ABSTRACT

JOHANNINGSMEIER, SUZANNE DUNN. Biochemical Characterization of Fermented Cucumber Spoilage using Non-targeted, Comprehensive, Two-dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry: Anaerobic Lactic Acid Utilization by Lactic Acid Bacteria. (Under the direction of Dr. Roger F. McFeeters.)

Cucumbers are preserved commercially by fermentation in brine with approximately 6% sodium chloride. Occasionally, fermented cucumbers spoil after the primary fermentation is completed. This spoilage is characterized by a decrease in lactic acid concentration and rise in brine pH caused by unidentified microorganisms. It was hypothesized that 1) Lactic acid degradation in fermented cucumbers can be initiated by lactic acid bacteria (LAB) that are able to maintain metabolic activity in the presence of sodium chloride and acid pH, and 2) A metabolomic approach to studying fermented cucumber spoilage will provide insight into biochemical changes that are yet unknown. Objectives were to 1) Determine the effects of NaCl and pH on anaerobic lactic acid utilization by spoilage microorganisms; 2) Determine the ability of LAB isolated from spoiled fermentations to initiate lactic acid degradation in fermented cucumbers; and 3) Develop and apply non-targeted, comprehensive, two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS) to detect biochemical changes during fermented cucumber spoilage.

Mixed cultures obtained from spoiled reduced NaCl and commercial cucumber fermentations as well as LAB isolated from those mixed cultures were evaluated for their ability to metabolize lactic acid in filter-sterilized fermented cucumber slurry (FCS). Loss of lactic acid and formation of major metabolites were measured using high performance liquid chromatography. Non-targeted GCxGC-ToFMS of volatile components and

trimethylsilyl (TMS) derivatives of non-volatile components was used to detect other metabolite changes that occurred during spoilage.

Spoilage organisms from reduced NaCl and commercial cucumber fermentations degraded lactic acid anaerobically in FCS in the range of pH 3.2 to 5.0 at NaCl concentrations up to 6% NaCl. Over 18 months incubation, only cucumbers fermented with 6% NaCl to a pH of 3.2 completely prevented anaerobic lactic acid degradation. A clear association between lactic acid utilization and increases in acetic acid, propionic acid and n-propanol was evident across treatments.

Among 13 LAB species isolated from spoilage, only *Lactobacillus buchneri* and *Lactobacillus parafarraginis* were able to metabolize lactic acid in FCS. *L. buchneri* was able to degrade lactic acid under both aerobic and anaerobic atmospheres in pH 3.8 FCS with up to 6% NaCl. *L. buchneri* was able to degrade both D- and L-lactic acid, and decreases in lactic acid were accompanied by increases in acetic acid, 1,2-propanediol, and ethanol. Another spoilage isolate, *Lactobacillus rapi*, converted 1,2-propanediol added to FCS to propionic acid and propanol.

Among 314 volatile components detected in fermented cucumber brine, 199 had peak areas with coefficients of variation below 30%. Peak identifications (214/314) established by mass spectral library matching were 92% accurate based on 63 authentic standards. In contrast, only 21% of TMS-metabolite peaks were identified by mass spectral match, and many of these identifications were incorrect. Analysis of variance of log2 peak areas combined with hierarchial clustering analysis revealed 62 volatile and 30 non-volatile metabolites that changed in concentration during spoilage with mixed cultures and isolated

L. buchneri ($P < 0.01$). These changes included decreases in sugars, amino acids, long chain fatty acids, aldehydes, and ketones and increases in several alcohols, butanoic and pentanoic acids. Most of the detected changes preceded lactic acid utilization.

This is the first documented evidence of the role of lactic acid bacteria in fermented cucumber spoilage. *L. buchneri* was capable of initiating lactic acid degradation under a wide range of environmental conditions that may be present in commercial cucumber fermentations. *L. rapi* may act syntrophically with *L. buchneri* to convert lactic acid to acetic acid, propionic acid and n-propanol, three of the major components found in spoiled commercial cucumber fermentations. Biochemical profiling using non-targeted GCxGC-ToFMS led to discovery of changes in several metabolites during spoilage that were previously unknown.

Biochemical Characterization of Fermented Cucumber Spoilage using Nontargeted, Comprehensive, Two-dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry: Anaerobic Lactic Acid Utilization by Lactic Acid Bacteria

by
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DEDICATION

Dedicated to my lovely ladies,

Victoria Marie, Rachel Renée, and Katherine Grace

Find joy in the pursuit of your dreams

and you will find success

BIOGRAPHY

Suzanne D. Johanningsmeier earned her Bachelor of Science degree in Food Science from Purdue University. She transferred to Purdue after completing her Associate in Arts degree at Vincennes University. It was there that she first learned about the field of Food Science from her advisor and organic chemistry professor, Jay Bardole. During her undergraduate studies at Purdue, she was involved with the department in a number of ways: President of the Food Science Club, Pilot plant and laboratory assistant and undergraduate researcher. Her experiences at Purdue and as a summer intern at A.E. Staley in Decatur, IL stimulated her interest in a career in research. Her undergraduate research project on the effect of grape skin contact time on resveratrol concentration in *Chambourcin* wine was quite rewarding, mostly because of the fine people that mentored her, Ellie Butz, Dr. Richard Vine, and Dr. Bruce Bordelon. This project marked her foray into high pressure liquid chromatography. Her favorite aspects of food science, unwavering to this day, included sensory science, fermentation, and chromatography. She continued her education at North Carolina State University where she completed her Master of Science degree in Food Science with a minor in Biochemistry under the direction of Dr. Henry P. Fleming. Her thesis research was on the malolactic activity of lactic acid bacteria and its effect on the sensory and chemical properties of fermented cabbage. Her project incorporated aspects of fermentation microbiology, method development for analysis of sulfur compounds by liquid

and gas chromatography, and descriptive sensory analysis. After completing her master's degree, Suzanne worked as a research technician for the USDA-ARS Food Science Research Unit located in the Department of Food Science at NC State. She conducted research on the sensory and chemical properties of fermented, acidified, and minimally processed vegetables, including sour taste chemistry, reduction of sodium chloride for sauerkraut fermentation, sensory optimization of process-ready fermented cucumber products, and fresh-cut sweet potato processing and storage. Suzanne entered the ARS student career experience program in order to continue working while completing the degree requirements for her doctoral degree in Food Science under the direction of Dr. Roger F. McFeeters. Her research is presented herein. After graduation, she plans to continue her career as a Research Food Technologist with the USDA-ARS Food Science Research Unit in Raleigh, NC. In addition to research, Suzanne also enjoys food and wine, reading with her children, making quilts and pottery, yoga, ballet, bicycling, jogging, swimming, and nature walks. She takes great pleasure in taking care of her family and engaging in the activities that they enjoy. She resides in Apex with her husband and three daughters.

