 0.2	3.8 ± 1.2	61.3 ± 3.0
16	7.4 ± 0.1	7.2 ± 0.4	2.6 ± 0.4	4.7 ± 0.4	18.5 ± 16.2
21	7.8 ± 0.1	6.6 ± 0.6	2.1 ± 0.4	4.3 ± 0.1	ND
32	8.3 ± 0.1	4.6 ± 1.2	ND	ND	ND
Incubated under anaerobic conditions for 3.5 months.					
Time (days)	**pH**	**Microbial counts (log CFU/mL)**			**Lactic acid (mM)**
		Total YMA Counts	**MY1 Counts from YMA**	**MY2 Counts from YMA**	
0	3.8 ± 0.0	3.2 ± 0.1	ND	ND	112.2 ± 2.2
4	3.8 ± 0.1	4.6 ± 0.1	ND	ND	109.7 ± 0.9
32	3.2 ± 0.1	2.1 ± 0.1	ND	ND	109.4 ± 2.2
39	3.9 ± 0.1	ND	ND	ND	107.5 ± 3.7
56	4.0 ± 0.1	1.3 ± 0.3	1.3 ± 0.3	ND	98.3 ± 3.7
106	4.3 ± 0.1	2.3 ± 0.3	1.9 ± 0.8	ND	74.3 ± 3.6

ND: not detected. Each time point represents the average of three independent replicates.

Table 2.4. Trends in pH, acetic and propionic acids formation, and lactic acid bacteria counts during secondary cucumber fermentation reproduced in the laboratory using commercial spoilage #4 as inoculum.

Incubated under aerobic conditions for 1 month.						
		Microbial counts (log CFU/mL)				
Time (days)	**pH**	**Total MRS Counts**	**MB1 Counts from MRS**	**MB2 Counts from MRS**	**Acetic Acid (mM)**	**Propionic Acid (mM)**
0	3.8 ± 0.1	3.8 ± 0.1	2.9 ± 0.1	ND	7.7 ± 0.1	ND
4	4.1 ± 0.1	4.1 ± 0.1	3.6 ± 0.1	ND	4.5 ± 0.5	ND
8	6.8 ± 0.1	6.8 ± 0.1	5.6 ± 0.1	0.5 ± 0.2	5.6 ± 1.1	ND
16	7.4 ± 0.1	7.4 ± 0.1	5.8 ± 0.5	1.9 ± 0.4	21.7 ± 7.5	11.4 ± 17.1
21	7.8 ± 0.1	7.8 ± 0.1	6.6 ± 0.3	1.9 ± 0.2	27.0 ± 3.3	17.5 ± 10.5
32	8.3 ± 0.1	8.3 ± 0.1	6.2 ± 0.1	2.2 ± 0.1	27.8 ± 5.3	17.7 ± 9.5
Incubated under anaerobic conditions for 3.5 months.						
		Microbial counts (log CFU/mL)				
Time (days)	**pH**	**Total MRS Counts**	**MB1 Counts from MRS**	**MB2 Counts from MRS**	**Acetic Acid (mM)**	**Propionic Acid (mM)**
0	3.8 ± 0.0	3.1 ± 0.1	ND	ND	7.7 ± 0.1	ND
4	3.8 ± 0.1	3.0 ± 0.1	ND	ND	7.1 ± 0.1	ND
32	3.2 ± 0.1	7.0 ± 0.1	ND	ND	11.8 ± 1.1	ND
39	3.9 ± 0.1	7.3 ± 0.1	4.0 ± 0.7	3.1 ± 0.5	14.9 ± 1.1	3.4 ± 0.4
56	4.0 ± 0.1	6.6 ± 0.4	3.1 ± 0.1	2.7 ± 1.1	19.9 ± 1.6	6.2 ± 0.7
106	4.3 ± 0.1	5.2 ± 0.3	3.8 ± 1.2	3.8 ± 0.2	30.5 ± 2.0	14.5 ± 1.1

ND: not detected. Each time point represents the average of three independent replicates

Table 2.5. Identification of selected isolates from the secondary cucumber fermentation based on colony morphology and *rRNA* sequencing.

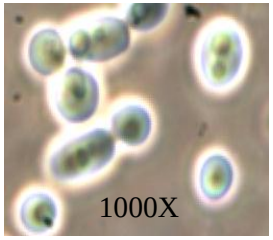
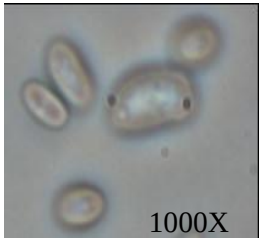
ID[1]	Colony Picture	Cell under the microscope	Identification[3]	Observed in[4]	Culture collection ID number[5]
MY1	 1 mm	 1000X	*Pichia manshurica*	Spoilage sample # 1, and 2, and outbreak 2010	Y88
MY2	 1.5 mm	 1000X	*Issatchenkia occidentalis*	Spoilage sample # 1, and 2, and outbreak 2010	Y89

Table 2.5 Continued

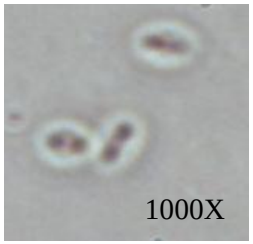
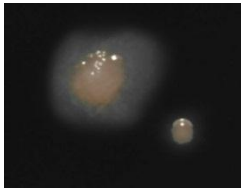
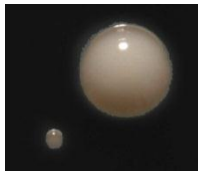
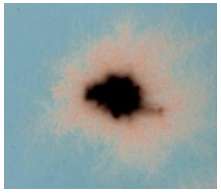
MY3	 0.3 mm	 1000X	*Candida cf. etchellsii*	Standard fermentation tanks	Y92
MB1	 1 mm	Gram positive Non spore-forming bacilli. Cells are spherical.	*Lactobacillus buchneri*	Spoilage sample # 1 and outbreak 2010	LA1149
MB2[2]	 1 mm	Gram positive coccus. Occurs in chains. Non spore-forming	*Pediococcus ethanolidurans*	Spoilage sample # 2 and outbreak 2010	LA1150

Table 2.5 Continued

MC1	 1 mm	Gram positive, spore-forming rod.	*Clostridium bifermentans or sordelii*	Spoilage sample # 4	B431

[1]Microorganisms designated with a MY#, MB#, and MC# were isolated from YMA, MRS, and DRCA agar plates, respectively.

[2] Small colony represents the isolate MB2. Bigger colony represents *L. plantarum* colony (LA 445, USDA-ARS culture collection). Approximately diameter for *L. plantarum* was 1 mm, while MB2 was about 1/10 of *L. plantarum*. Both colonies are shown here for comparison in size.

3 Based on *16S* or *26S rRNA* for bacteria and yeasts, respectively.

[4] The colony morphology was observed in different commercial secondary cucumber fermentation brines.

[5]All isolates are stored at the USDA-ARS Food Science Research Unit, North Carolina State University, Raleigh, NC

Table 2.6. Allyl isothiocyanate (AITC) inhibition of lactic acid degradation caused by MY1 (*Pichia manshurica*) and MY2 (*Issatchenkia occidentalis*) isolates

Time (d)	pH		Lactic acid (mM)		Acetic acid (mM)	
	+AITC[1]	- AITC[2]	+AITC	- AITC	+AITC	- AITC
0	4.1 ± 0.01	4.2 ± 0.02	ND*	ND	23.0 ± 0.05	23.6 ± 0.05
4	3.5 ± 0.01	3.2 ± 0.01	37.2 ± 0.05	61.7 ± 0.9	23.1 ± 0.03	20.2 ± 0.3
7	3.3 ± 0.01	3.2 ± 0.00	51.4 ± 0.04	66.6 ± 0.01	22.9 ± 0.2	20.1 ± 0.3
15	3.2 ± 0.00	3.1 ± 0.02	61.9 ± 0.5	72.6 ± 1.7	22.4 ± 0.2	13.9 ± 0.2
20	3.1 ± 0.02	6.3 ± 0.01	65.9 ± 0.5	29.8 ± 0.9	23.5 ± 0.3	1.6 ± 0.03
30	3.1 ± 0.04	6.9 ± 0.03	68.5 ± 1.2	6.32 ± 0.2	24.2 ± 0.6	1.3 ± 0.1
60	3.2 ±0.01	6.9 ± 0.03	70. ± 1.1	ND	24.3 ± 0.5	ND

[1]+AITC: Cucumber juice media supplemented with 100 ppm AITC

[2]- AITC: Cucumber juice media NOT supplemented with AITC

*ND: Not detected

Each time point represents the mean of two replicates.

Figure 2.1. Lactic and acetic acid in commercial spoiling tanks ranked according to pH

A total of 66 commercial fermentation tanks were monitored. Eighteen of them were standard fermentation tanks kept under storage for 1 to 12 months with a stable pH of 3.3 ± 0.1. Twenty- eight spoiling tanks included in the box plots shown in the figure had pH values ranging from 3.6 ± 0.1 to 3.8 ± 0.1; 17 ranging from 3.9 ± 0.1 to 4.1 ± 0.1; and only 4 were ranked at pH of 4.2 ± 0.2. An average temperature of 31.6 ± 0.6°C was recorded from the 66 fermentation tanks monitored at the time of sampling. Significant differences ($P > 0.05$) in lactic and acetic acids were observed when means from spoiling tanks were grouped by Duncan's multiple range test and compared with the standard fermentation samples. The box plot shows: Median (Middle black line); Sample maximum (Upper box line); Sample minimum (Lower box line); Quartile (Fainted black line); Outliers (Black dotes).

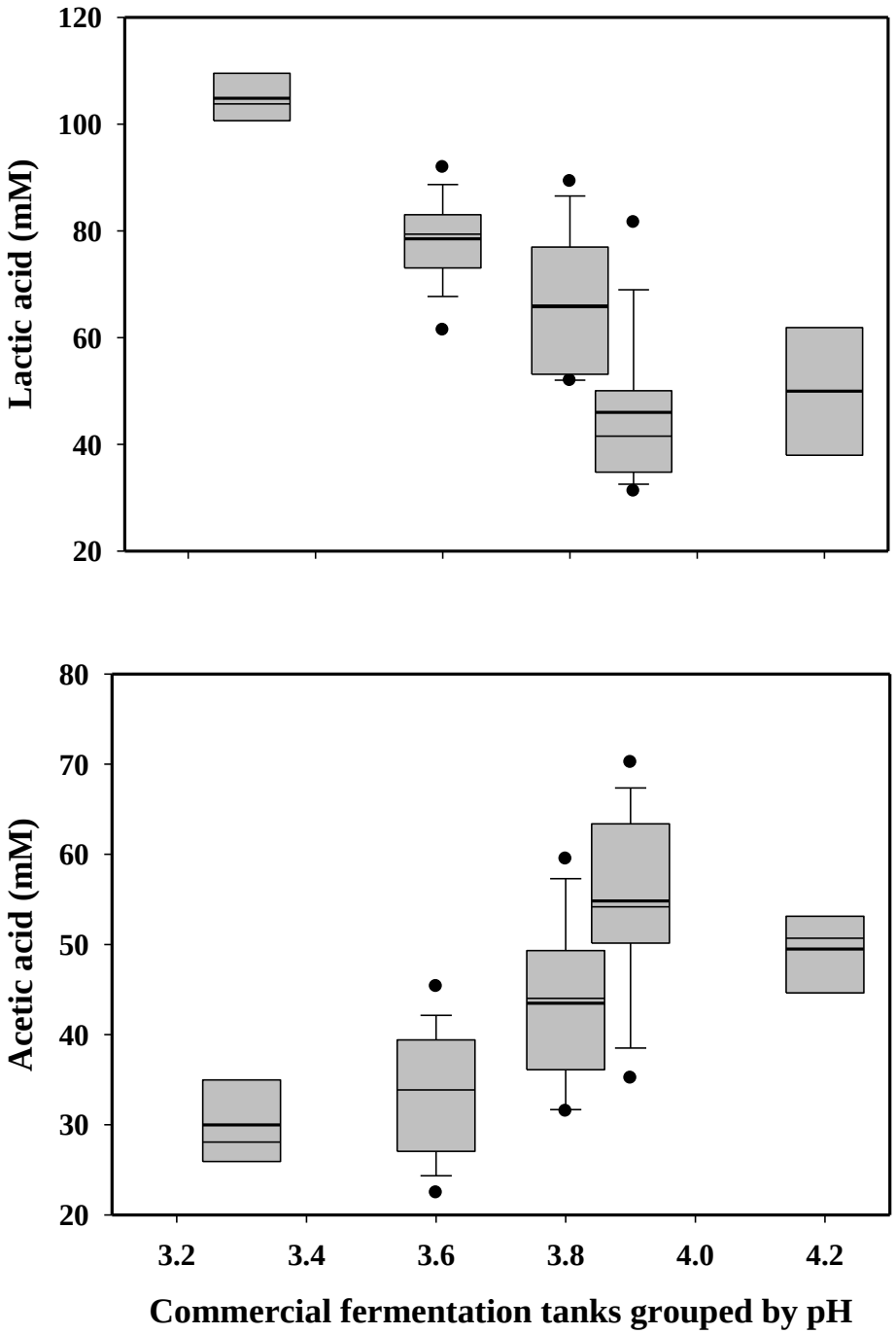

120
100
80
60
40
20
Lactic acid (mM)
80
70
60
50
40
30
20
Acetic acid (mM)
3.2
3.4
3.6
3.8
4.0
4.2
Commercial fermentation tanks grouped by pH

Figure 2.2. Redox potential measurements in standard and spoiled commercial cucumber fermentation tanks

A total of 66 tanks were monitored from which 18 were standard fermented cucumber tanks that had been in storage between 1 and 12 months, with a stable pH of 3.3 ± 0.1. While 18 spoiling tanks at pH 3.6 ± 0.1 to 3.9 ± 0.1 were included in calculation of the medians, only 8 and 5 tanks were monitored at pH 4.1 ± 0.1 and 4.2 ± 0.2, respectively. An average temperature of 31.61 ± 0.61 □C was recorded from the 66 fermentation tanks monitored on the sampling day. A significant difference between the Eh at pH between 3.3 to 4.1 and 4.2 was determined by the Duncan's multiple range test ($P > 0.05$).

The box plot shows: Median (Middle black line); Sample maximum (Upper box line); Sample minimum (Lower box line); Quartile (Fainted black line); Outliers (Black dotes).