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Many individuals have shaped my life and contributed to this success. To all of you whom supported me: Thank you for believing in me and helping me achieve this dream.

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Chapter 1

Introduction

Introduction

Cucumber fermentation and spoilage. Cucumber fermentation and storage in bulk tanks is one method for preparation of pickle products that results in a unique food product and aids in the preservation of the cucumber fruit for extended periods of time. Fresh cucumbers of various sizes are typically brined in sodium chloride (NaCl) solutions so that the equilibrated concentration of NaCl is between 5 - 8% (w/w). This concentration of salt inhibits softening enzymes (Bell and Etchells 1961) and favors the growth of the naturally occurring lactic acid bacteria (LAB), particularly the homofermentative species, such as *Lactobacillus plantarum* (Etchells and Jones 1946; Pederson and Ward 1949; Costilow and others 1956; Pederson and Albury 1956). Cucumber fruits typically contain about 2% sugars, primarily glucose and fructose (Jones and Etchells 1943; Fleming and others 1973; Lu and others 2002). These sugars are metabolized by LAB to predominately lactic acid with small amounts of acetic acid, thus reducing the pH and the readily available energy sources. The combination of NaCl, acid pH, and lack of sugars results in a naturally preserved product that can typically be held for many months prior to final product processing (Figure 1.1). However, as early as 1940, it was noted that spoilage of fermented cucumbers during storage was a common problem for pickle manufacturers (Jones 1940).

One disadvantage of this fermentation process is the high concentration of NaCl in the waste stream. Efforts to reduce the NaCl used in the fermentation and storage of cucumbers have resulted in increased incidence of fermented cucumber spoilage. This

spoilage has been characterized by an initial normal lactic acid fermentation followed by depletion of lactic acid, rise in pH, and increases in acetic, propionic, and butyric acids and n-propanol during storage (Fleming and others 1989; Fleming and others 2002). Similar patterns of spoilage have been reproduced with mixed cultures in a model system under anaerobic conditions (Kim and Breidt 2007). The production of volatile acids and increased pH compromise both the quality and safety of the product, necessitating early processing of the tank or resulting in wasted product. It has been shown that addition of hydrochloric acid to reduce the pH to 3.5 after fermentation can substantially slow the utilization of lactic acid in fermented cucumbers where the terminal pH was between 3.6 and 3.8 (Fleming and others 2002). Attempts to isolate the responsible microorganisms have proven difficult (Fleming and others 1989; Kim and Jang 2004; Breidt and others unpublished), and the causative agents and environmental conditions required for lactic acid utilization in fermented cucumbers have not yet been fully elucidated. The currently unpredictable nature of this spoilage has resulted in increased production costs for the pickling industry, mainly in the form of increased monitoring of fermentation tanks. In the case where microbial instability is not detected early, additional costs are incurred from product losses and disposal of the wasted product.

Lactic acid metabolism by lactic acid bacteria (LAB). Lactic acid (2-hydroxy-propanoic acid) is the primary end product of anaerobic glycolysis. It is produced by both

homofermentative and heterofermentative LAB. While lactic acid is typically considered an end product of microbial metabolism, it can also be a substrate of metabolism for many microorganisms, including some species of LAB (Table 1.1). Aerobic utilization of lactic acid as an energy source by *Lactobacillus plantarum* has been demonstrated with acetic acid as the accumulated end product (Murphy and others 1985). This metabolic activity was inhibited by 6 and 8% NaCl and altered under anaerobic conditions (Bobillo and Marshall 1991). Malleret and others (1998) suggested that a similar mechanism for aerobic L-lactate utilization was present in *Lactobacillus sakei*. Anaerobically, *L. plantarum* fermented glucose to lactic acid with subsequent co-depletion of lactic and citric acids and increases in acetic, formic, and succinic acids and carbon dioxide (Lindgren and others 1990). Similarly, *L. buchneri* and *L. brevis* have been shown to exhibit anaerobic growth with lactate as an energy source, and growth was significantly increased in the presence of glycerol (Viega-da-Cunha and Foster 1992). *L. bifermentans* produced acetate, ethanol, carbon dioxide and hydrogen gas at the expense of lactate under conditions of glucose deprivation and pH > 4.0. This metabolic activity by *L. bifermentans* was believed to cause cracking defects in Edam and Gouda cheeses (Kandler and others 1983). In contrast, lactic acid degradation by *L. buchneri* has been demonstrated as a favorable secondary metabolism in silage, resulting in increased aerobic stability. Inoculation of silage with *L. buchneri* resulted in decreased lactic acid content and increased concentrations of acetic acid, propionic acid, and propanol (Driehuis and others 1999). The anaerobic degradation of lactic acid by *L. buchneri* in pure

cultures required induction by acidic conditions, did not require an external electron acceptor, and was found to produce acetic acid and 1,2-propanediol as accumulated end products (Oude Elferink and others 2001). In contrast to other studies on lactate utilization, this particular metabolism in *L. buchneri* was not found to support cell growth. A new lactobacillus species, *L. dioliverans*, that was able to degrade 1,2-propanediol to propionic acid and propanol, was isolated from aerobically stable silages that had been treated with *L. buchneri* (Krooneman and others 2002). Accordingly, the use of *L. buchneri* as an inoculant to enhance silage preservation and the proposed metabolic pathway for lactic acid degradation has been recently reviewed (Holzer and others 2003).

Metabolomic approach. Given the potential diversity of chemical species in a food fermentation system, a discovery based approach may be useful for studying the chemistry of fermented cucumber spoilage where much is yet unknown. Metabolomics has been defined as the field of research that involves characterization, including identification and quantification, of the complete collection of small molecule metabolites in a biological system. In practice, metabolomic studies apply a nontargeted approach to the analytical chemistry and statistical analyses used to discover changes in metabolites that occur related to some criteria of interest (i.e. a disease state, changes in a food due to processing treatments, changes in human plasma related to consumption of a food, etc…). The criteria that were set forth for metabolomic studies stated that the sample preparation, analytical

method, and data analysis must include all classes of compounds; have high recovery; be robust, sensitive, reproducible, matrix independent and universal; and have a plan for identifying unknowns (Fiehn 2001). Achieving these goals with a single technology is a significant challenge given the extraordinary diversity of chemical species that make up the metabolome. The technologies that are primarily being used for metabolomic investigations include nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS). Each of these techniques has advantages and limitations, and a single analytical technique to comprehensively study the metabolome is not yet readily available (Wishart 2008). Nonetheless, rapid developments in analytical and data handling technologies have dramatically increased the ability to perform extensive metabolite profiling.

Despite the limitation of requiring a volatile metabolite or a volatile metabolite derivative, two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS) is a powerful tool for obtaining metabolite information. Advances in GC-MS systems and data collection capability provide the potential to carry out separations of volatile chemical components using two separation mechanisms by connecting columns of different bonded phases in series. The benefits and challenges associated with this technology have been the subject of recent reviews (Marriott and Shellie 2002; Adachour and others 2008). This GCxGC methodology has been widely applied to the analysis of food volatiles. An increased number of compounds were detected, and significantly higher mass

spectral similarity values were obtained for butter volatiles and grape monoterpenoids, providing evidence of the increased separation efficiency of GCxGC-ToFMS as compared to 1D GC-MS (Adachour and others 2005; Rocha and others 2007). Analysis of lavender essential oil using a 2D separation consisting of a nonpolar 1st dimension column followed by a polar 2nd dimension column, resulted in a 25 fold increase in sensitivity and 3 fold increase in the number of resolved components as compared to traditional GC analysis (Shellie and others 2001). Similarly, GCxGC-ToFMS may be applied for the study of nonvolatile components that are first chemically derivatized with one or more trimethylsilyl (TMS) group(s) to make them volatile, allowing the analysis of a number of chemical classes including mono and disaccharides, sugar alcohols, sugar phosphates, organic acids, amino acids, and long chain fatty acids. One-dimensional GC-MS based metabolomic platforms have been developed and applied for metabolite profiling in plants (Roessner and others 2000; Gullberg and others 2004; Weckworth and others 2004; Lisec and others 2006; Zörb and others 2006; Rudell and others 2008), microorganisms (Barsch and others 2004; Strelkov and others 2004; Bölling and Fiehn 2005; O'Hagan and others 2005; Koek and others 2006; van der Werf and others 2008), and human fluids and tissues (Jiye and others 2005; O'Hagan and others 2005; Denkert and others 2008; Pasikanti and others 2008; Begley and others 2009; Mal and others 2009). Two-dimensional GC-ToFMS has been less extensively used for metabolomic investigations due to the challenges associated with the large volumes of data produced and the greater expense of the instrumentation itself.

However, Welthagan and colleagues (2005) clearly demonstrated the advantages of GCxGC-ToFMS for biomarker discovery in spleen tissue extracts. GCxGC-ToFMS required approximately 10 fold less sample than GC-ToFMS, exhibited excellent peak resolution, and separated 7 fold more peaks with S/N > 50 and high quality mass spectra. Similarly, a GCxGC-ToFMS metabolomic platform was applied to rice extracts in a study of phenotypic variation. Peak responses were double that observed with the corresponding one-dimensional GC method, and approximately 620 peaks were observed in each sample (Kusano and others 2007). Twenty-six metabolites that distinguished fermenting yeast cells from respiring yeast cells were identified from among more than 2500 peaks detected using nontargeted GCxGC-ToFMS metabolite profiling (Mohler and others 2006). Similar platforms have been developed for metabolomic investigations in rye grass (Hope and others 2005), microbial metabolomics (Guo and Lidstrom 2008), and biomarker discovery for diabetes mellitus (Li and others 2009). Optimization of instrumental analytical parameters for a nonpolar/polar column combination resulted in the detection of more than 1800 metabolite peaks in human serum (O'Hagan and others 2007). Most of the previously mentioned 2D GC studies used a nonpolar/polar column combination. In contrast, Koek and others (2008) found that a polar/nonpolar column combination resulted in better resolution of components and greater use of the separation space with similar separation efficiency. The aforementioned studies have clearly demonstrated the usefulness of GC-MS for nontargeted metabolite profiling in a variety of matrices.

Combining the information obtained from both volatile and nonvolatile analyses using a nontargeted GCxGC-ToFMS platform will allow a much more comprehensive view of the chemical changes occurring during fermented cucumber spoilage than has been possible with high performance liquid chromatography (HPLC) coupled to ultraviolet and refractive index detectors.

Hypotheses

1) Lactic acid degradation in fermented cucumbers can be initiated by lactic acid bacteria that are able to survive and maintain metabolic activity in the presence of 4 - 6% sodium chloride, high acid pH, and anaerobic conditions.
2) A GCxGC-ToFMS metabolomic approach to studying fermented cucumber spoilage will provide insight into biochemical changes that are yet unknown.

Objectives

The objectives of this research study were as follows:

1) Test the ability to transfer successively and reproduce fermented cucumber spoilage with mixed cultures of unknown identity obtained from reduced salt and commercial fermentations that spoiled spontaneously.

2) Determine the effects of pH and NaCl concentration on anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms from reduced salt and commercial fermentations.
3) Determine the ability of lactic acid bacteria isolated from spoiled fermentations to initiate lactic acid degradation in fermented cucumbers
4) Develop nontargeted GCxGC-ToFMS methods for studying the biochemical changes that take place during fermented cucumber spoilage.
5) Determine the biochemical changes that take place during spoilage of fermented cucumbers.
6) Compare spoilage metabolite profiles of bacteria isolated from spoilage brines to that which occur using mixed culture inocula from spontaneously spoiled fermented cucumbers.