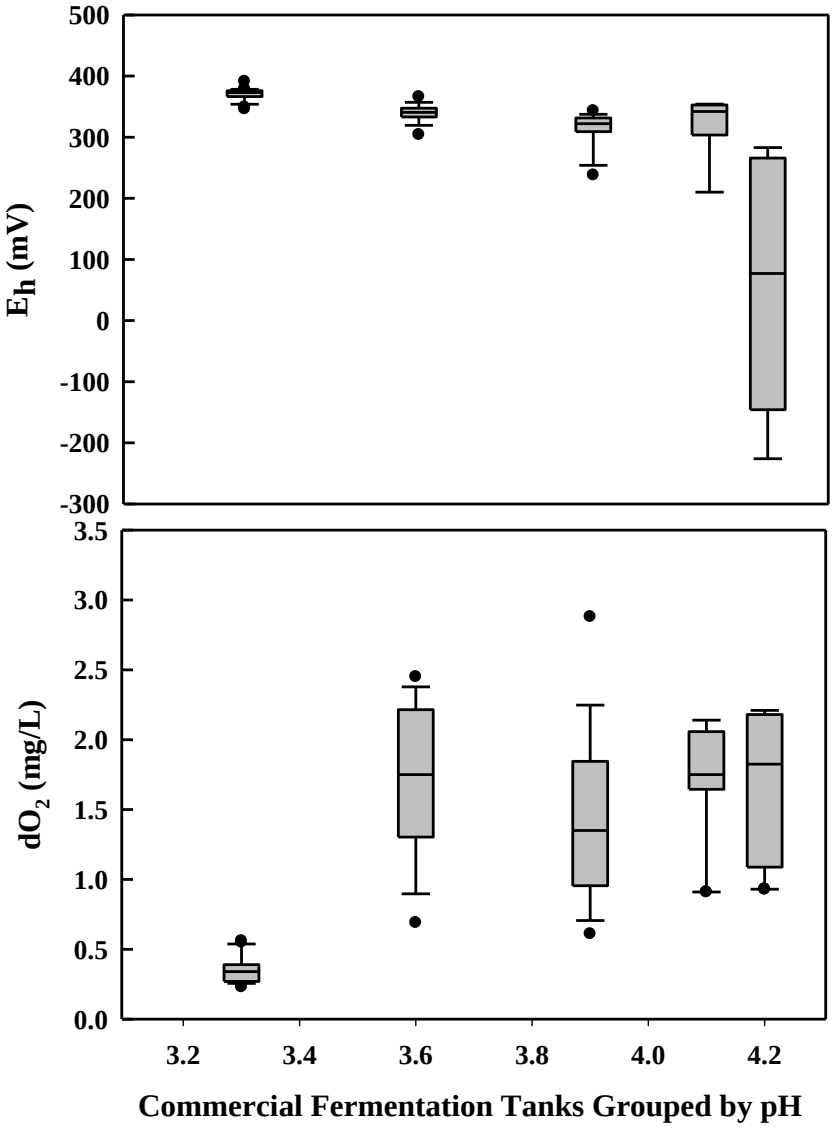
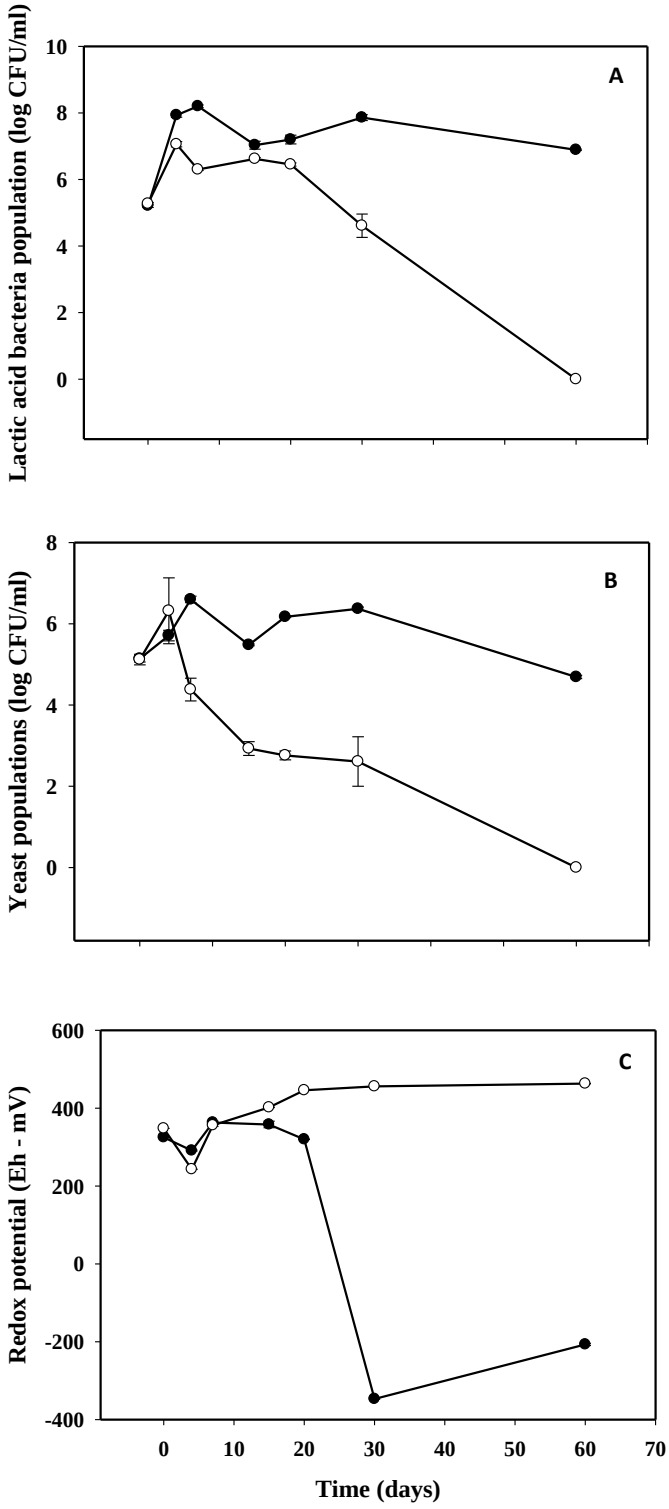

500
400
300
200
100
0
-100
-200
-300
E_h (mV)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
dO_2 (mg/L)
3.2
3.4
3.6
3.8
4.0
4.2
Commercial Fermentation Tanks Grouped by pH

Figure 2.2. Microbiological and environmental changes observed during the inhibition of lactic acid degradation caused by *Pichia manshurica* and *Issatchenkia occidentalis*

Changes in lactic acid bacteria populations (panel A), yeast populations (panel B), and redox potential (panel C) observed during air-purged incubation of cucumber juice media supplemented with AITC (○) and without AITC (●) and inoculated with spoilage yeasts (*Pichia manshurica* and *I. occidentalis*) and *L. plantarum* mixtures.


A
Lactic acid bacteria population (log CFU/ml)
10
8
6
4
2
0
B
Yeast populations (log CFU/ml)
8
6
4
2
0
C
Redox potential (Eh - mV)
600
400
200
0
-200
-400
0
10
20
30
40
50
60
70
Time (days)

References

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Chapter 3 Development of a model system for studying secondary cucumber fermentation associated with spoilage of the fermented fruits during long term storage

Submitted to the Journal of Food Science – August 2011

Abstract

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

Introduction

The study of secondary cucumber fermentation has represented a challenge for many years, primarily because of the sporadic occurrence of the event at the commercial scale in tanks containing cover brine solutions with 1.03 M (6%) NaCl, and the inability to predict the development of the spoilage after the primary fermentation completes. Additionally, a limited number of studies have based the characterization of the event under anaerobic conditions, low NaCl, and elevated initial brine pH (Kim and Breidt 2007; Johanningsmeier and others 2011), conditions which do not fully mimic the current commercial fermentation conditions.

The study of a secondary cucumber fermentation outbreak in 2010 provided an opportunity to document biochemical and microbiological characteristics (Franco and others 2011) which suggested that both oxidative yeasts, such as *Pichia manshurica* and *Isstachenkia occidentalis,* and spoilage lactic acid bacteria (LAB), such as *Lactobacillus buchneri,* are involved in the development of the phenomenon. However, no specific role was associated to any of these organisms under the prevailing conditions once the primary fermentation is completed. The acidic conditions, lack of sugars and the irregularities in the air purging routine applied at the commercial scale after the primary fermentation is completed (Pérez-Díaz 2010) suggest that the organisms capable of initiating the spoilage of the fermented product are able to utilize organic acids as a source of energy and are microaerophilic or aerobic. Thus, it was hypothesized that it should be possible to develop the secondary cucumber fermentation in the laboratory by introducing an air-purging routine

to fermentation vessels during and after the primary fermentation. The successful development of such model systems for the study of the secondary cucumber fermentation in the laboratory is essential to gain a better understanding of the event and in optimizing the table salt free cucumber fermentations at the commercial scale.

Materials and Methods

Model system

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

Vessels were inoculated with a mixture of three *Lactobacillus plantarum* strains, LA445, LA98, and LA 89, (USDA-ARS Food Science Research Unit, Raleigh, N.C., U.S.A., culture collection) to 10^5 CFU/mL. A constant temperature of 30 °C was maintained in the fermentation vessels using a re-circulating chiller (NESLAB Merlin M-75, Thermo Electron

Co., Newington, N.H., U.S.A.). Control experiments followed the same experimental design but were kept anaerobic by flushing nitrogen into the head space of the fermentation vessels. Parameters such as redox potential (Pt4805-DPAS-SC-K8S/200 redox electrode; Mettler-Toledo, Bedford, M.A., U.S.A.), dissolved oxygen (Inpro 6830/220 electrode; Mettler-Toledo) and pH (pH electrode model 405-DPAS-SC-K8S/225; Mettler-Toledo) were monitored over time. The measured redox potentials were converted to the redox potential against the standard hydrogen electrode (E_h) by adding 203.4 mV (30 °C) according to manufacturer instructions. Electrodes for all of these measurements were calibrated, rinsed with 70% ethanol and secured into fittings on the head plate of each fermentation vessel.

Microbiological analysis

Aseptically collected samples were serially diluted in 0.85% saline solution, and spiral plated using an Autoplate 400 (Spiral Biotech, Norwood, M.A., U.S.A.) on Lactobacilli deMan Rogosa and Sharpe agar (MRS, Becton Dickinson and Co., Franklin Lakes, N.J., U.S.A.) supplemented with 1% cycloheximide (0.1% solution, OXOID, New England); yeast and molds agar (YMA, Becton, Dickinson and Co.) supplemented with 0.01% chloramphenicol (Sigma-Aldrich, St. Louis, M.O., U.S.A.) and 0.01% chlortetracycline (Sigma-Aldrich); violet red bile agar supplemented with 1% glucose (VRBG, Becton, Dickinson and Co.); and differential reinforced clostridia agar (DRCA, Becton) supplemented with 1% cycloheximide (OXOID, New England) for the enumeration of lactic acid bacteria, yeast and molds, enterobacteria, and *Clostridium* spp., respectively. Purple and pink colonies after 24 h incubation on VRBG plates were recorded as

presumptive enterobacteria. Black colonies after 48 h anaerobic incubation on DRCA plates were recorded as *Clostridia* spp.

The bacteria and yeast isolated were identified using partial *16S* and/or *26S rRNA* gene sequencing, respectively. Bacterial chromosomal DNA was obtained using the DNeasy genomic extraction and purification kit (DNeasy, Qiagen, Valencia, C.A., U.S.A.) while yeast chromosomal DNA was obtained using the MasterPure™ Yeast DNA Purification Kits (Epicentre Biotechnologies, Madison, W.I., U.S.A.). The PCR mix contained 2X master mix (Biorad, Hercules, C.A., U.S.A.), chromosomal DNA and forward and reverse primers, which were 27F (5-AGAGTTTGATCCTGGCTCAG-3') (Barrangou and others 2002) and 1492r (5'-GGTTACCTTGTTACGACTT-3') (Wilson and others 1990) for the bacterial isolates, and NL-1 (5'-GCCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG) for yeasts (Kurtzman and Robnett 1997). All primers were obtained from Integrated DNA Technologies (Coralville, I.A., U.S.A.). PCR products were purified using the Qiagen PCR purification kit and sequenced by Eton Bioscience Inc. (Raleigh, N.C., U.S.A.). The sequences obtained were subjected to the basic local alignment search tool (BLAST) (Altschul and others 1990) in the GenBank (Benson and others 2000) database to determine the identity of the strains.

Chemical analysis

Cover brine solution samples were aseptically collected, spun for 10 minutes at 12,000 rpm using an Eppendorf 5810R Centrifuge (Eppendorf North America, Inc., Westbury, N.Y., U.S.A.) and the supernatants diluted 10X prior to HPLC analysis. The

concentrations of organic acids and sugars in the supernatants were measured using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, C.A., U.S.A.) (McFeeters and Barish 2003). The column was heated to 37 °C and eluted with 0.03N sulfuric acid at a flow rate of 0.6 mL/min. A Thermo Separations UV6000 diode array detector (Spectra System Thermo Scientific, Waltham, M.A., U.S.A.) 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, M.A., U.S.A.) 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.

Results and Discussion

The primary fermentation of cucumbers brined as described above proceeded as expected and was followed by growth of yeasts and selected lactic acid bacteria under both air-purging and anaerobic conditions for both NaCl and $CaCl_2$ brines. Under air-purged conditions, the completion of primary fermentation was evident by day 6 due to complete sugar utilization, the production of 83.6 ± 6.1 mM lactic acid, and a pH of 3.2 in the $CaCl_2$ fermentations (Table 3.1, Panel A), and 81.5 ± 0.6 mM lactic acid, and same final pH in the NaCl fermentations (Table 3.1, Panel B). The fermented product for both brines remained stable in the control vessels in which a nitrogen head space was maintained (Table 3.1). However, under air-purged conditions a secondary fermentation or spoilage was initiated and characterized by the rapid oxidation of the lactic acid produced during the primary fermentation, which, in turn, increased the brine pH. Organic acid disappearance seems to be

the result of yeast metabolism (Tables 3.1 and 3.4) that also leads to the reduction of the environment (E_h = -100 mV, Ag/AgCl, 3M KCl) (Table 3.2) to such levels at which bacteria, other than the lactic acid bacteria responsible for the primary fermentation (Table 3.4), become established and produce butyric, propionic, and acetic acids (Table 3.3). Organisms presenting morphologies representative of those previously isolated from commercial spoilage samples were observed in air-purged $CaCl_2$ and NaCl fermentations (Franco and others 2011). Three stages were noticed during this secondary fermentation. The first stage was characterized by a continuous increase in brine pH, and the disappearance of lactic and acetic acids (Table 3.1), which are characteristics observed from the commercial secondary fermentation as well. During this period the yeast population increased in both NaCl and $CaCl_2$ brines to populations about 6 log CFU/mL (Table 3. 2). As yeast population increased, the environment was further reduced as evidenced by decreases in redox potential to negative values (Table 3.2). Reduction of the environment occurred at a faster rate in $CaCl_2$ brines, which achieved a value of -228.6 mV by day 9 (Table 3.2). In NaCl brines, the reduction of the environment was noticed concomitantly with the increase in pH once primary fermentation was completed. Yeast population started to decline 9 d post-packing in $CaCl_2$ brines and 18 d in NaCl brines. Although the declining trends in E_h continued up to the end of the experimentation (30 d), yeast counts were, on average, 5.4 log CFU/mL and 4.0 log CFU/mL for $CaCl_2$ and NaCl brines, respectively.

In the fermentations brined with 100 mM $CaCl_2$, day 9 marked the beginning of a tertiary fermentation stage, characterized by the production of butyric and propionic acids, an

E_h of -82.6 mV, dissolved oxygen (dO_2) of 5.2 mg/L, and increases in the counts for enterobacteria and presumptive *Clostridium* spp. to 1.9 and 2.0 log of CFU/mL, respectively (Table 3.3, Panel A). While the numbers of the enterobacteria remained at 5 log of CFU/mL until the end of the experimentation, the population of presumptive clostridia increased to 5.9 log of CFU/mL concomitantly with the production of butyric acid (Table 3.3, Panel A). In NaCl fermentations, the tertiary fermentation was observed later, on day 22, with the production of propionic acid only (Table 3.3, Panel B). The production of the organic acids was concomitant with a significant increase in enterobacteria population above 4 log CFU/mL. No *clostridium* species were identified during the course of the experimentation in NaCl brines which was also in agreement with the absence of butyric acid (Table 3.3, Panel B).

Although, the LAB population remained close to 8 and 7 log CFU/mL for $CaCl_2$ and NaCl fermentation, respectively, it is important to notice that upon initiation of the tertiary fermentation, the LAB microflora changes and becomes a mixed culture (Table 3.4). Morphologies B1 and B2, which are representative of *Lactobacillus buchneri* and *Pediococcus ethanolidurans* among other LAB (Franco and others 2011), become predominant.