Significance/Impact

The biochemical characterization of post-fermentation lactic acid utilization in fermented cucumbers using nontargeted, comprehensive, two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS) will enhance our knowledge of a less well studied metabolism of lactic acid bacteria. This knowledge may contribute to the development of novel uses of LAB in foods as well as the control of this metabolic activity where it is undesirable. Determination of the pH and salt concentrations

which are permissive or restrictive of this undesirable metabolism of lactic acid in fermented cucumbers directly benefits the fermented cucumber industry. This information will have immediate impact in the control of bulk preservation of cucumbers.

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Table 1.1 Utilization of lactic acid by lactic acid bacteria[1]

Organism	Atmo-sphere	NaCl (%)	pH	Media	Essential Cofactor	End products	Growth
L. plantarum	Aerobic	0	7.0/6.6	MRS/TY	O_2	Acetate	Yes
L. plantarum	Aerobic	0, 6	4.3 to 5.0	MRS	NR[2]	Acetate	NR
L. plantarum	Anaerobic	0, 6	3.8 to 4.7	MRS	NR	Acetate	NR
L. plantarum	Anaerobic	0	5.0	CSM	Citrate	Acetate, formate, succinate, & CO_2	NR
L. brevis	Anaerobic	0	NR	MLM	Glycerol	Acetate & 1,3-propanediol	Yes
L. buchneri	Anaerobic	0	NR	MLM	Glycerol	Acetate & 1,3-propanediol	Yes
L. buchneri	Anaerobic	0	3.8, 4.0 & 4.3	MRS-MOD	NR	Acetate & 1,2-propanediol	No
L. parabuchneri	Anaerobic	0	3.8, 4.0 & 4.3	MRS-MOD	NR	Acetate & 1,2-propanediol	No
L. bifermentans	Anaerobic	0	> 4.0	MRS	NR	Acetate, Ethanol, CO_2, H_2	NR

[1] Kandler and others 1983; Murphy and others 1985; Lindgren and others 1990; Bobillo and Marshall 1991; Veiga Da Cunha and Foster 1992; Oude Elferink and others 2001; [2] NR = not reported in this study

Figure 1.1 Flow diagram of commercial cucumber fermentation

Chapter 2

Environmental and microbial factors affecting anaerobic lactic acid utilization during fermented cucumber spoilage

Abstract

Cucumbers are preserved commercially by fermentation in brine with 5-8% sodium chloride (NaCl). Occasionally, fermented cucumbers spoil after the primary fermentation is completed. This spoilage is characterized by decreases in lactic acid and rise in pH caused by unidentified microorganisms. The objectives of this study were to determine the effects of NaCl and pH on anaerobic lactic acid utilization by spoilage microorganisms and to determine the ability of lactic acid bacteria (LAB) spoilage isolates to initiate lactic acid degradation in fermented cucumbers. Cucumbers fermented with 0, 2, 4, and 6% NaCl were blended into slurries (FCS) and adjusted to pH 3.2, 3.8, 4.3, and 5.0 prior to centrifugation, sterile-filtration, and inoculation with spoilage brines. Organic acids and pH were measured after 3 wk, 2, 6, 12, and 18 months anaerobic incubation at 25°C. Anaerobic lactic acid degradation occurred in FCS at pH 3.8, 4.3 and 5.0 regardless of NaCl concentration. At pH 3.2, reduced NaCl concentrations resulted in increased susceptibility to spoilage. Over 18 months incubation, only cucumbers fermented with 6% NaCl to pH 3.2 completely prevented anaerobic lactic acid degradation. A clear association between lactic acid utilization and increases in acetic acid, propionic acid and propanol was evident across treatments. Among several LAB species isolated from fermented cucumber spoilage, *Lactobacillus buchneri* was unique in its ability to metabolize lactic acid in FCS with concurrent increases in acetic acid and 1,2-propanediol. Therefore, *L. buchneri* may be one of multiple organisms that play a role in fermented cucumber spoilage.

Introduction

Cucumber fermentation and storage in bulk tanks is one method for preparation of pickle products that results in a unique food product and aids in the preservation of the cucumber fruit for extended periods of time. Fresh cucumbers of various sizes are typically brined in sodium chloride (NaCl) solutions so that the equilibrated concentration of NaCl is between 5 - 8% (w/w). This concentration of salt inhibits softening enzymes (Bell and Etchells 1961) and favors the growth of the naturally occurring lactic acid bacteria (LAB), particularly the homofermentative species, such as *Lactobacillus plantarum*. Cucumber fruits typically contain about 2% sugars, primarily glucose and fructose (Lu and others 2002). These sugars are metabolized by LAB to predominately lactic acid with small amounts of acetic acid, thus reducing the pH and the readily available energy sources. The combination of NaCl, acid pH, and low concentration of residual sugars results in a naturally preserved product that can typically be held for many months prior to final product processing. Occasionally, tanks of commercially fermented cucumbers spoil after the primary fermentation. Fleming and others (1989) observed this spoilage in pilot scale (4500 L) closed tank fermentations carried out in reduced salt brines. They also found spoilage of small cucumbers increased when the pH of samples of fermented cucumbers from commercial tanks (salt concentration 4.8% to 5.5%) was raised from 3.6-3.8 to pH 4.0 and decreased when pH was reduced to 3.5 (Fleming and others 2002). However, at reduced NaCl concentrations, spoilage potential was demonstrated at pH 3.5 within three months of anaerobic incubation

(Kim and Breidt 2007). This type of spoilage has been characterized by a normal lactic acid fermentation followed by depletion of lactic acid, rise in pH, and increases in acetic, propionic, and butyric acids and n-propanol during prolonged storage (Fleming and others 1989; Fleming and others 2002). Commercially, the production of volatile compounds and increased pH can compromise both the quality and safety of the product, necessitating premature processing of tanks where spoilage is detected early or discarding product from tanks where spoilage raises the pH to 4.6 or above. Attempts to isolate the responsible microorganisms have proven difficult (Fleming and others 1989; Breidt and others unpublished), and the causative organisms and environmental conditions required for lactic acid utilization in fermented cucumbers have not yet been fully elucidated. The unpredictable nature of this spoilage has resulted in increased production costs for the pickling industry, mainly in the form of increased monitoring of fermentation tanks. In the case where microbial spoilage is not detected early, additional costs are incurred from product losses and disposal of the spoiled product.