Dissolved oxygen (dO_2) in air-purged experiments remained about 5.3 and 3.2 mg/L in $CaCl_2$ and NaCl brines, respectively, (Table 3.2) the first 6 d of experimentation. A reduction in the dO_2 levels was observed immediately after adding the cucumber cubes to the fermentation vessels, presumably due to the respiration of the fruits. The dO_2 levels stabilize

after equilibration of the cucumbers and cover brine solution constituents (Potts and Fleming 1979). Once the lactic acid utilization starts, dO_2 measurements decreased significantly, even when the air flow was kept constant at 5 mg/L. Oxygen uptake during and after primary fermentation is attributed to yeast populations that become established in high numbers in air-purged fermentations (Potts and Fleming 1979).

The characteristics of the secondary fermentations reported here are similar to the outbreak study described by Franco and others (2011), with special emphasis on the events observed towards the end of the experimentation. In $CaCl_2$ experiments, lactic acid was depleted after 20 d of fermentation and although acetic acid was also utilized during this period; minimal amounts of acetic acid were produced after lactic acid was depleted (Table 3.1). A total of 16, 4, and 14 mM butyric, propionic, and acetic acids were produced after 30 d of experimentation with $CaCl_2$ brines. Final brine pH, E_h and dO_2 were 5.6, -290.6 mV, and 0.38 mg/L, respectively. In NaCl experiments, lactic acid was not completely utilized, and after 30 d of experimentation about 6 mM remained. In agreement with the $CaCl_2$ experiments, small amounts (about 3 mM) of acetic acid were produced. Final NaCl brine pH, E_h, and dO_2 were 5.6, -295.1 mV, and 4.1 mg/L, respectively. Interestingly, in NaCl experiments propionic acid production was about 10 mM more than in $CaCl_2$ brines while butyric acid was not detected. At the commercial scale, half of the spoilage samples reported showed the presence of propionic acid, and only a few had butyric acid (Franco and others 2011). Although, the ability of the production of secondary products is related with the initial microbial load in the cucumber fruit, it is possible that the presence of NaCl favors certain

bacteria which are able to utilize lactic acid and convert it to propionic acid (Johanningsmeier and others 2011). Although, *Clostridium* spp. are somehow tolerant to a wide range of NaCl concentrations (Spielberg 1944), it seems that the combination of the preservative with low pH and carbon limiting sources is effective in decreasing the chances of the bacterium to produce butyric acid in fermented cucumbers. About 3% NaCl has been reported as an inhibitor of toxin production and other metabolic activities in *Clostridium botulinum*, but it is not a limiting factor for outgrowth and cell multiplication (Boyd and Southcott 1971).

Morphological characteristics of the commercial spoilage isolates, such as YI, Y2, B1 and B2, were also exclusively detected from the fermentation vessels subjected to air-purging. Such morphologies have been associated with *Issatchenkia occidentalis*, *Pichia manshurica*, *Lactobacillus buchneri*, and *Pediococcus ethanolidurans*. Moreover, the morphology designated as Y3, previously isolated from standard commercial fermentations only, was exclusively isolated from the vessels maintained under a nitrogen head space. A different yeast morphology was observed in NaCl brines (Y4). The yeast colony in YMA plates (48 h incubation at 30 °C) is white, with an entire margin, smooth and raised in the middle. The colony can be clearly distinguished from others due to its "mount-like" morphology. The cells occur in buds and have an ellipsoidal shape with the presence of ascopores. The isolated colony was identified as *Candida tropicalis*. Colonies showing morphologies similar to that of *L. plantarum* were observed predominantly during primary fermentation in all the fermentation vessels scrutinized. Morphologies B1 and B2 isolated previously from commercial spoilage samples were observed concomitant with propionic

acid production. Selected LAB have been suggested as able to initiate the spoilage of fermented cucumbers (Johanningsmeier and others 2011); however, lactic acid consumption proceeds at a slower rate in the absence of yeasts and/or under anaerobic conditions (Franco and others 2011).

Aside from noticing the same morphologies observed from the commercial secondary fermentations, four additional morphologies were targeted. Three of these morphologies were detected on the VRBG plates inoculated with the samples from the air-purged vessels and were identified by the partial sequencing of the *16S* rRNA as *Enterobacter cloacae*, *Enterobacter aerogenes* and a member of the *Enterobactereceae* family. Enterobacteria are usually inhibited as lactic acid is produced and pH decreases during the primary fermentation process (Etchells and others 1945). However, recent studies have indicated enterobacteria, such as *E. cloacae*, as a source of contamination and potential spoilage problems in naturally fermented table olives (Bevilacquia and others 2009).

The isolate targeted from the DRCA plates inoculated with the spoilage samples was identified as *Clostridium bifermentans/sordelli*. *Clostridium* spp. have been reported as responsible for lactic acid conversion into butyric acid (Fleming and others 1989; Yang and others 2009) primarily at pH above 5.0 under anaerobic conditions (Fleming and others 1989). However, the data from $CaCl_2$ air-purged experiments indicates that butyric acid is produced in reduced environmental conditions (negative E_h values) but lower pH values. Further studies are necessary to test the ability of *Cl. bifermentans* to produce these changes at a lower pH.

Conclusion

Based on the information gathered in this study, we suggest the following succession of events during the fermentation of cucumber pickles fermented in $CaCl_2$ brines under air-purged conditions. Primary fermentation dominated by *L. plantarum* is completed, converting glucose into lactic acid with the concomitant decrease in brine pH. Unidentified triggers, including oxygen availability, induce growth of yeasts, such as the film formers *I. occidentalis,* and *P. manshurica,* after primary fermentation. Yeast metabolism results in not only organic acids (lactic and acetic) utilization and the corresponding increase in brine pH, but also on further reduction of the environment as dissolved oxygen is utilized. Reduced environments and increased pH lead to the growth of other microorganisms that are able to further utilize lactic acid and produce either propionic, butyric and/or acetic acids, such as *C. bifermentans/sordelii, L. buchneri, P. ethanolidurans,* and enterobacteria.

Table 3.1. Changes in pH, lactic and acetic acids observed in $CaCl_2$ and NaCl cucumber fermentations with and without air-purging in a 2L vessel BioFlo110 system

Time	pH		Lactic acid (mM)		Acetic acid (mM)	
	A[1]	NA[2]	A	NA	A	NA
Panel A: $CaCl_2$ fermentation						
Day 1	4.0 ± 0.2	4.2 ± 0.1	1.8 ± 1.4	15.7 ± 1.6	24.1 ± 0.6	24.8 ± 1.9
Day 6	3.2 ± 0.1	3.1 ± 0.0	83.6 ± 6.1	71.6 ± 2.9	16.6 ± 4.3	27.1 ± 0.8
Day 9	3.4 ± 0.1	3.1 ± 0.0	70.8 ± 0.6	71.0 ± 7.1	10.8 ± 1.7	25.8 ± 1.3
Day 18	4.5 ± 0.7	3.2 ± 0.0	32.1 ± 1.1	86.9 ± 2.3	3.5 ± 0.5	27.7 ± 2.8
Day 22	4.7 ± 0.4	N/A	N/D	N/A	9.4 ± 2.6	N/A
Day 30	5.6 ± 0.03	N/A	N/D	N/A	18.5 ± 3.4	N/A
Panel B: NaCl fermentation						
Day 1	4.1 ± 0.0	4.1 ± 0.1	N/D	N/D	27.5 ± 0.1	27.1 ± 0.7
Day 6	3.2 ± 0.0	3.2 ± 0.0	81.5 ± 0.6	95.1 ± 3.5	24.6 ± 0.0	22.8 ± 0.3
Day 9	3.5 ± 0.0	3.2 ± 0.0	74.4 ± 0.1	96.3 ± 2.9	18.7 ± 0.3	20.2 ± 0.0
Day 18	4.4 ± 0.2	3.2 ± 0.0	17.7 ± 0.3	98.0 ± 1.5	11.7 ± 0.5	23.4 ± 0.3
Day 22	5.0 ± 0.1	3.2 ± 0.0	15.7 ± 0.4	97.0 ± 4.2	12.8 ± 0.3	25.6 ± 0.4
Day 30	5.6 ± 0.0	3.2 ± 0.0	6.3 ± 0.3	94.7 ± 3.3	14.7 ± 0.7	20.6 ± 7.1

[1] Air-purged fermentation

[2] Non-aerated fermentation

N/D: Not detected

N/A: Data not available

Values represent mean ± standard deviation of three independent replicates

Table 3.2 Changes in redox potential (E_h), dissolved oxygen, and colony counts for LAB and yeasts observed in $CaCl_2$ (Panel A) and NaCl (Panel B) cucumber fermentations with and without air-purging in a 2L vessel BioFlo110 system

Time	dO_2 (mg/L)		E_h (mV)		Total LAB (log CFU/ml)		Total yeasts (log CFU/ml)	
	A[1]	NA[2]	A	NA	A	NA	A	NA
$CaCl_2$ fermentation								
Day 0	0.5 ± 0.1	1.7 ± 0.1	186.4 ± 17.2	169.5 ± 5.6	5.1± 0.1	5.0 ± 1.2	3.1 ± 0.3	3.2 ± 0.6
Day 1	0.2 ± 0.1	N/D	1.4 ± 1.4	189.3 ± 44.3	8.5 ± 0.3	8.5 ± 0.3	4.9 ± 0.3	4.3 ± 0.1
Day 6	5.3 ± 0.2	N/D	-82.6 ± 11.5	210.0 ± 0.1	8.8 ± 0.1	7.4 ± 0.1	6.0 ± 0.5	5.1 ± 0.0
Day 9	5.2 ± 0.5	N/D	-228.6 ± 8.8	216.5 ± 2.1	7.1 ± 0.3	6.1 ± 0.2	5.9 ± 0.3	5.5 ± 0.0
Day 18	1.8 ± 0.1	N/D	-178.6 ± 14.5	194.2 ± 4.5	8.1 ± 0.5	5.7 ± 0.1	5.7 ± 1.1	5.5 ± 0.0
Day 22	1.9 ± 0.2	N/A	-290.6 ± 9.9	N/A	8.5 ± 0.1	N/A	5.2 ± 0.7	N/A
Day 30	0.8 ± 0.1	N/A	194.2 ± 4.5	N/A	8.0 ± 0.3	N/A	4.9 ± 0.6	N/A
NaCl fermentation								
Day 0	3.2 ± 0.1	2.6 ± 0.1	188.1 ± 0.1	157.4 ± 0.0	5.3 ± 0.2	5.2 ± 0.0	2.1 ± 0.1	2.4 ± 0.1
Day 1	4.8 ± 0.0	N/D	295.4 ± 9.9	250.0 ± 0.1	8.3 ± 0.0	6.1 ± 0.3	3.1 ± 0.2	3.2 ± 0.3
Day 6	3.2 ± 1.2	N/D	347.4 ± 0.0	247.4 ± 0.0	8.1 ± 0.0	8.4 ± 0.6	6.1 ± 0.2	5.3 ± 0.0
Day 9	3.5 ± 0.0	N/D	3.9 ± 0.7	324.4 ± 0.0	7.8 ± 0.2	8.2 ± 0.0	6.0 ± 0.0	5.7 ± 0.0
Day 18	1.2 ± 0.0	N/D	-107.4 ± 0.0	299.0 ± 0.0	7.4 ± 0.3	6.4 ± 0.1	6.1 ± 0.0	4.7 ± 0.0
Day 22	1.2 ± 0.0	N/D	-229.6 ± 0.0	277.4 ±0.0	7.3 ± 0.1	7.4 ± 0.0	4.3 ± 1.0	3.9 ± 0.0
Day 30	4.1 ± 0.0	N/D	-295.1 ± 0.0	292.4 ± 0.0	7.0 ± 0.2	6.4 ± 0.1	4.0 ± 0.5	2.8 ± 0.1

Table 3.2 continued
[1]Air-purged fermentation

[2] Non-aerated fermentation (Control)

N/A: Data not available

N/D: Not detected

Values represent mean ± standard deviation of three independent replicates

Table 3.3 Colony counts for *Enterobacteria* and *Clostridium* spp. and secondary products detected in $CaCl_2$ (Panel A) and NaCl (Panel B) cucumber fermentations with and without air-purging in a 2L vessel BioFlo110 system

Time (day)	VRBG Counts Log (CFU/ml)	DRCA Counts Log (CFU/ml)	Propionic acid (mM)	Butyric acid (mM)
Panel A: $CaCl_2$ fermentation				
1	N/D	N/D	N/D	N/D
6	N/D	N/D	N/D	N/D
9	1.9 ± 0.5	2.0 ± 0.1	N/D	5.3 ± 0.2
18	5.5 ± 0.2	5.9 ± 0.2	N/D	7.7 ± 0.8
22	5.8 ± 0.3	5.8 ± 0.3	4.4 ± 0.5	29.3 ± 3.7
30	5.2 ± 0.1	4.7 ± 0.1	4.4 ± 0.6	19.3 ± 4.9
Control[1]	ND	ND	N/D	N/D
Panel B: NaCl fermentation				
1	N/D	N/D	N/D	N/D
6	N/D	N/D	N/D	N/D
9	0.5 ± 0.1	N/D	N/D	N/D
18	4.8 ± 0.2	N/D	N/D	N/D
22	5.6 ± 0.0	N/D	12.8 ± 0.2	N/D
30	5.5 ± 0.1	N/D	13.8 ± 1.4	N/D
Control[1]	N/D	N/D	N/D	N/D

Table 3.3 continued

N/D; Not detected

[1] Control samples represent the same experimental design but excluding aeration. For control samples the head space of the fermentation vessels were purged with nitrogen to give the experiment an anaerobic environment

Values represent mean ± standard deviation of three independent replicates

Table 3.4 Morphologies observed in MRS and YMA plates during $CaCl_2$ (Panel A) and NaCl (Panel B) cucumber fermentations with and without air-purging in a 2L vessel BioFlo110 system

Time (days)	Air-purged fermentation				Anaerobic fermentation	
	Morphologies observed in MRS	Morphologies observed in YMA	Morphologies Observed in VRBG	Morphologies Observed in DRCA	Morphologies Observed in MRS	Morphologies Observed in YMA
Panel A: $CaCl_2$ fermentation						
1	B0	Y2, Y3	E1	N/D	B0	Y3
6	B0	Y2, few Y1 and Y3	E1, E3	N/D	B0	Y3
9	B0	Y3, Y2, Y1	E1, E2	C1	B0	N/D
18	B0, B1, B2	Y3, Y1, Y2	E2, E1, E3	C1	B0	N/D
22	B1, B2, few B0	Y3, Y1, Y2	E2, E1, E3	C1	N/A	N/A
30	B1, B2, few B0	Y3, Y1	E2, E1, E3	C1	N/A	N/A
Panel B: NaCl fermentation						
1	B0	Y3	E1	N/D	B0	Y3
6	B0	Y3, Y4, Y2, few Y1	E1, E3	N/D	B0	Y3, Y4
9	B0, B2	Y4, Y3	E2, E1	N/D	B0	Y3, Y4
18	B2, B0	Y4, Y3	E2, E1, E3	N/D	B0	Y3
22	B2, B1, few B0	Y4, Y3, Y2	E2, E1, E3	N/D	B0	Y3
30	B2, B1, few B0	Y4, Y3, Y4	E2, E1, E3	N/D	B0	Y3

Table 3.4 continued

B0: Morphology similar to *L. plantarum*; B1: Morphology similar to *L. buchneri*; B2: Morphology similar to *Pe. ethanolidurans*; Y1: Morphology similar to *Pichia manshurica;* Y2: Morphology similar to *I. occidentalis;* Y3: Selected isolates identified as *Candida tropicalis;* Y4: Selected isolates identified as *Pichia membranifaciens*; C1: Morphology similar to *Cl. bifermentans*; E1: Morphology similar to *E. aerogenes*. E2: Morphology similar to *E. clocace*, E3: Morphology similar to *Enterobactereceae* spp.

NA: Data not available; ND: Not detected

bifermentans

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Chapter 4 Role of oxidative yeasts and selected spoilage bacteria in spoilage of fermented cucumbers

Abstract

Changes during the spoilage of fermented cucumber pickles have been attributed to the metabolism of yeasts, gram positive, and gram negative bacteria. In this study six organisms isolated from commercial spoiled cucumber pickles were evaluated for their possible role in the primary and secondary (spoilage) 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. The heterofermentative lactic acid bacteria *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 the medium (3.2). The formation of secondary (spoilage) products was related to the metabolism of the spore-forming bacteria *Clostridium bifermentans* and the gram negative bacterium, *Enterobacter cloacae*. Butyric and propionic acids production at the expense of lactic and acetic acids was pH dependent. In pure cultures *C. bifermentans* and *E. cloacae* produced butyric and propionic acids when the pH od the starting media was 5.0. Individually, the selected spoilage microorganisms were able to produce changes associated with secondary fermentations of fermented cucumbers. The fact that oxidative yeasts were able to produce the chemical changes associated with the initiation of the spoilage process indicates that the prevention of the secondary fermentation could be achieved by inhibiting these organisms.

Impact statement: Deterioration of fermented cucumber pickles represents an important economic loss for the pickle industry. In this study, the onset of the spoilage process was correlated with the metabolic activity of oxidative yeasts and spoilage LAB. The identification of the role of potential microbes capable of initiating the secondary fermentations may assist the pickle industry in developing monitoring strategies to prevent the establishment of these undesirable microbes and thus prevent the spoilage of the product.

Introduction

The secondary fermentation (spoilage) of fermented cucumber pickles has been described as the result of lactic acid degradation, increases in cover brine 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 2011; Franco and others 2011). Organisms naturally present in the cucumber fruit, such as yeasts, 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 2011; Franco and others 2011; Johanningsmeier and others 2011). The spoilage has been reproduced in cucumber pickles fermented with reduced sodium chloride under anaerobic conditions (Kim and Breidt 2007; Johanningsmeier and others 2011), and at faster rates under aerobic conditions in which sodium chloride (NaCl) in cover brine solutions was replaced with calcium chloride ($CaCl_2$) (Franco and Pérez-Díaz 2011). Organisms presenting morphologies similar to those of isolates from commercial spoilage samples were observed in laboratory reproduced spoilage using $CaCl_2$ fermentations as a model system. Together these studies

aided in the identification of a succession of events and microorganisms potentially involved in the development of secondary fermentations in fermented cucumber pickles. Among the isolated microorganisms, the yeasts *Pichia manshurica* and *Issatchenkia occidentalis*, the lactic acid bacteria *Lactobacillus buchneri* and *Pediococcus ethanolidurans*, the gram negative *Enterobacter cloacae*, and the anaerobe *Clostridium bifermentans* were repeatedly observed in commercial spoilage samples and laboratory experiments.

In previous research it was observed that certain changes were necessary to trigger the production of secondary products, among which were an increase in brine pH and the chemical reduction of the fermentation (Franco and Pérez-Díaz 2011). The aerobic metabolic activity of the yeasts, *P. manshurica* and *I. occidentalis*, has been suggested as a key factor for these changes to occur. Different yeast genera have been reported as able to utilize an array of organic acids present in table olives 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 (Etchells and Bell 1950.; Binsted and others 1962*). P. manshurica* and *I. occidentalis*, commonly observed and isolated from fermented cucumber spoilage samples, were suggested to be related to utilization of lactic acid and increases in cover brine solutions pH (Franco and Pérez-Díaz 2011; Franco and others 2011).

L.buchneri and *Pe. ethanolidurans* were frequently observed in the commercial spoilage and laboratory reproduced secondary fermentations (Franco and Pérez-Díaz 2011; Franco and others 2011; Johanningsmeier and others 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 anaerobic environments (Johanningsmeier and McFeeters 2011; Johanningsmeier and others 2011), 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 (*Pe. ethanolidurans*) studied here, has been reported as suited for persisting in harsh conditions characterized by high ethanol and low sugar concentrations (Liu and others 2006), but the ability of the bacterium to utilize other carbon sources, such as organic acids, has not been documented.

Clostridium tertium (Fleming and others 1989) and *Clostridium bifermentans* (Franco and Pérez-Díaz 2011) have been isolated and suggested to be responsible for butyric acid production during spoilage of fermented pickles. *Clostridium* species are ubiquitous in 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. However, tolerance of this organism to low pH as well as its ability to sporulate-germinate and produce undesirable metabolites in fermented vegetable products has not been reported.

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 (Etchells and other 1945; Bevilacquia and others 2009). 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 the growth of these organisms in fermented is the production of acid and low 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 and others 1989; Franco and Pérez-Díaz 2011). Although at the commercial scale, a fermentation brine that has reached these pH levels it is discarded, it is of scientific interest to strudy the factors and variables that lead to this change and the following events that might be triggered. 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 and others 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 medium. Given the interest of the pickle industry to move towards NaCl free fermentations, the $CaCl_2$ fermentation matrix proposed by Franco and others (2011) to study secondary cucumber fermentations was also used during the experimentation.

Materials and Methods

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 one hour at 10,000 x g (Sorbal RC58, DuPont Instuments, Red Oak, IA, U.S.A.). 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 mix contained 25 mM acetic acid as vinegar and 100 mM anhydrous calcium chloride ($CaCl_2$). The mixture was filter sterilized using 0.2 aPES filter membranes (Nalgene, Nalge Nunv International, Rochester, NY, U.S.A.). 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 *L. plantarum* strains LA 445, LA 98, and LA 285 (USDA-ARS Food Science Research Unit, Raleigh, NC, U.S.A, Culture Collection). The inoculated CJM was incubated at 30 °C for 7 to 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 xg, 15 min, Sorbal) and the supernatant filter-sterilized (Nalgene) after a pH of 3.2 or lower was measured and low (<1 mM) quantities of sugar 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.

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, North Carolina, U.S.A. Isolates were streaked from frozen stock cultures in the agar plates indicated in Table 1 and manipulated as described. Organisms from broth cultures were harvested at 10,000 xg 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., U.S.A.).

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 initial pH was adjusted to 4.0, 4.5, and 5.0 using 5N 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* LA445 into CJM to simulate a natural fermentation.

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, Mass., U.S.A.) and a rubber septum for taking brine samples using sterile syringes. The proper functioning of the redox electrode probes was tested in buffered pH calibration solutions (4 and 7) saturated with quinnhydrone (Acros Organics, NJ, U.S.A.). 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 equiped 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 to 27 °C).

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

Microbiological Analysis

Collected samples were serially diluted in 0.85% saline solution, and spiral plated (Autoplate 400, Spiral Biotech, Norwood, MA, U.S.A.). Lactic acid bacteria and *Clostridia* enumeration was done using the deMann Rogosa and Sharp Lactobaciili 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. 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 % chlortetracycline (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.

Chemical analysis

The pH of the samples was measured with a Fisher Accumet pH meter, model 825MP, (Pittsburgh, PA., U.S.A.). Sample components such as sugars, organic acids, and ethanol were determined by HPLC on a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) (McFeeters and Barish 2003). The column was heated to 37 °C and eluted with 0.03N sulfuric acid at a flow rate of 0.6 ml/min. A Thermo Separations UV6000 diode array detector (Spectra System Thermo Scientific, Waltham, Mass., U.S.A.) set to collect data at 210 nm was used to detect malic, lactic, acetic, propionic, and butyric acids. A Waters

model 410 refractive index detector (Waters Corp.,Millipore Corp., Billerica,Mass., U.S.A.) connected in series with the diode array detector was used to measure glucose, fructose, and ethanol. External standardization of the detectors was done using 4 concentrations of the standard compounds.

Statistical analyses

Experiments conducted in the fermentation vessels were performed in duplicated in two independent runs, while experiments conducted to characterize the single isolates and the yeast-bacteria interactions were performed 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, N.C.).

Results

Characterizing the spoilage yeasts

The metabolic activity of the spoilage yeasts incubated in cucumber juice medium (CJM) under aerobic and anaerobic conditions is shown in Table 4.2. Under anaerobic conditions yeasts were inhibited and no significant changes in cell population, substrates use, 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 aerobic environments 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. As expected the samples inoculated with the *L. plantarum* culture had high levels of lactic acid (Table 4.2). In terms of growth in CJM both yeasts showed similar behavior. For simplicity figure 4.1 shows the changes in pH and cell population in CJM for the isolate *I. occidentalis*. The highest yeast population (about 8 log CFU/ml) was achieved after 5 days of incubation (Figure 4.1). After 20 days of experimentation samples inoculated with *I. occidentalis* reached a pH value above 5.8 while samples inoculated with *P. manshurica* reached a final pH close to 5.0.

Both yeasts showed a similar behavior when co-cultured with *L. plantarum* in cucumber juice media (CJM) (Table 4.3). For simplicity the results shown in table 4.3 represent the changes observed for the isolate *P. manshurica*. As expected the LAB rapidly utilized the sugar in the CJM and produced lactic acid with the concomitant decrease in medium pH regardless of the environmental conditions. However, under aerobic conditions and once sugar were depleted lactic and acetic acid concentrations declined with time in the presence of the yeasts. Organic acid utilization led to increases in medium pH and yeast populations above 7 log CFU/mL. Under anaerobic conditions, lactic acid production was associated with increases in LAB populations. The fermented medium remained stable once sugars were depleted such that after 1 year the pH was 3.2 and more than 80 mM lactic acid remained. In anaerobic experiments, yeasts were not detected after 30 d of experimentation.

Yeasts' metabolic activity in fermented cucumber juice medium (FCJM) purged with air resulted in organic acid utilization with the concomitant increase in brine pH (Figure 4.2,

Panel A and C). The spoilage yeasts were able to deplete more than half of the initial lactic acid concentration in about 3 to 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 (Figure 4.2, Panel A). During active lactic acid utilization yeast populations were close to 7 log CFU/mL. In a similar fashion, the yeast isolates were able to utilize acetic acid present in the fermented media (Figure 4.2, Panel B). Acetic acid utilization took place at a faster rate, and in 4 days about 98% of the initial acetic acid was depleted. Similarly to the experiments conducted in CJM, the metabolic activity of the yeast isolates was inhibited under anaerobic conditions, 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).

As organic acids were utilized in the FCJM, the environment inside the experimental jars became reduced, as shown by decreases in redox potential, E_h (Figure 4.3). During the first two days E_h decreased but remained positive while pH and the population of yeasts increased gradually. After 5 days the yeast population reached its maximum and the redox potential decreased to - 100 mV. Then declined to its lowest level of – 110 mV after 10 days, but then increased slightly as yeast populations began to decrease. The environment remained reduced at – 10 mV after 15 days of incubation (Figure 4.3).

Characterization of spoilage lactic acid bacteria

Both spoilage LAB showed similar behavior when incubated in cucumber juice medium (CJM) under both aerobic and anaerobic conditions. The sugars contained in the medium were rapidly utilized and the pH decreased (Table 4.2). *Pe. ethanolidurans* 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. bucheneri* was 3.1 ± 0.01 under both aerobic and anaerobic conditions. In the presence of oxygen, *Pe. ethanolidurans* produced 22 ± 0.7 mM of lactic acid more than *L. buchneri*. During anaerobic incubation both spoilage LAB produced less lactic acid than under aerobic conditions, and about 1o mM acetic acid was also produced by each organisms. Nor propionic or butyric acids were detected for any isolate under neither 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). *Pe. ethanolidurans* produced 14 mM of lactic acid, similar to *L. plantarum* control experiments. Only 6 mM 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 4.4). 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. *L. buchneri* population increased by 0.5 log CFU/mL during lactic acid utilization (data not shown). Similar changes were observed under anaerobic conditions. Neither *Pe. ethanolidurans* nor *L. plantarum* were able to utilize the lactic acid present in the fermented medium (Table 4.4).

Characterization of *Clostridium bifermentans*

Growth and metabolic activity of *C. bifermentans* was only observed when the isolate was grown in the medium with a starting pH above 4.5. In the fresh cucumber juice medium (CJM), with an initial pH of 4.2, the bacterium was not able to utilize the sugar and no changes in pH were observed (Table 4.2). Furthermore, no growth was detected and cells died off. *C. bifermentans* also died off in FCJM with an intial pH of 3.2 (Table 4.5). In medium adjusted to pH 4.5, the bacterium was able to utilize remaining sugars and produce lactic acid, but growth was inhibited by the decreasing brine pH (Table 4.5), so there was no lactic acid utilization or butyric acid production. Lactic acid utilization was observed by the bacterium in FCJM in which the initial pH was adjusted to 5.0. Once sugars were depleted, the bacterium started to utilize lactic acid and produced butyric acid with the concomitant increase in brine pH. About 50% of the lactic acid was consumed and 25.1 ± 0.4 mM of butyric acid were produced in 14 days. The final medium pH increased to 5.9 ± 0.1 (Table 4.5).

Characterization of *Enterobacter cloacae*

The enterobacter isolate was not able to survive in CJM (Table 4.2) 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 was able to utilize lactic acid and in turn produce propionic and acetic acids and increase the medium pH (Table 4.6).

Discussion

The spoilage of fermented cucumber pickles occurs when the 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. The microorganisms, in order to make these changes, 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*) were able to utilize lactic acid at the acidic conditions characteristic of the fermented product once primary fermentation was completed, and therefore the isolates are able to initiate the secondary fermentations that will lead to spoilage of the product. However, the metabolic activity of the yeasts is more rapid than of *L. buchneri* so that in aerobic conditions yeasts are probably the primary microorganisms responsible for raising the pH to 4.5 and above. Due to the potential for *Clostridium botulinum* spores to germinate and growth above pH 4.6, tanks must be destroyed. Additionally 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 *Enterobactereceae*. Increased brine pH (above 4.5) and reduced conditions are necessary for *C. bifermentans* and *E. cloacae*, studied here, to utilize lactic and acetic acids contained in fermented brines and produce butyric and propionic acids.

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 *Pichia manshurica* utilized lactic and acetic acids from fermented brines under aerobic conditions. This metabolic activity led to the chemical

reduction of the fermentation matrix. In previous studies we have reported that the aerobic spoilage of fermented cucumbers is the result of growth a series of microorganisms (Franco and Pérez-Díaz 2011). 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.

Similarl to the behavior reported under anoxic conditions in silage (Oude-Elferink and others 2001) and fermented brines (Johanningsmeier and others 2011), *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, *Pe. ethanolidurans*, which has been also observed and isolated from different spoilage samples (Franco and others 2011; Johanningsmeier and others 2011), was not able to utilize the lactic acid in fermented brines. Although, a wide variety of LAB have been isolated from spoilage samples (Franco and others 2011; Johanningsmeier and others 2011) only *L. buchneri* and probably *L. parafarraginis* have been shown capable of initiating lactic acid utilization in the low pH and high acid conditions that prevail once a normal primary fermentation is completed under both aerobic and anaerobic conditions.

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 fermented cucumbers before they can produce butyric and propionic acids (Franco and Pérez-Díaz 2011). Butyric acid production by *C. tertium* has been previously reported in fermented brines with initial pH of 5.0 incubated under anaerobic conditions (Fleming and

others 1989). The same result was observed for *C. bifermentans* studied here. Although, enterobacteria are inhibited by the environmental changes produced during the primary fermentation of the cucumber fruits (Etchells and others 1945), once pH increases sufficiently these organisms may grow (Franco and Pérez-Díaz 2011). The metabolic activity of these organisms has been reported previously to result in gas production and softening of fermented cucumber tissue (Etchells and others 1945; Bevilacquia and others 2009). In this study we found that *E. cloacae* is also able to produce propionic acid while utilizing lactic acid as carbon source when the brine pH is between 5.0 and 6.0.