Lactic acid is the primary end-product of anaerobic glycolysis. It is produced by both homofermentative and heterofermentative LAB. Although lactic acid is typically considered an end product of fermentation, it can also be a substrate of metabolism for many microorganisms, including some species of LAB. Aerobic utilization of lactic acid as an energy source by *Lactobacillus plantarum* has been demonstrated with acetic acid as the accumulated end-product (Murphy and others 1985). This metabolic activity was inhibited

by 6 and 8% NaCl and less evident under anaerobic conditions (Bobillo and Marshall 1991). Anaerobically, *L. plantarum* fermented glucose to lactic acid with subsequent co-depletion of lactic and citric acids accompanied by increases in acetic, formic, and succinic acids and carbon dioxide (Lindgren and others 1990). Similarly, *L. buchneri* and *L. brevis* have been shown to exhibit anaerobic growth with lactate as an energy source, and growth of these bacteria was significantly increased in the presence of glycerol (Viega-da-Cunha and Foster 1992). Lactic acid degradation by *L. buchneri* has been demonstrated to be a favorable secondary metabolism in silage that results in increased aerobic stability of the silage (Driehuis and others 1999). Furthermore, *Lactobacillus* species have been previously isolated from fermented cucumber spoilage samples (Breidt and others unpublished). Therefore, we hypothesized that lactic acid degradation in fermented cucumbers may be initiated by lactic acid bacteria that are able to survive and maintain metabolic activity in the presence of 4 - 6% sodium chloride (NaCl), acid pH, and anaerobic conditions. The objectives of this study were (1) to determine the effects of environmental variables (pH and NaCl concentration) on anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms; and (2) to test the ability of lactic acid bacteria spoilage isolates to initiate lactic acid degradation in fermented cucumbers.

Materials and Methods

Production of anaerobic spoilage at varying NaCl concentrations and pH was conducted as follows: Pickling cucumbers (size 2B) were fermented with 0, 2, 4, and 6% NaCl equilibrated concentrations. *Lactobacillus plantarum* MOP3 starter culture (Culture Collection ID LA0219, USDA-ARS Food Science Research Unit, Raleigh, NC) was added to each fermentation at 10^6 cfu/g. Fermentations were conducted in triplicate 1 gallon sized glass jars with a 55:45 pack-out ratio (weight cucumbers to volume brine). Fermentation progress was monitored by pH and HPLC analysis of acids and sugars. Fermented cucumbers were blended into slurry and stored at -10°C. To prepare media for inoculation with spoilage microorganisms, fermented cucumber slurries with 0, 2, 4, and 6% NaCl were thawed and then pressed through three layers of cheesecloth to remove large particulates prior to centrifugation in 250 ml bottles at 23,400 x g for 15 min. For each NaCl concentration, clarified slurry was divided into four aliquots. One aliquot had no pH adjustment. The other 3 aliquots were adjusted to pH 3.8, 4.3 and 5.0 with 6N NaOH. The resulting clarified, pH-adjusted fermented cucumber slurries (FCS) were each 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). FCS (12 mL) was aseptically transferred into sterile 15 mL conical tubes and placed into the Coy anaerobic chamber for at least two days prior to inoculation with spoilage brine. Samples were aseptically withdrawn immediately after inoculation and after 21 days, 2, 6, 12, and 18 months incubation at

ambient temperature (21 - 25°C). Samples were stored at -20°C until analysis. High performance liquid chromatography (HPLC) quantification of glucose, fructose, glycerol, ethanol, propanol, malic, succinic, lactic, acetic, propionic, and butyric acids was used to measure anaerobic lactic acid degradation and formation of possible spoilage metabolites.

Spoiled fermented cucumbers from three sources were used for anaerobic reproduction of spoilage (Table 2.1). Briefly, an aliquot of brine from a 5 gal pail of brined cucumbers (2% NaCl) that had fermented normally, but then spontaneously spoiled after several months of storage was used for isolation and identification of microorganisms and also as an inoculum for controlled production of spoilage. This spoilage source is referred to in these experiments as the reduced NaCl spoilage culture. A second spoilage culture that originated from a commercial cucumber fermentation tank that had spoiled was obtained from Breidt and colleagues (unpublished) and designated commercial spoilage 1M. This spoilage culture had been transferred sequentially in fermented cucumber slurry adjusted to pH 5.0 and 2.0% NaCl and then frozen at -80°C with glycerol as the cryoprotectant, so the M was added to the designation to indicate that the spoilage source was modified from when it was originally obtained. During the course of experimentation, another commercial cucumber fermentation tank underwent spoilage (commercial spoilage 2), and a brine sample was obtained and used directly as a source of potential spoilage organisms. These spoilage sources were handled and used as inocula as described in Figures 2.1, 2.2, and 2.3. Data that describes lactic acid utilization in these experimental

spoilage productions is available as Supplement 1 in Appendix 1 (Tables A1.1, A1.2, and A1.3).