Given the nature of cucumber fermentations and the variability of cucumber fruits used for the fermentation process it is reasonable to assume that other microbiota 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 and others (2011) reported that *actobaciullus 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 might contribute to the secondary fermentations. Given that both yeasts (*P. manshurica* and *I. occidentalis*) and *L. buchneri* are able to utilize 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 morphologies similar to *I.*

occidenlatilis, Pichia manshurica and *L. buchneri* in addtin to pH and acidity to prevent the onset of the spoilage process.

Conclusion

Specific, spoilage organisms isolated from commercial fermented cucumber spoiled samples were shown to take part in the secondary fermentations that lead to the spoilage of the fermented product. The studied yeasts and the bacterium *L. buchneri* were able to utilize lactic acid at low pH levels characteristic of fermented cucumber pickles. The changes produced by these organisms included increase in pH and reduction of the environment in case of yeasts. These changes were necessary for the onset a second stage in the secondary fermentation above pH 4.5 that is characterized by propionic and butyric acid production by bacteria such as . *cloacae* and *C. bifermentans*, respectively. Based on the results presented here we suggest the monitoring of morphologies similar to the yeast isolates and *L. buchneri* to prevent the initiation of spoilage at the commercial scale.

Table 4.1. Cultures used in this study

Microorganism	Culture collection	Source	Culture media	Incubation temperature	Incubation time
L. plantarum	LA 0219	Fermented cucumbers	MRS	30 °C	48 h
L. plantarum	LA 98	Fermented cucumbers	MRS	30 °C	48 h
L. plantarum	LA 445	Fermented cucumbers	MRS	30 °C	48 h
Pichia manshurica	Y88	Spoiled $CaCl_2$ fermented brine – Commercial	YMA	30 °C	48 h
Issatchenkia occidentalis	Y89	Spoiled $CaCl_2$ fermented brine – Commercial	YMA	30 °C	48 h
Lactobacillus buchneri	LA1149	Spoiled $CaCl_2$ fermented brine – Commercial	MRS	30 °C	48 h
Pediococcus ethanolidurans	LA1150	Spoiled $CaCl_2$ fermented brine – Commercial	MRS	30 °C	48 h
Clostridium bifermentans	B431	Spoiled NaCl fermented brine – Commercial	DRCA	30 °C - Anaerobic	48 h
Enterobacter cloacae	B432	Spoiled $CaCl_2$ fermented brine – Laoratory reproduced	VRBG	37 °C	24 h

Table 4.2 Continued

Table 4.2. Metabolism of selected spoilage isolates incubated in cucumber juice medium (CJM)

Sample	Δ pH ($pH_f - pH_i$)[1]		Lactic acid (mM)		Acetic acid (mM)	
	Aerobic	**Anaerobic**	**Aerobic**	**Anaerobic**	**Aerobic**	**Anaerobic**
Control[2]	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 *	67.6 ± 2.2 *	25.0 ± 0.7	22.2 ± 0.5
Pichia manshurica	1.5	N/C[3]	N/D §	N/D §	N/D §	5.7 ± 0.02 *§
Isstachenckia occidentalis	1.8	0.1	N/D §	N/D §	N/D §	1.0 ± 0.3 *§
Lactobacillus bunchenri	- 0.9	-0.9	59.7 ± 1.1 * §	74.0 ± 0.1 *	10.3 ± 0.4*§	9.1 ± 0.3 *§
Pediococcus ethanolidurans	- 0.7	- 1.3	92.6 ± 0.4 *	77.2 ± 0.3 *	25.2 ± 0.3	26.1 ± 0.1
Clostridium bifermentans	0.0	0.0	N/D	N/D	25.4 ±0.02	25.5 ± 0.0
Enterobacter cloace	0.0	0.0	N/D	N/D	25.4 ±0.02	25.5 ± 0.0

Table 4.2 Continued

Values represent the mean ± standard deviation of six replicates after 20 days under aerobic conditions (30 ± 2 °C) and 12 months under anaerobic conditions (25 ± 2°C)

[*] Indicates a significant difference compared to uninoculated sample ($\alpha = 0.05$)

[§] Indicates a significant difference compared to *L. plantarum* inoculated sample ($\alpha = 0.05$)

[1] pH_i: Initial pH, pH_f: final pH. (+) indicates an increase in media pH, and (-) indicates a decrease in media pH.

[2] 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 the termination of the experimentation.

[3] N/C: No significant change observed

[4]N/D: Non detected

Table 4.3. Changes in organic acids, pH, and cell population observed in cucumber juice medium (CJM) inoculated with *Pichia manshurica* and *Lactobacillus plantarum*

Aerobic and static incubation at 30 °C					
Time (days)	**Lactic acid (mM)**	**Acetic acid (mM)**	**pH**	**Log CFU/ml in YMA**	**Log CFU/ml in MRS**
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
Anaerobic and static incubation at 30 °C					
0	N/D	25.3 ± 0.01	4.13 ± 0.01	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

N/D Not detected

Values represent the mean ± standard deviation of four replicates

Table 4.4. 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	Δ pH[1]	Δ Lactic acid (mM) [1]	Δ Acetic acid (mM) [1]	Δ Ethanol (mM) [1]
Aerobic				
Control [2]	3.2 ± 0.0	84.9 ± 0.3	26.9 ± 2.4	N/D
L. buchneri	0.2 ± 0.0	-10.4 ± 0.1	5.1 ± 1.3	N/D
Pe. ethanolidurans	-0.2 ± 0.0	14.0 ± 0.23	0.3 ± 0.6	N/D
Anaerobic				
Control [2]	3.2 ± 0.0	85.1 ± 0.2	26.9 ± 1.6	2.3 ± 1.5
L. buchneri	0.4 ± 0.0	-36.4 ± 0.2	19.1 ± 0.1	8.5 ± 0.0
Pe. ethanolidurans	-0.1 ± 0.0	14.7 ± 1.2	1.1 ± 0.0	0.6 ± 0.0

[1] Changes represent the difference of the sample final value minus control sample value

[2] Control samples represent un-inoculated media. The values presented in the table correspond to measurements collected when the experiments were concluded. Changes in metabolites and pH were calculated taken these control values for comparison.

N/D: Non detected

Values represent mean ± standard deviation of six replicates (3 samples two independent experiments

Table 4.5. Lactic acid utilization and product formation by *Clostridium bifermentans* in fermented cucumber juice medium (FCJM) adjusted to different initial pH

Medium	pH	Characteristics after 14 days anaerobic incubation					
		Glucose	Fructose	Lactic Acid	Acetic acid	Butyric Acid	Ethanol
		(mM)					
Control[1]	3.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	100.2 ± 1.3	25.4 ± 0.1	N/D	N/D
pH 3.2	3.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	100.2 ± 1.3	25.4 ± 0.1	N/D	N/D
pH 4.0	4.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	100.2 ± 1.3	25.4 ± 0.1	N/D	N/D
pH 4.5	4.0 ± 0.1	N/D	N/D	102.8 ± 0.5	37.9 ± 0.3	N/D	1.5 ± 0.3
pH 5.0	5.9 ± 0.1	N/D	N/D	50.3 ± 0.6	10.5 ± 0.1	25.1 ± 0.4	1.2 ± 0.3

[1] Control: non-inoculated samples subjected to the same experimental conditions.

N/D: Not detected

Table 4.6. Lactic acid utilization and products formation of *Enterobacter cloacae* inoculated in fermented cucumber juice medium (FCJM) adjusted to different pH

FCJM Media	Characteristics after 20 days of aerobic incubation			
	Final pH*	Δ Lactic acid (mM)[1]	Δ Acetic acid (mM)[1]	Δ Propionic acid (mM)[1]
Control[2]	3.2 ± 0.2	84.8 ± 0.5	26.4 ± 0.2	N/D
pH 3.2	3.2 ± 0.2	84.8 ± 0.5	26.4 ± 0.2	N/D
pH 4.0	4.0 ± 0.2	84.8 ± 0.5	26.4 ± 0.2	N/D
pH 4.5	4.5 ± 0.1	84.8 ± 0.5	26.4 ± 0.2	N/D
pH 5.0	6.9 ± 0.1	-39.3 ± 1.2	+9.3 ± 0.7	+14.0 ± 0.5
pH 6.0	7.0 ± 0.1	-51.7 ± 0.7	+17.6 ± 1.2	+19.1 ± 0.4

* After 20 days of incubation under aerobic static conditions.

[1]Positive sign (+): production of the compound. Negative sign (-): Utilization of the compound. As compared to control samples

[2] Control: non-inoculated samples subjected to the same experimental conditions.

N/D: Not detected

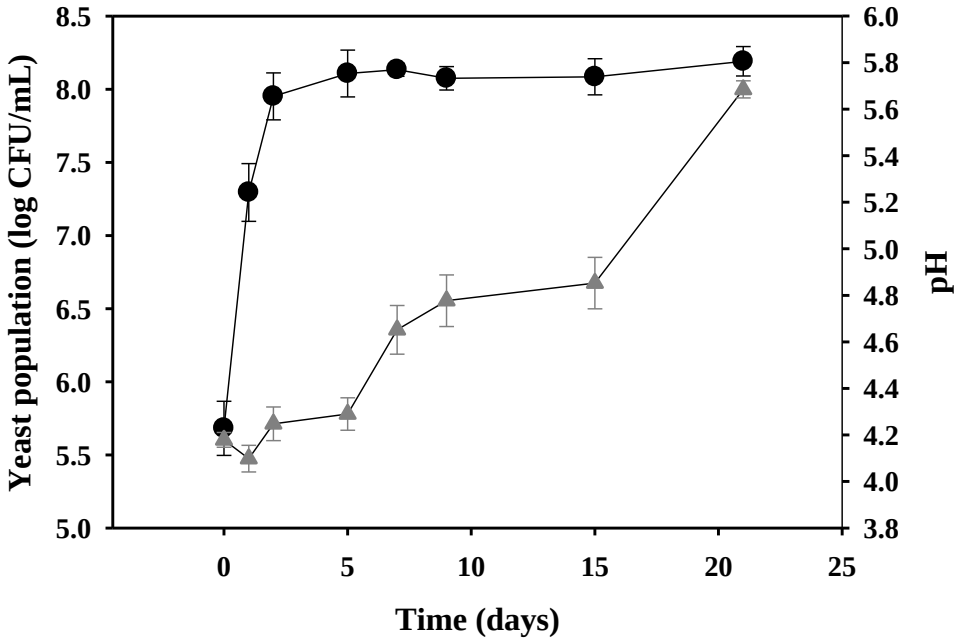


Figure 4.1. Changes in Cucumber Juice Medium pH and *I. occidentalis* population under aerobic conditions

pH (▲) and population (●) changes of the spoilage yeast isolate *I. occidentalis* grown in cucumber juice media (CJM) at 30 °C, under aerobic and static conditions. Values represent the mean and standard deviation of six replicates.

Figure 4.2. Changes in organic acids, pH, and populations during incubation of spoilage yeasts (*I. occidentalis* and *P. manshurica*) 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 experiments replicates

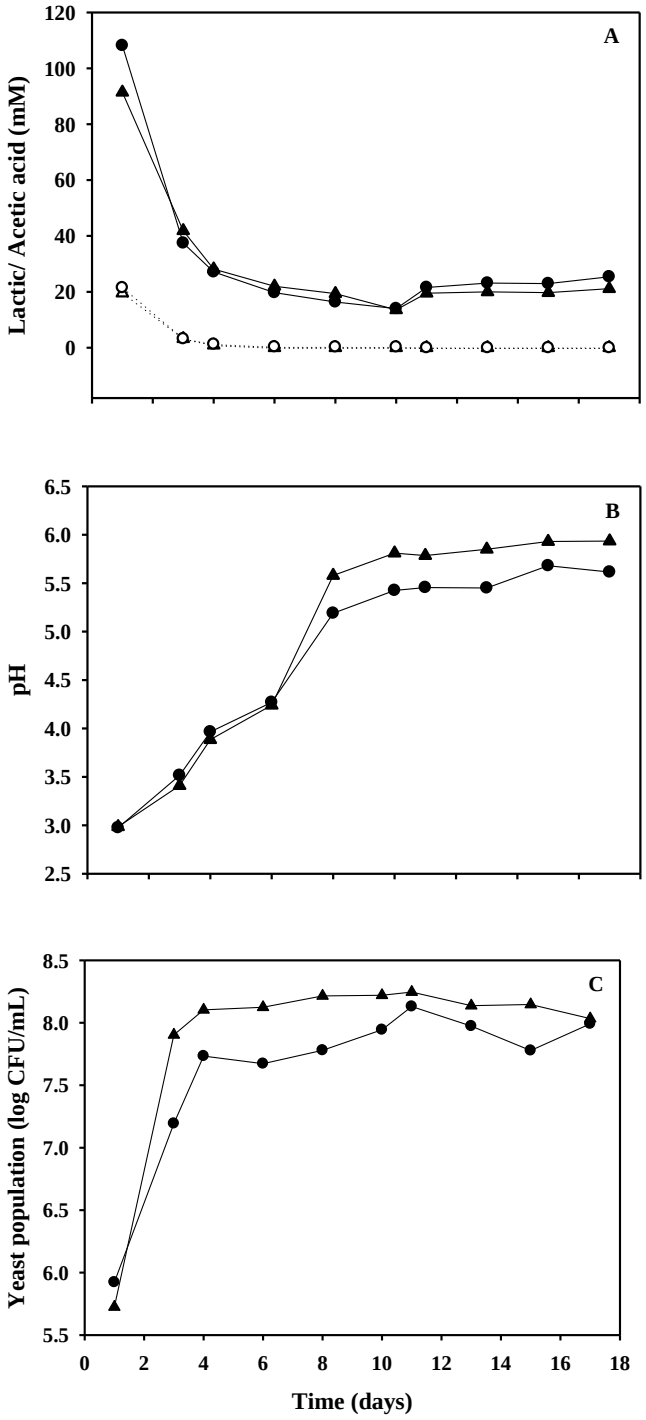
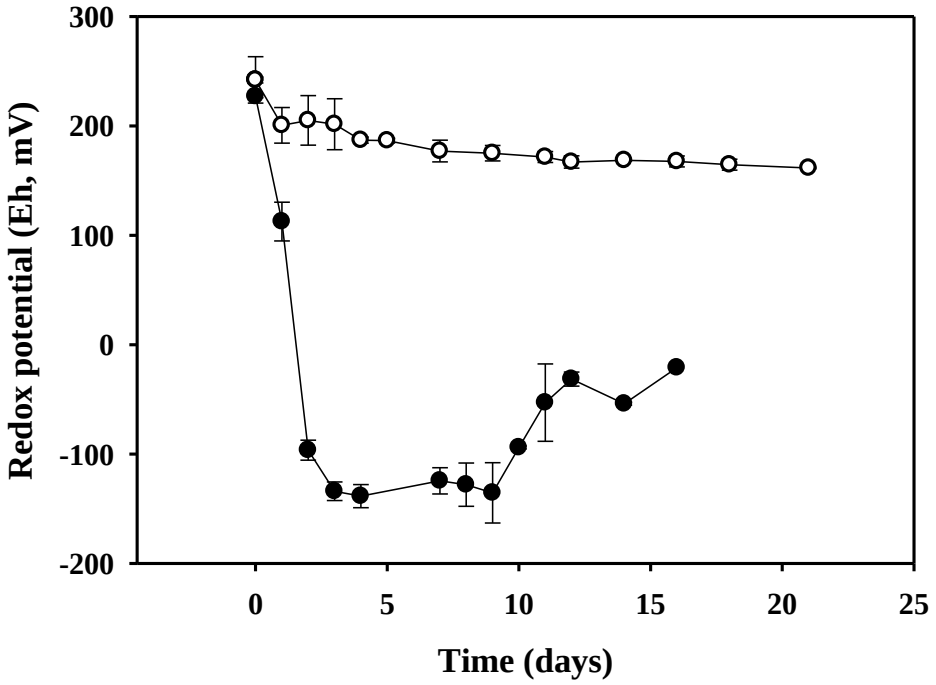

A
B
C
Lactic/ Acetic acid (mM)
pH
Yeast population (log CFU/mL)
Time (days)
120
100
80
60
40
20
0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
8.5
8.0
7.5
7.0
6.5
6.0
5.5
0
2
4
6
8
10
12
14
16
18

Figure 4.3. Redox potential changes during aerobic incubation of the spoilage yeasts in fermented cucumber juice medium

Effect in redox potential by spoilage yeasts *Issatchenkia 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 replicates.


300
200
100
0
-100
-200
Redox potential (Eh, mV)
0
5
10
15
20
25
Time (days)

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Chapter 5 Microbial Interactions Associated with Secondary Cucumber Fermentations

Abstract

A number of microorganisms have been associated with commercial and laboratory scale secondary cucumber fermentations. The interactions among selected yeasts and Gram (+) and Gram (-) bacteria were studied in a cucumber juice model system. A complete secondary cucumber fermentation proceeded when relevant microbes were inoculated in a cucumber fermentation vessel using a succession approach. 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. Such metabolic activity resulted in increases in brine pH and the chemical reduction of the fermentation matrix to levels at which other spoilage organisms were able to 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 after the pH had increased to 4.6. Yeast and bacteria interactions favored the survival of *C. bifermentans*, but did not enhanced butyric acid production at the acidic medium pH (3.2) characteristic of fermented cucumbers. While growth of *E. cloacae* was inhibited by the medium acidity, the bacterium was able to continue its metabolic activity and convert lactic acid into propionic acid when co-cultured with other microorganisms, including yeasts. The successive microbial inoculation approach used in this study confirmed that a complex microbiota is responsible for the changes observed during fermented cucumber secondary fermentation.

Introduction

Observations from the analysis of commercial fermented cucumber spoilage showed that during bulk storage the fermented product underwent a secondary fermentation in which lactic acid was converted into acetic and propionic acids, and. in extreme cases butyric acid (Franco and others 2011). The degradation of lactic acid led to increases in brine pH which made the product unstable and suitable for undesirable organisms able to produce aroma or off odors compounds associated with spoilage of the fermented product. Yeasts, such as *Pichia manshurica* and *Issatchenckia 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 and others 2011; Johanningsmeier and others 2011). Microorganisms presenting colony morphologies similar to those representative of the microbes listed above were observed in the model system developed by Franco and Pérez-Díaz (2011a) for the study of secondary fermentation in fermented cucumbers. The observations from that study led to the hypothesis that a succession of microorganisms grow and cause chemical changes that are typical in secondary cucumber fermentations (Fig 5.1).

Later the ability of selected yeast and bacteria (pure cultures) to utilize lactic and acetic acids present in fermented cucumber medium was evaluated (Franco and Pérez-Díaz 2011b). The spoilage yeasts *P. manshurica* and *I. occidentalis* were able to utilize lactic and acetic acids in the presence of oxygen. Organic acids utilization led to the

chemical reduction of the environment and an increase of the medium pH. It seems that these changes could favor the growth of spoilage undesirable bacteria, such as *L. buchneri*, *C. bifermentans*, and *E. cloacae* which later were found responsible of converting lactic acid into acetic, butyric and propionic acids, respectively (Franco and Pérez-Díaz, 2011b). Acetic acid production, observed at low pH, 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 commercial fermentation of cucumbers is not a sterile process and it is likely to have a complex microbiota, therefore the objectives of this study were to evaluate interactions of selected yeasts and bacteria and their effect on cell growth, organic acid utilization, and spoilage product formation. In addition, the succession of microorganisms proposed in previous research (Franco and Pérez-Díaz 2011a) 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 – juice maker (Juiceman, Jr., Juiceman, Bedford Heights, OH, U.S.A.). Cucumber juice was

centrifuged for one hour at 10,000 x g (Sorbal RC58, DuPont Instuments, Red Oak, IA, U.S.A.). The supernatant was then collected and stored at -20 °C before use. Fresh medium, labeled as cucumber juice medium (CJM) was prepared by mixing (50:50 – w/w) cucumber juice with cover brine solution, such that after equilibration the mixture contained 25 mM acetic acid added as vinegar (20%) and 100 mM $CaCl_2$. The mixture was filter sterilized using 0.2 µ PES filter membranes (Nalgene, Nalge Nunv International, Rochester, NY, U.S.A.). Filter sterilized CJM was stored at 4 °C before use. Fermented cucumber juice medium (FCJM) was obtained by 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, U.S.A, Culture Collection). The mixture was incubated at 30 °C for a time that allowed the conversion of sugars into lactic acid. As denoted by HPLC analysis and decreased medium pH. The fermented medium was also filter-sterilized (Nalegene). Ten ml aliquots were aseptically transferred to 50-ml and 15-ml conical tubes (Corning Inc., NY, U.S.A.), 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, North Carolina, U.S.A (Table 5.1). Isolates from frozen stock cultures were streaked in the appropriate media as described in Table 5.1. Organisms from broth-cultures were then harvested at 10,000 x 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. 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., U.S.A.).

Evaluation of yeast and bacteria growth responses in mixed cultures

Bacteria and yeast interactions were evaluated by inoculating FCJM with a mixture of *P. manshurica* Y88 and *I. occidentalis* Y89 (USDA-ARS Culture collection) with 5 log CFU/mL. Pure bacterial cultures were individually inoculated in the FCJM to 5 log CFU/mL. Triplicate experimental tubes were incubated at 30 °C under static and aerobic conditions for *L. buchneri* and *E. cloacae* (facultative anaerobes) and under static and anaerobic conditions for *C. bifermentans* (Coy Laboratory Products, Inc., Grass Lake, MI, U.S.A.).

Evaluation of secondary fermentation by mixed cultures in cucumber juice medium (CJM)

In order to test the ability of the isolates to reproduce the chemical changes characteristic of secondary cucumber fermentation, as determined from commercial (Franco and others 2011) and experimental samples (Franco and Pérez-Díaz 2011a), the isolates were inoculated into fresh cucumber juice medium (CJM) following a successive inoculation approach. Two liters (2 L) of filter sterilized CJM were put into

2 L glass water jacketed fermentation vessels of a BioFlo110 Modular Benchtop Fermentor (New Brunswick Scientific Co., Inc., Edison, NJ, U.S.A.). The vessels were air-purged at a rate of 5 mL/min controlled by a Matheson PG-1000 (U001) flowmeter (Matheson Instruments, Montgomeryville, PA). 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, U.S.A.). Parameters such as redox potential (Pt4805-DPAS-SC-K8S/200 redox electrode; Mettler-Toledo, Bedford, MA), dissolved oxygen (Inpro 6830/220 electrode; Mettler-Toledo) and pH (pH electrode model 405-DPAS-SC-K8S/225; Mettler-Toledo) were continuously monitored. 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 instructions. Electrodes for all of these measurements were calibrated, rinsed with 70% ethanol and secured into fittings on the head plate of each fermentor vessel.

First, three *L. plantarum* strains LA 445, LA 98, and LA 85 (USDA-ARS Culture collection) were inoculated along with *P. manshurica* Y88 and *I. occidentalis* Y89 to 5 log CFU/mL, each. The inoculated medium was incubated for one week to allow for the completion of the primary fermentation. *L. buchneri* LA1149 (5 log CFU/mL) was inoculated 10 days into the fermentation while *C. bifermentans* B431 and *E. cloacae* B432 inocula were added (5 log CFU/mL, each) on day 14. The time course selected for the inoculation of the respective spoilage isolates corresponded to the

microbiological changes (colonies in agar plates) observed in the model system proposed by Franco and Pérez-Díaz (a2011).

Impact of microbial metabolism on secondary cucumber fermentation

Size 3B (39 to 51 mm diameter) cucumbers from a local processor were washed with tap water to remove debris. Fruits with visual damage (cuts and bruises) were discarded. Fruits and cover brine (50:50 – w/w) were packed into four - 50 L plastic containers such that after equilibration the mixture contained 100 mM anhydrous $CaCl_2$ and 25 mM acetic acid added as vinegar (20%). A mixture of three *L. planatarum* strains, LA 445, LA 98, and LA 385 (USDA-ARS Culture collection), was inoculated (5 log CFU/mL) 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 growth of film forming yeasts. Containers were exposed to the UV light from sunlight 5 hours a day. The primary fermentation was stopped once the cover brine pH approached 3.2 ± 0.3 and sugars were significantly depleted as determined by HPLC analysis (< 1 mM).

The fermented brine was centrifuged at 10,000 x g for 30 min (Sorvall RC-5B, DuPont Instruments,Wilmington, DE, U.S.A.). The supernatant was then filter-sterilized using a 0.22 µm bottle top filter apparatus (Nalegene). Three liters (3L) 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 (Coy Laboratory Products, Inc., Grass Lake, MI, U.S.A.). Gas flows for the purged jars were kept constant at 5 mL/min by a Matheson PG-1000 (U001) flow meter (Matheson Instruments, Montgomeryville, PA). The jars of fermented cucumber juice were inoculated with a mixed culture composed by the spoilage yeasts, *P. manshurica* Y88 and *I. occidentalis* Y89 (USDA-ARS Culture collection) to 5 log CFU/mL and incubated at 30 ± 3 °C.

The secondary fermentation was stopped once the pH of the cover brine solution was 4.5 to 5.0. The cover brine solution was spun at 10,000 x g for 15 min and filter sterilized (Nalgene). Two hundred ml (200 ml) aliquots of the filter sterilized brine were then added into 8 oz sterile glass jars. Each jar was equipped with a gas inlet and outlet and a rubber septum for sample collection. Experimental jars were inoculated with *L. buchneri* LA1149,*C. bifermentans* B431, *E. cloacae* B432, or a mixture of the three bacteria. Cultures were added to the experimental jars at cell populations of about 5 log CFU/mL. Each experimental treatment was incubated under aerobic and anaerobic conditions (Coy, Laboratoty Products Inc.) in triplicate.

Chemical and microbiological analysis

Samples were aseptically collected during the incubation periods. The concentrations of organic acids and sugars were measured by High Performance Liquid Chromatography (HPLC) analysis using a 30-cm HPX-87H column (Bio-Rad

Laboratories, Hercules, CA) for the separation of components (McFeeters and Barish 2003). The column temperature was held at 37 °C and samples components were eluted with 0.03N sulfuric acid at a flow rate of 0.6 mL/min. A Thermo Separations UV6000 diode array detector (Spectra System Thermo Scientific, Waltham, MA) set to collect data at 210 nm was used to quantify malic, lactic, acetic, propionic, and butyric acids. A Waters model 410 refractive index detector (Waters Corp., Millipore Corp., Billerica, MA) connected in series with the diode array detector was used to measure glucose, fructose, and ethanol. External standardization of the detectors was done using four concentrations of the standard compounds.

Aseptically collected samples were serially diluted in 0.85% saline solution and spiral plated using an Autoplate 400 (Spiral Biotech, Norwood, M.A., U.S.A.) on Lactobacilli deMan Rogosa and Sharpe agar (MRS, Becton Dickinson and Co., Franklin Lakes, N.J., U.S.A.) supplemented with 1% cycloheximide (0.1% solution, OXOID, New England); yeast and molds agar (YMA, Becton, Dickinson and Co.) supplemented with 0.01% chloramphenicol (Sigma-Aldrich, St. Louis, M.O., U.S.A.) and 0.01% chlortetracycline (Sigma-Aldrich) ; violet red bile agar supplemented with 1% glucose (VRBG, Becton, Dickinson and Co.); and differential reinforced clostridia agar (DRCA, Becton) supplemented with 1% cycloheximide (OXOID, New England) for the enumeration of presumptive lactic acid bacteria, 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

at 37 °C on VRBG plates were recorded as presumptive enterobacteria. Black colonies after 48 h of anaerobic incubation at 30 °C on DRCA plates were recorded as *Clostridia* spp.

Results and Discussion

Interaction of Bacteria and Yeasts in fermented cucumber juice medium (FCJM)

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 (Figure 5.2, Panel A). A total of 41.7 ± 1.5 mM lactic acid was consumed, and 27.4 ± 1.9 mM acetic acid produced, corresponding to a final medium pH of 4.5. *L. buchneri* population increased more than one log in the first week of incubation (Figure 5.2, Panel B). Increase in the bacteria population could be attributed to the utilization of the remaining sugars in the medium and to the ability of the cells to achieve higher cell densities when co-cultured with the yeasts. Higher cell populations were reported for LAB co-cultured with *Saccharomyces cerevisiae* in sourdough and experimental medium in comparison to pure cultures (Sieuwerts and others 2011). Stimulation of growth was associated with a factor secreted by the yeast. In pure culture *L. buchneri* has been reported as able to convert lactic acid contained in FCJM into acetic acid; however, the onset of the organic acid utilization takes longer (about 10 days) and the bacteria decreased significantly in population after 20 days of incubation (Franco and Pérez-Díaz 2011b) suggesting that the co-culture with yeasts improves the ability of the bacterium to cope with the environmental stress by providing nutrient factors that allow

longer survival. Similarly, in *S. cerevisiae* and *L. bulgaricus* mixed cultures, the presence of the yeast was associated with the down regulation of genes encoding for amino acid and polysaccharide biosynthesis and up-regulation of long chain fatty acid biosynthesis in the bacterium (Sieuwerts and others 2011). There was little effect of *L. buchneri* on the spoilage yeasts except for slight decreases in cell population at the beginning of the experimentation (Figure 5.2, Panel B). However, after 15 days of incubation the yeast populations increased, as expected, as the lactic acid was utilized.

During the lactic acid utilization stage, *L. buchneri* remained at about 6.5 log CFU/mL, suggesting that this phenotype might be related to a stress survival strategy. *L. buchneri* LMG6892 has been reported as able to utilize lactic acid contained in experimental medium and silage and to produce acetic acid and 1,2 propanediol under anaerobic conditions (Oude-Elferink and others 2001). The conversion of lactic acid into another organic acid of lower pKa and an alcohol appears to help the bacterium to cope with environmental stress, but does not support growth. Similar behavior was observed for the *L. buchneri* strain studied here. Even when the bacterium was able to utilize lactic acid no significant changes in cell population were observed (Figure 5.2, Panel B). Different stress response systems that allow LAB to withstand harsh conditions have been reported (Van de Guche and others 2002) 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. The bacterium utilized sugars present in the fermented medium and increased cell

population at the initial pH of 3.8 in the co-culture with the yeasts and at lower pH in pure culture (Franco and Pérez-Díaz 2011b; Johanningsmeier and McFeeters 2011), but once the carbohydrates were depleted growth ceased and lactic acid utilization is initiated. Glucose starvation in LAB induces an increased resistance to many stress conditions, including oxidative and osmotic stress (Van de Guche and others 2002). Therefore, it might contribute to longer survival of the bacterium in commercial fermented cucumbers. Conversely, the changes observed in the *L. buchneri* and yeasts in mixed culture were similar to those found in commercial spoilage samples (Franco and others 2011) in which decreased lactic acid was associated with increases in brine pH and acetic acid concentration. In this study, about 45% of the initial lactic acid disappeared, presumably due to the metabolic activity of both the bacteria and yeasts. In commercial spoilage samples, mixtures of yeasts and bacteria, including morphologies similar to *L. buchneri,* were observed (Franco and other, 2011). Our findings suggest that this mixture will aid in the longer survival of the bacterium and increase the chances to induce the secondary fermentation.