Lactic acid bacteria (LAB) were isolated as described in Table 2.2 from original samples of reduced NaCl and commercial spoilage 2 brines and after anaerobic reproduction of spoilage, as outlined in Figures 2.1 and 2.3. For the reduced NaCl spoilage-R4 and commercial spoilage 2-R4, LAB were isolated on MRS after 22 weeks anaerobic spoilage of fermented cucumber slurry (initial pH 3.8) at varying NaCl concentrations. Inoculated FCS samples where lactic acid utilization was demonstrated (Table A1.3) were spiral plated on MRS and incubated anaerobically for 4-6 days at 30°C. Isolates were selected for identification based on differences in colony morphology (Photographs are available as Supplement 2 in Appendix 1). LAB isolates from original samples and anaerobic spoilage experiments were identified by polymerase chain reaction (PCR) and sequencing of 16S rRNA as follows: Genomic DNA was isolated using commercially available kits (Bio-Rad Laboratories, Hercules, CA or Qiagen, Inc, Valencia, CA) following the directions provided by the manufacturers. Amplification of the 16S rRNA by polymerase chain reaction (PCR) was conducted using the following universal primer pair: 8f (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1492r (5'- GGT TAC CTT GTT ACG ACT T -3'). Amplicons were purified and sent to GeneWiz (LaJolla, CA) or the NC State Genomic Sciences laboratory (Raleigh, NC) for sequencing. All sequences were examined using the original chromatographs and edited with ABI Sequence Scanner (Applied Biosystems, Carlsbad, CA). The resulting sequences

were compared by BLAST analysis to the nucleotide collection of the National Center for Biotechnology Information (NCBI, Bethesda, MD) to determine their similarity to other 16S rRNA sequences. All BLAST matches reported had 98-100% identity with the query sequence.

Lactic acid utilization by LAB Isolates was tested by inoculating FCS (pH 3.8, 6% NaCl) with approximately 10^6 cfu/ml of each organism. LAB isolates were grown in either MRS or modified cucumber slurry media (mCS). Modified cucumber slurry media was prepared from fresh size 2B cucumbers blended into slurry and processed as described above for fermented cucumber slurry. After centrifugation, NaCl, yeast extract, peptone and water were added to give final concentrations of 67% fresh cucumber, 4% NaCl, 1% yeast extract, and 1% peptone. The resulting growth media (mCS) was sterile-filtered with a 0.22 µm bottle top filter and stored at 4°C. LAB isolates (Table 2.2) were grown at 30°C until visual turbidity was observed. Cells were spun down, resuspended in FCS, and inoculated into FCS at ambient temperature in the anaerobic chamber. Inoculated FCS was aseptically sampled at time intervals throughout the incubation period to measure pH and lactic acid concentration.

HPLC analysis was conducted with minor modification of the method published by McFeeters and Barish (2003). Briefly, components of samples were separated on an Aminex HPX-87H resin column (300 x 7.8 mm, Bio-Rad Laboratories, Hercules, CA) with 0.03 N H_2SO_4 eluent at a flow rate of 0.6 mL/min. The column temperature was held at 37°C to

separate propionic acid, a potential spoilage metabolite, from an unknown component that frequently occurs in fermented cucumbers. Sugars and alcohols were quantified in the same analysis using a refractive index detector connected in series. Measurement of pH was done at ambient temperature with an Accumet AR25 pH meter (Catalog #13-636-AR25A) equipped with a gel-filled combination pH electrode (Catalog #13-620-290, Fisher Scientific, Pittsburgh, PA) that was calibrated with certified standards of pH 2.00, 4.00, and 7.00 (Fisher Scientific).

Results and Discussion

Prior attempts to reproduce fermented cucumber spoilage under controlled laboratory conditions and isolate the responsible microorganisms have been challenging. There is a diversity of organisms present in vegetable fermentations, so the potential for multiple stages in the spoilage process exists. In this study, controlled reproduction of lactic acid degradation (spoilage) in fermented cucumber slurry (FCS) was achieved using spoilage cultures obtained from three sources. Lactic acid bacteria (LAB) were isolated and identified from reduced NaCl and commercial spoilage brines and from spoilage produced experimentally with these sources in filter-sterilized FCS. Anaerobic lactic acid degradation was observed in FCS inoculated with the reduced NaCl spoilage culture regardless of NaCl concentration at pH 3.8, 4.3, and 5.0 with the greatest extent of spoilage occurring at pH 3.8. At pH 3.2, lactic acid degradation occurred in 0, 2, and 4% NaCl, but not in 6% NaCl FCS.

The rate and extent of lactic acid degradation in FCS at pH 3.2 was significantly greater with 0 and 2% NaCl than in 4% NaCl (Figure 2.4). This increased susceptibility to spoilage at reduced NaCl concentrations is consistent with previous reports (Fleming and others 2002; Kim and Breidt 2007) and provides evidence for the first time that lactic acid can be degraded with a pH as low as 3.2. Kim and Breidt (2007) demonstrated that spoilage microorganisms could degrade lactic acid in reduced NaCl fermented cucumber slurries at pH 3.5 but not at pH 3.0. The current study shows that the pH limit for lactic acid utilization in reduced NaCl fermented cucumbers is 3.2 or lower. Decreases in lactic acid were accompanied by increases in acetic acid, propionic acid, and propanol for all NaCl and pH conditions. The only condition where anaerobic lactic acid degradation (spoilage) was completely prevented was in 6% NaCl fermented cucumbers at pH 3.2 (Figure 2.4). Under these conditions, no lactic acid was degraded even upon extended incubation to 18 months.

A commercial spoilage sample (commercial spoilage 1M) previously characterized by Breidt and colleagues (unpublished) was used for comparison of these results to prior studies of fermented cucumber spoilage. Interestingly, NaCl and pH affected lactic acid utilization in fermented cucumber slurry differentially depending on which spoilage culture was used (data available in Appendix 1 Table A1.1). In the latter case, lactic acid utilization occurred more rapidly at pH 5.0 and 4.3 and butyric acid was produced in some (pH 5.0, 2 and 4% NaCl) but not all NaCl and pH combinations. These differences may have been due to selection of different microorganisms (as evidenced by different spoilage metabolites at