While the co-culture of *E. cloacae* and the spoilage yeasts did not have any positive effect on the bacterial growth response (Figure 5.3, Panel A), the mixed culture of *C. bifermentans* and the spoilage yeasts enhanced the survival of the bacterium at the lower initial pH of the FCJM (3.8) (Figure 5.3, Panel B). In pure cultures, the low pH of FCJM resulted in die off of *C. bifermentans* so that after 2 days of incubation the bacterium was not detected in the medium (Franco and Pérez-Díaz 2011b). Butyric acid

production by *C. bifermentans* was not detected in the mixed culture. Production of butyric acid by C*lostridium* spp. and other anaerobic organisms has been reported as dependent on the presence of other metabolites, such as acetic acid (Bhat and Barker 1947; Bourriard and others 2005). In our experiments yeasts rapidly utilized acetic acid present in the fermented medium. This might prevent the formation of butyric acid by the *Clostridia* isolate. Regardless of butyric acid production, *C. bifermentans* populations increased one log and remained at that level during 30 days of incubation in the fermented medium while the spoilage yeast counts decreased with time presumably due to the lack of oxygen required by *C. bifermentans* to grow (Figure 5.2, Panel C).

Effect of mixed cultures on the development of secondary fermentation in cucumber juice medium (CJM)

Interactions between the microorganisms potentially involved in the spoilage were successively inoculated as pure cultures as described in Table 5.2. Lactic acid produced during the primary fermentation by *L. plantarum* was consumed by the yeasts, which increased to more than 6 log CFU/mL within the first week. This in turn increased the medium pH and lowered the redox potential of the medium (Table 5.2). Propionic acid production was first detected at day 14 at pH 3.7, suggesting that either *L. buchneri, L. plantarum* or the spoilage yeasts are able to produce the acid. 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 5.2). The production of butyric acid in this proposed model seems to be independent of lactic acid utilization.

As is shown in Table 5.2, butyric acid is detected once lactic acid is depleted. Different carbon sources can be used by *Clostridium* spp. for the production of butyric acid including amino acids (Yang and others 2009). These compounds have been detected in fermented cucumber commercial spoilage samples (Johanningsmeier and others 2011) and might be the source of the limited amounts of butyric acid detected in the experimental medium.

In contrast to what was previously reported by Franco and Pérez-Díaz (a2011), propionic acid was produced before butyric acid (Table 5.2). It seems that production of propionic acid is not dependent on the chemical reduction of the fermentation matrix 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 and others 2011). About 50% of the studied commercial samples had detectable levels of propionic acid, while only 2 out of 42 samples contained butyric acid. One sample had a pH of 4.2 and the other 4.7. Moreover, negative E_h values were only observed in samples in which butyric acid was detected, while the other spoiling samples had positive E_h values. The pathway proposed for the production of propionic acid in fermented cucumber medium suggests the complementary activity of two spoilage LAB under anaerobic conditions (Johanningsmeier and others 2011). *L. buchneri* converts lactic acid into 1,2 propanediol and acetic acid, and later the alcohol is used as a substrate for the production of propionic acid by *L. rapi*. Similar synergistic activity was observed in silage, in where *L. dioliverans* was found able to convert 1,2

propanediol produced by *L. buchneri* into the short fatty acid (Krooneman and others 2002). Additionallly, other opportunistic bacteria, such as members of the *Enterobactereacea* family, might able to convert lactic acid into propionic acid when the proper conditions are available (Franco and Pérez-Díaz, b2011).

Based on the characterization of commercial samples and laboratory experiments the chemical reduction of the fermentation matrix is strongly related to high yeast populations (above 5 log CFU/mL). The presence of high yeast populations at the commercial scale could be associated with abnormal primary fermentations and low sugar content in the cucumber fruits which in turn did not allow for a proper acidification of the fermentation matrix or to an abnormally high load of yeast population on the fresh fruit prior to packing.

Mixed culture secondary fermentation with spoilage yeasts increased the survival of bacteria with low pH tolerance (Table 5.3). Although, *E. cloacae* populations decreased 0.6 log CFU/mL after inoculation, probably due to the acid stress, the bacterium population increased to 5.4 log CFU/mL, while *C. bifermentans* populations increased after inoculation by about 0.6 log CFU/ml.

Impact of the microbial metabolism in the secondary cucumber fermentation

Although, a secondary fermentation was observed when the selected isolates were successively inoculated in CJM, the changes observed could not be attributed to a specific microorganism or to the overall community effect. Therefore, in this section a similar successive inoculation approach was followed by removal of one microorganism

before inoculation of the next one. Following the approach described in Fig. 5.1 and once the expected change was observed the experimental medium was spun to remove the microorganisms responsible for the change and then filter-sterilized. The cell free medium was inoculated with the corresponding microorganism for further experimentation.

As expected a primary fermentation was observed when the cucumber fruits were inoculated with the *L. plantarum* culture (Table 5.4). 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 to 90.3 ± 4.3 mM of lactic acid. The population of natural yeasts increased 1 log during the progression of the primary fermentation. No yeast films were observed in any of the experimental containers. As the environment became more acidic, enterobacteria populations, naturally present at the beginning of the experimentation, disappeared (Table 5.4).

The oxidative metabolism of the spoilage yeasts was observed only when the experimental jars containing filter sterilized medium were air-purged (Table 5.5). About 30 mM of lactic acid were utilized during aerobic yeast metabolism, which in turn increased the medium pH to 4.5. Acetic acid, originally at about 25 mM decreased to a final concentration of 17.6 ±0.1 mM by day 14th (Table 5.5). No significant changes in pH and organic acids were observed in nitrogen-purged jars or in those kept under anaerobic conditions, confirming that the oxidative metabolism is only present when

oxygen is available (Franco and Pérez-Díaz 2011a; Franco and Pérez-Díaz 2011b). 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 2011b). Suggesting that the aerobic conditions provided by the air-purging practices at the commercial scale might play a risk factor for the initiation of secondary fermentations

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 5.6). 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 2011b; Johanningsmeier and others 2011). *C. bifermentans* was able to utilize lactic acid when incubated under anaerobic conditions (Franco and Pérez-Díaz 2011b). As lactic acid was degraded by the bacterium the medium pH increased and butyric acid was formed (Table 5.6). Interestingly, under air-purged conditions, small amounts of lactic acid were degraded concomitant with butyric and acetic acids production. In commercial spoilage samples, butyric acid, usually associated with the presence of *Clostridia* spp., was detected in fermentation tanks with reduced environments (negative E_h values) but also small amounts were detected in oxidized environments

(Franco and others 2011). It has been reported that certain *Clostridium* spp. are able to metabolize oxygen present in growth medium using an NADH/NADPH dependent mechanism (Kawasaki and others 1998). The mechanism along with a set of oxygen scavenger enzymes may allow the bacterium to eliminate oxygen in the medium and continue with cell growth. The survival strategy reported for *C. acetobutylicum* resulted in the formation of acetic acid (O'Brien and Morris 1971). It is possible that some *C. bifermentans* cells were able to utilize the oxygen as an electron acceptor to derive energy or to scavenge some oxygen dissolved in the medium, but since oxygen supply was kept constant, the bacterium was not able to keep up with the oxygen up-take and eventually died off.

Regardless of redox potential, butyric acid was detected, at the commercial scale, only when the pH of cover brine solutions was above 4.2. The regulation for acidified and acid food products establish that the limiting pH to declare the food product safe should be less than 4.6 (Code Food Regulations). At this acidic pH, the germination of *C. botulinum* spores is controlled. Although, it was not expected to It was not expected to

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). *E. cloacae* strains isolated from

spontaneous fermentations of Italian table olives were reported to grow in nutrient medium supplemented with citric, ascorbic and lactic acid (Bevilacquia and others 2009). Growth and metabolic activity was not inhibited when the medium was supplemented with lactic acid at a final pH of 4.5, but lower growth rates were reported as compared to growth in citric or ascorbic acid supplemented medium (Bevilacquia and others 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 and others 1945). However, *E. cloacae* populations have been isolated from air-purged fermentations once the medium pH is above 4.5 (Franco and Pérez-Díaz 2011a) 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 non culturable state (VBNC) is triggered 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 (Franco and Pérez-Díaz, not published).

Lactic acid utilization and increase in medium pH were similar under air-purged and anaerobic conditions for the mixed culture experiments (Table 5.6). 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 CFU/mL under air-purged and anaerobic conditions, respectively. *C. bifermentans* on the other hand decreased about 2 logs when incubated under air-purged conditions, but did not disappeared after 30 days of incubation. Under anaerobic conditions the bacterium increased 0.8 log CFU/mL. Significantly higher *C. 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 under both conditions, suggesting that in mixed culture the bacterium might be using a survival (no growth) mechanism. The changes observed in the mixed culture experiments were similar to the chemical characteristics reported for commercial spoilage samples (Franco and others 2011). In commercial samples, lactic acid concentrations were decreased, but rarely depleted while acetic acid concentrations were increased. In samples at which butyric and propionic acids were detected, the brine pH was above 4.0.

Conclusions

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 a successive inoculation approach of selected isolates, the matrix in which cucumbers are fermented and stored at the commercial scale is more complex suggesting that other microorganisms might also be involved in the spoilage. For instance, propionic acid production in fermented cucumber medium was reported as the catabolic activity of *L. rapi* (Johanningsmeier and others 2011; Johanningsmeier 2011). It is clear that synergistic activities are necessary to trigger the post-packing 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. Therefore, strategies to prevent the development of these yeasts should be encouraged in the fermented cucumber industry. Especially now that commercial pickle processors are evaluating the introduction of calcium chloride ($CaCl_2$) fermentations in place of sodium chloride (NaCl) fermentations. The use of $CaCl_2$ fermentation decreases by about 80% the chloride content in waste waters used for the fermentation of cucumber fruits, allowing the industry increase production of fermented cucumber products while at the same time complying with the Environmental Protection Agency regulations regarding chlorides disposal (Environmental Protection Agency 1987). However, $CaCl_2$ fermentations are more likely to spoil when air-purging routines are followed due to growth ox oxidative yeasts and spoilage LAB.

Figure 5.1. Proposed microbial succession and environmental changes produced during primary fermentation and storage of the fermented cucumbers in $CaCl_2$ brines

Primary fermentation carried out by *L. plantarum* converts sugars into lactic acid with concomitant increase in cover brine solutions pH. Oxidative yeasts, such as *P. manshurica* and *I. 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 reduction of the environment allow other bacteria, such as *L. buchneri, Cl. bifermentans,* and *E. 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, a2011).

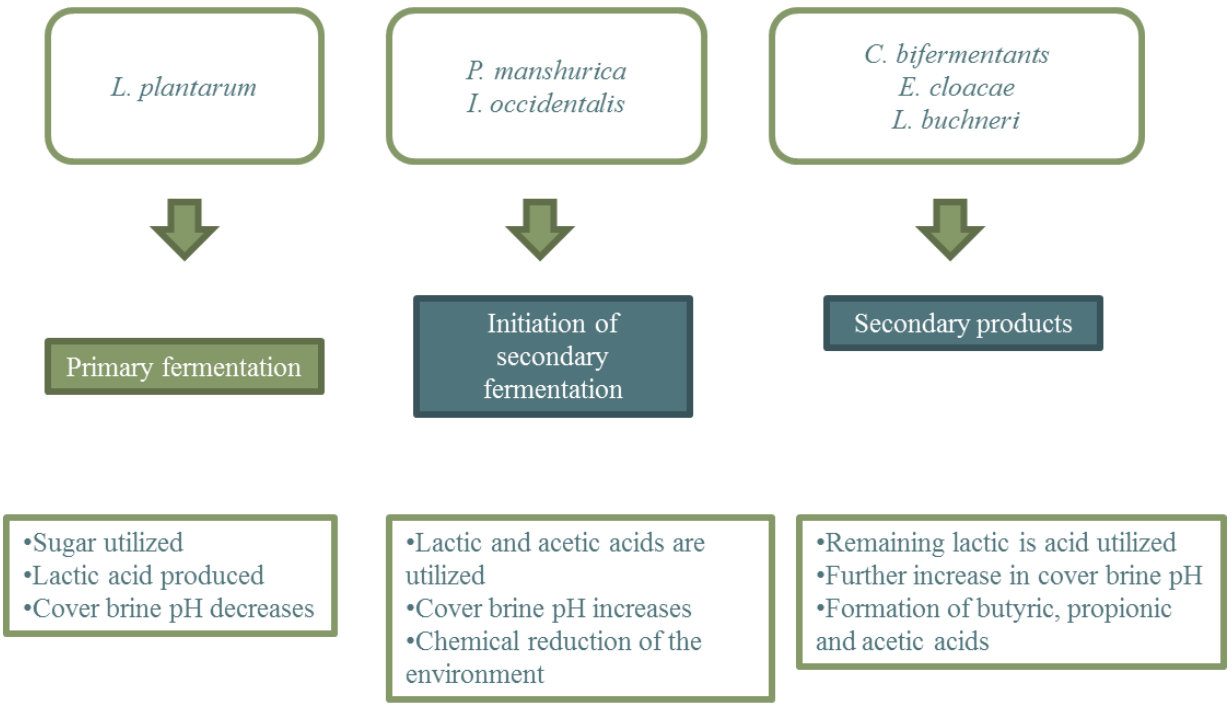
L. plantarum
P. manshurica
I. occidentalis
C. bifermentants
E. cloacae
L. buchneri
Primary fermentation
Initiation of secondary fermentation
Secondary products
•Sugar utilized
•Lactic acid produced
•Cover brine pH decreases
•Lactic and acetic acids are utilized
•Cover brine pH increases
•Chemical reduction of the environment
•Remaining lactic is acid utilized
•Further increase in cover brine pH
•Formation of butyric, propionic and acetic acids

Figure 5.2. Characteristics of *L. bucnheri* and spoilage yeasts in mixed culture in fermented cucumber juice medium (FCJM) with an initial pH of 3.8.

Panel A: Changes in lactic (●) and acetic (○) acids. Panel B: Changes in *L. buchneri* (▲) and spoilage yeasts (Δ)

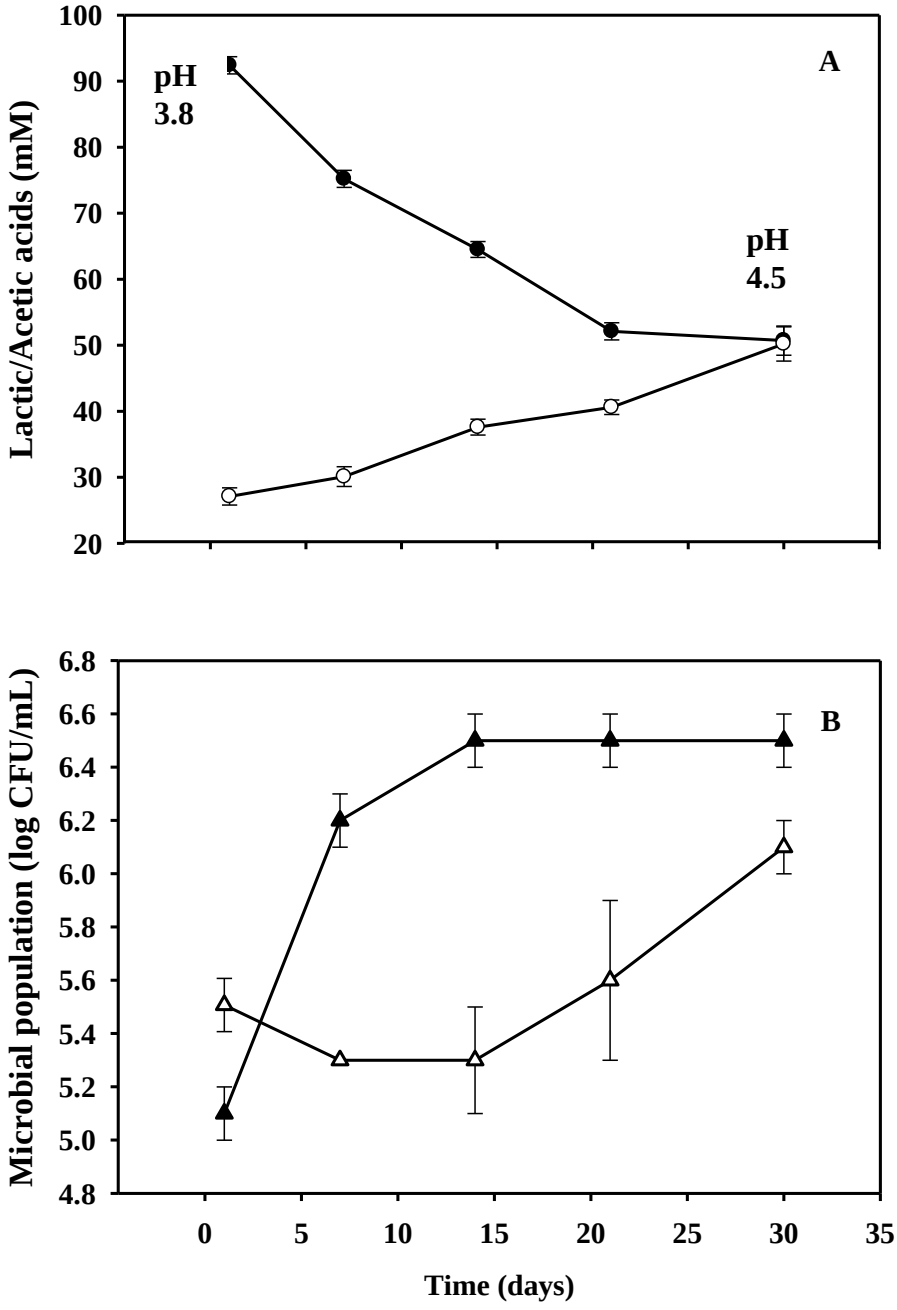
A
pH
3.8
pH
4.5
Lactic/Acetic acids (mM)
100
90
80
70
60
50
40
30
20
B
Microbial population (log CFU/mL)
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
0
5
10
15
20
25
30
35
Time (days)

Figure 5.3. Characteristics of mixed cultures in fermented cucumber juice medium with an initial pH of 3.8.

Panel A: Changes in *E. cloacae* (▲) and yeast populations (△) lactic (●) and acetic (○) acids. Panel B: Changes in *C. bifermentans* (▼) and yeast (▽ populations, lactic (□) and acetic (■) acids.

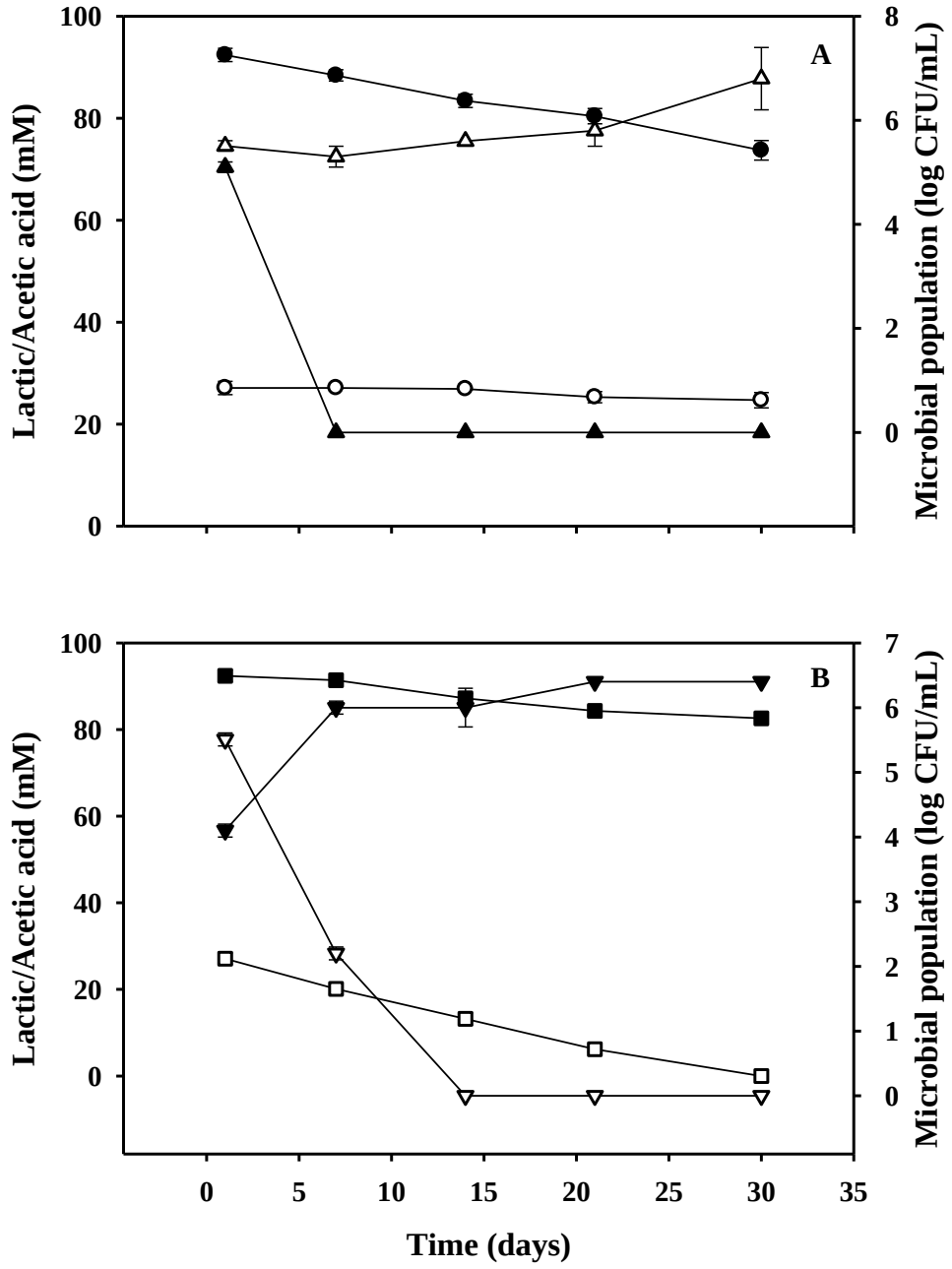
A
B
Lactic/Acetic acid (mM)
Microbial population (log CFU/mL)
Time (days)
0
20
40
60
80
100
0
2
4
6
8
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35

Table 5.1. Isolates used in this study

Microorganism	Culture collection	Source	Culture media	Incubation temperature	Incubation time
Lactobacillus plantarum	LA 0219	Fermented cucumbers	MRS	30 °C	48 h
Lactobacillus plantarum	LA 98	Fermented cucumbers	MRS	30 °C	48 h
Lactobacillus plantarum	LA 445	Fermented cucumbers	MRS	30 °C	48 h
Pichia Manshurica	Y88	Spoiled $CaCl_2$ fermented brine – Commercial	YMA	30 °C	48 h
Issatchenkia occidentalis	Y89	Spoiled $CaCl_2$ fermented brine – Commercial	YMA	30 °C	48 h
Lactobacillus buchneri	LA1149	Spoiled $CaCl_2$ fermented brine – Commercial	MRS	30 °C	48 h
Pediococcus ethanolidurans	LA1150	Spoiled $CaCl_2$ fermented brine – Commercial	MRS	30 °C	48 h
Clostridium bifermentans	B431	Spoiled NaCl fermented brine – Commercial	DRCA	30 °C - Anaerobic	48 h
Enterobacter cloacae	B432	Spoiled $CaCl_2$ fermented brine – Lab. reproduced	VRBG	37 °C	24 h

Table 5.2. 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 Figure 5.1

Time (days)	Organisms present at the sampling point	pH	Lactic acid	Acetic acid	Propionic acid	Butyric acid	dO_2 (mg/L)	Redox potential (E_h, mV)
			(mM)					
0	LP, PM, IO	4.2 ± 0.04	N/D	23.6 ± 0.3	N/D	N/D	N/D	322.0 ± 3.1
7	LP. PM, IO	3.3 ± 0.0	72.6 ± 1.7	14.0 ± 0.2	N/D	N/D	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	N/D	0.5 ± 0.1	4.1 ± 0.2	3.3 ± 0.03	N/D	-110.5 ± 14.8
40	LP, PM, IO, LB, CB, EC	6.0 ± 0.01	N/D	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	N/D	0.5 ± 0.1	4.0 ± 0.3	3.8 ± 0.02	0.6 ± 0.01	-41.0 ± 2.8
Anaerobic Control[1]	LP, PM, IO, LB, CB, EC	3.3 ± 0.1	81.5 ± 0.2	28.7 ± 0.1	N/D	N/D	N/A	-382.5 ± 1.5

Table 5.2 Continued

LP: *L. plantarum;* PM: *P. manshurica*, IO: *I. occidentalis*; LB: *L. buchneri*; CB: *C. bifermentans*; EC: *E. cloacae*

[1]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.

NA: Not available

ND: Not detected

Table 5.3. 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[1]	*L. plantarum*[1]	Yeasts[1]	*L. buchneri*[2]	*E. clocae*[3]	*C. bifermentans*[3]
		Log CFU/ml				
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[2]	LP, PM, IO, LB, CB, EC	1.2 ± 0.1	N/D	1.5 ± 0.1	N/D	N/D

[1] Organisms were successively inoculated as explained in materials and methods section following the succession approach proposed in Figure 1. LP: *L. plantarum;* PM: *P. manshurica*, IO: *I. occidentalis*; LB: *L. buchneri*; CB: *C. bifermentans*; EC: *E. cloacae.*

[2]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.

N/D: Not detected

Table 5.4. Changes in substrates, products, and microbial populations during laboratory primary fermentation of 2A cucumber fruits in cover brines containing 100 mM $CaCl_2$ and 25 mM acetic acid

Variable	Initial conditions	After primary fermentation
pH	4.2 ± 0.2	3.1 ± 0.1
Glucose (mM)	12.9 ± 1.6	1.3 ± 0.2
Fructose (mM)	18.0 ± 2.0	5.2 ± 0.3
Lactic acid (mM)	N/D	90.3 ± 4.3
Acetic acid (mM)	25.4 ± 0.3	23.4 ± 3.2
LAB populations (log CFU/mL)	5.3 ± 0.1	8.3 ± 0.4
Yeast populations (log CFU/mL)	3.0 ± 0.0	4.1 ± 0.2
Enterobacteria populations (log CFU/mL)	3.3 ± 0.1	N/D
Clostridia populations (log CFU/mL)	N/D	N/D

N/D: Not detected

Table 5.5. Changes in organic acids, pH, redox potential, and yeasts populations observed during secondary fermentations under air and nitrogen – purging 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	N/A[1]
Lactic acid (mM)	89.7 ± 1.2	60.6 ± 0.9	89.1 ± 1.2	89.1 ± 1.2
Acetic acid (mM)	23.8 ± 1.3	17.6 ± 0.1	23.1 ± 1.9	23.2 ± 1.1
Yeast population (log CFU/mL)	5.3 ± 0.1	7.1 ± 0.2	3.1 ± 0.1	3.2 ± 0.2

[1]Not available

Table 5.6. Lactic and acetic acid utilization by *L. buchneri, C. bifermentans,* and *E. 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 (mM)	59.6 ± 1.8	49.1 ± 0.2	47.3 ± 0.3
Acetic acid (mM)	15.5 ± 1.6	22.3 ± 0.1	21.7 ± 0.1
Propionic acid (mM)	N/D	N/D	N/D
Population (log CFU/mL)	5.1 ± 0.0	5.6 ± 0.4	5.2 ± 0.1
C. bifermentans			
pH	4.6 ± 0.1	4.7 ± 0.1	5.9 ± 0.2
Lactic acid (mM)	60.1 ± 1.2	55.9 ± 0.1	25.2 ± 0.1
Acetic acid (mM)	15.1 ± 1.3	18.1 ± 0.5	10.5 ± 0.2
Butyric acid (mM)	N/D	3.1 ± 0.5	38.1 ± 0.1
Propionic acid (mM)	N/D	N/D	N/D
Population (log CFU/mL)	4.9 ± 0.0	N/D	5.3 ± 0.0
E. cloacae			
pH	4.6 ± 0.1	6.9 ± 0.1	7.0 ± 0.1
Lactic acid (mM)	60.1 ± 1.2	21.2 ± 0.6	23.1 ± 0.4
Acetic acid (mM)	17.1 ± 1.3	26.5 ± 0.0	27.1 ± 0.1
Propionic acid (mM)	N/D	14.0 ± 0.1	15.1 ± 0.2
Butyric acid (mM)	N/D	N/D	N/D
Population (log CFU/mL)	5.2 ± 0.0	7.1 ± 0.1	6.9 ± 0.3

Table 5.6 Continued

Mix culture: L. buchneri, Cl. bifermentans,* and *E. cloacae			
pH	4.6 ± 0.1	5.9 ± 0.3	5.7 ± 0.0
Lactic acid (mM)	60.2 ± 0.1	25.7 ± 0.4	27.1 ± 0.3
Acetic acid (mM)	17.1 ± 1.2	28.3 ± 0.3	23.1 ± 0.5
Butyric acid (mM)	N/D	4.5 ± 1.2	10.1 ± 0.1
Propionic acid (mM)	N/D	17.1 ± 0.2	15.3 ± 0.2
LAB population (log CFU/mL)	5.1 ± 0.0	6.5 ± 0.0	6.4 ± 0.0
Enterobacteria population (log CFU/mL)	5.3 ± 0.1	5.3 ± 0.0	5.4 ± 0.0
Clostridia populations (log CFU/mL)	4.9 ± 0.0	3.0 ± 0.0	5.7 ± 0.0

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Chapter 6 Conclusion and Future Directions

The secondary fermentation that leads to the spoilage of fermented cucumbers is a complex phenomenon associated with microbial and chemical changes. Based on the microbiological and chemical observations made during this study, we conclude that the secondary fermentation can be initiated by spoilage yeasts with morphologies and metabolic activity similar to *Pichia manshurica* and *Issatchenkia occidentalis*, and the spoilage LAB, *Lactobaciullus buchneri*. The metabolic activity of these organisms will result in lactic acid utilization with the concomitant increase in cover brine pH. Additionally, the oxidative yeast's metabolic activity aids with the chemical reduction of the fermentation matrix. These favorable conditions will benefit the growth of opportunistic microorganisms, such as *Clostridium bifermentans* and *Enterobacter cloacae*. These bacteria are able to utilize remaining lactic acid in the cover brine and produce butyric and propionic acid, respectively. However, these metabolic activities were only observed at medium pH above 4.5.

The pickle industry is looking for alternative fermentation matrices to reduce/eliminate sodium chloride during the primary fermentation and later storage of the fermented cucumbers. The calcium chloride fermentation matrix studied here is an interesting alternative, which can be applied by the processors, provided control and monitoring of morphologies similar to *P. manshurica. I. occidentalis,* and *L. buchneri* along with changes in pH to prevent the development of the secondary fermentation.

The observations, results and conclusions drawn in this study were based on culturable techniques, however the microbiota associated with primary and secondary fermentation of cucumber fruits is much more complex, and therefore further studies are

necessary for its complete characterization. In that sense, the use of non-culturable techniques might bring an opportunity for the identification of different agents (or the confirmation of the ones observed using culturable techniques). A complete picture of the phenomenon will aid in finding specific preventive measures to control the onset of the secondary fermentation by targeting specific microbiota.