pH 5.0) and possibly a preconditioning effect (as evidenced by the longer lag time but formation of similar metabolites at pH 3.8). This particular commercial spoilage culture had been serially passaged through pH 5.0 and 2% NaCl fermented cucumber media prior to freezing and storage. In contrast, the reduced NaCl spoilage culture was obtained directly from fermented cucumber brine (2% NaCl) that had undergone spontaneous spoilage and had not experienced any freezing or other processes that might have selected for or against microorganisms present as spoilage occurred. These differential results indicate that multiple organisms capable of metabolizing lactic acid are likely to be present in fermented cucumber brines. It is reasonable to expect that spoilage brines from different sources may contain different organisms and increasing the pH experimentally can allow organisms to produce metabolic products that may not normally occur in commercial spoilage situations. For example, *Propionibacteria* sp are known to convert lactic acid to acetic and propionic acids, but this metabolism has only been reported at pH 4.5 and above (Plastourgos and Vaughn 1957; Hsu and Yang 1991; Rehberger and Glatz 1998). Similarly, *Clostridia* sp are known to produce butyric acid, and this has been demonstrated in fermented cucumbers where the pH had been raised to pH 5.0 and above with NaOH (Fleming and others 1989; Franco and Pérez-Díaz unpublished). Therefore, such organisms may be present in commercial fermentation environments and play a role in the latter stages of fermented cucumber spoilage after the pH has risen, but these organisms would not be able to initiate lactic acid utilization under the conditions that typically prevail prior to spoilage (2-6% NaCl

and pH 3.2-3.8). We have provided evidence that fermented cucumber spoilage can be reproduced in the laboratory in 6% NaCl FCS at pH 3.8. This set of conditions is within the range of NaCl and pH that may be encountered in commercial fermentation tanks prior to spoilage. Therefore, these conditions were selected for continued experimental production of spoilage and testing LAB spoilage isolates for the ability to initiate lactic acid degradation in FCS.

Lactic acid bacteria were present in significant numbers during anaerobic spoilage of 6% NaCl fermented cucumber slurry adjusted to pH 3.8 and inoculated with reduced NaCl spoilage and commercial spoilage 2 cultures. Initially, numbers of LAB were lower for the commercial spoilage treatment but increased to 7.3 log cfu/ml prior to the initiation of lactic acid degradation. Although the number of LAB were higher in the reduced NaCl spoilage inoculum, total LAB counts increased only 1.5 log from 6.1 to 7.6 log cfu/ml and reached the highest numbers 2 days after inoculation. The utilization of residual glucose appeared to be related to the increases in LAB (Figure 2.5). The approximately 3 mM residual glucose present in FCS was depleted in 8 days by the reduced NaCl spoilage organisms and 24 days by the commercial spoilage organisms. Regardless of the differences during the initial incubation period, anaerobic lactic acid degradation began approximately 24 days after inoculation for both spoilage cultures (Figure 2.6). The rate of lactic acid utilization by the reduced NaCl spoilage inoculum was greater than for the commercial spoilage source. Nevertheless, decreases in lactic acid concentration were accompanied by

increases in acetic and propionic acids (Figure 2.6) and rise in pH to 4.57 ± 0.01 and 4.33 ± 0.05 for the reduced NaCl and commercial spoilage reproductions, respectively. LAB remained viable during the course of lactic acid utilization (127 days), but did not increase in total number (Figure 2.5).

A variety of lactic acid bacteria (LAB) were isolated from spoiled fermented cucumbers and experimental spoilage produced in sterile-filtered, fermented cucumber slurry adjusted to pH 3.8 (FCS) (Table 2.2). Organisms were primarily from the Lactobacillus genus. *L. plantarum*, *L. brevis*, and *Pediococcus* sp. have been previously documented as part of the normal flora of fermented cucumbers (Etchells and Jones 1946; Pederson and Ward 1949; Pederson and Albury 1950; Costilow and others 1956; Pederson and Albury 1956). *L. plantarum* and *L. brevis* were isolated from both the reduced NaCl spoilage brine and the commercial spoilage sample in this study. Although these organisms have been shown to degrade lactic acid (Murphy and others 1985; Lindgren and others 1990; Bobillo and Marshall 1991; Viega-da-Cunha and Foster 1992), there was no evidence of lactic acid degradation by *L. plantarum* and *L. brevis* spoilage isolates in FCS (pH 3.8 with either 2 or 6% NaCl) even during extended incubation for 146 days (data not shown). Therefore, we concluded that these organisms do not contribute to the initiation of fermented cucumber spoilage, since they did not utilize lactic acid in FCS under conditions typical of those encountered in commercial cucumber fermentations. *Pediococcus ethanolidurans* is a newly proposed pediococci species that was first isolated from a distilled spirits cellar in

China and shown to grow under stressful environmental conditions (Liu and others 2006). In addition, a number of *Lactobacillus* species less commonly associated with cucumber fermentations were found, including *L. buchneri*, *L. camelliae*, *L. casei*, *L. coryniformis*, *L. harbinensis*, *L. parafarraginis*, and *L. rapi*. Of these, *L. buchneri*, *L. casei*, and *L. coryniformis* have been the most well studied and have been frequently associated with food fermentations. *L. camelliae* is a homofermentative organism genetically related to *L. casei* that produces only the L-isomer of lactic acid and was originally isolated from fermented tea leaves in Thailand (Tanasupawat and others 2007). This organism did not grow in modified cucumber slurry with 4% NaCl (mCS), so it was not tested for lactic acid degradation in FCS. *L. harbinensis* has been isolated from traditional Chinese fermented vegetables, brewery environments, Korean rice wine (Jianbo and others 2008) and spoiled carbonated beverages and was proposed as a new species related to the *L. casei*-Pediococcus group by Miyamoto and colleagues (2005). *L. parafarraginis* was isolated from distilled shochu residue compost and was found to be related to *L. buchneri* (Endo and Okada 2007). Similarly, *L. rapi* was recently isolated from sunki, a traditional Japanese pickle fermented without NaCl, and described as a new species in the *L. buchneri* group of the lactobacilli (Watanabe and others 2009). In all, 13 different LAB species were isolated from fermented cucumber spoilage samples, including multiple strains of *L. buchneri*.

Despite the diversity of LAB that was present in spoiled fermented cucumber samples, *Lactobacillus buchneri* was unique in its ability to degrade lactic acid in FCS at pH

3.8 and 6% NaCl under anaerobic conditions. Decreases in lactic acid accompanied increases in acetic acid and 1,2-propanediol in FCS (2% NaCl, pH 3.8) inoculated with the *L. buchneri* strain isolated from reduced NaCl spoilage (Figure 2.7). These chemical changes were consistent with the utilization of lactic acid by *L. buchneri* in acidified laboratory media without NaCl observed by Oude Elferink and others (2001). However, 1,2-propanediol was not detected in the experimental production of spoilage with mixed spoilage cultures, indicating that one or more other organisms were involved. In silage, lactic acid was converted to acetic acid, propionic acid, and propanol when *L. buchneri* was added as a starter culture adjunct (Driehuis and others 1999). However, *L. buchneri* does not produce propionic acid and propanol in pure culture. Accordingly, Krooneman and others (2002) isolated *L. dioliverans* from treated silage and proposed that it was responsible for converting 1,2-propanediol produced by *L. buchneri* to propionic acid and propanol. Therefore, the LAB species isolated from the commercial spoiled brine and *P. ethanolidurans* were inoculated into FCS in combination with *L. buchneri*, but no propionic acid or propanol were formed. The same amount of 1,2-propanediol accumulated in FCS inoculated with *L. buchneri* regardless of the presence of the other LAB species (data not shown). *L. rapi* and *L. parafarraginis* were isolated for the first time from anaerobic spoilage of FCS in recent experiments, and therefore, were not tested for the ability to utilize lactic acid and/or 1,2-propanediol in this study.

Only 1 of 7 *L. buchneri* isolates from the original reduced NaCl fermented cucumber spoilage demonstrated measurable anaerobic lactic acid degradation in fermented cucumber slurry (pH 3.8, 2% NaCl) within 56 days. This suggests that the ability to metabolize lactic acid in fermented cucumbers may be a strain-related characteristic. Although *L. buchneri* was not directly isolated from the commercial spoilage sample in this study, it was detected by non-culture based identification of microorganisms (Breidt and others unpublished). *L. buchneri* was also isolated from FCS that had been inoculated with the commercial spoilage culture and showed decreased lactic acid concentration during 22 weeks of anaerobic incubation (Table 2.2). Based on subsequent work, we believe that the shorter MRS incubation time used for the commercial sample precluded the isolation of *L. buchneri* from the original spoilage brine due to its relatively slow growth rate on MRS compared to the other species that were present. Furthermore, the presence of *L. buchneri* in several commercial spoilage samples was indicated by 16S rRNA sequence analysis of PMA-treated bacterial DNA, and this was the LAB most consistently identified (Breidt and others unpublished). The repeated identification of *L. buchneri* in spoilage samples in this study and in spoiled commercial cucumber fermentations (Franco and others unpublished) combined with its demonstrated ability to use lactic acid in acid pH, 6% NaCl FCS under anaerobic conditions indicates that *L. buchneri* may be a significant contributor to fermented cucumber spoilage.

Conclusions

Among several lactic acid bacteria species isolated from spoilage fermentations, *Lactobacillus buchneri* was unique in its ability to initiate lactic acid degradation in fermented cucumbers and may play an important role in fermented cucumber spoilage. In the absence of oxygen, cucumbers fermented with 6% sodium chloride to a terminal pH of 3.2 were not subject to spoilage during long term storage. However, lactic acid was degraded by spoilage microorganisms in cucumbers fermented with 0, 2, and 4% NaCl, even with a terminal pH as low as 3.2. Ongoing efforts to reduce sodium in commercial brining operations will need to include measures for controlling the increased susceptibility to spoilage of cucumbers fermented and stored with lower NaCl concentrations. *L. buchneri* may be an appropriate target for development of such control measures.

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Table 2.1 Spoiled fermented cucumber sources for anaerobic reproduction of spoilage and isolation and identification of lactic acid bacteria (LAB)

Spoilage ID	Source Description	Storage
Reduced NaCl	Brine from a 5 gal pail of brined cucumbers (2% NaCl) that had fermented normally, but then spontaneously spoiled after several months of storage as described by Johanningsmeier and McFeeters (2011). Residual lactic acid was 10 mM and pH was 4.1. Acetic, propionic and butyric acids were 80 mM, 40 mM, and undetected, respectively. Yeasts were not detected and LAB count on MRS was 1.19×10^6 cfu/ml.	-80°C with glycerol
Commercial 1M	Spoiled brine from a commercial cucumber fermentation that had been inoculated into 2% NaCl fermented cucumbers that were adjusted to pH 5.0 and held anaerobically at ambient temperature until evidence of spoilage activity was demonstrated (Breidt and others unpublished). The resulting spoiled brine was stored in individual aliquots with glycerol at -80°C.	-80°C with glycerol
Commercial 2	Spoiled brine from a commercial cucumber fermentation where the lactic acid had been completely depleted, pH was 4.9, and acetic, propionic and butyric acids were 32 mM, 36 mM, and 52 mM, respectively. NaCl was estimated at 4.3% at the time the sample was received. Yeast count on YMA was 6.13×10^4 cfu/ml and LAB count on MRS was 7.15×10^5 cfu/ml.	4°C

Table 2.2: Lactic acid bacteria isolated from fermented cucumber spoilage

Organism ID (prevalence)	Culture Collection ID[1] for Representative Isolate	Spoilage Source	Isolation Media	Temp-erature	Time (days)
Lactobacillus brevis (3/13)	N/A	Reduced NaCl fermented cucumber spoilage	M17 lactic & BHI	25°C	7
Lactobacillus buchneri (7/13)	LA 1147	Reduced NaCl fermented cucumber spoilage	M17 lactic & MRS	25°C	7
Lactobacillus plantarum (1/13)	N/A	Reduced NaCl fermented cucumber spoilage	M17 lactic	25°C	7
Pediococcus sp (2/13)	N/A	Reduced NaCl fermented cucumber spoilage	M17 lactic & MRS	25°C	7
Lactobacillus buchneri (12/20)	LA 1138	R2: Anaerobic spoilage in 6% NaCl FCS with Reduced NaCl spoilage source	MRS	30°C	6

Table 2.2 Continued

Pediococcus ethanolidurans (8/20)	LA 1139	R2: Anaerobic spoilage in 6% NaCl FCS with Reduced NaCl spoilage source	MRS	30°C	6
Lactobacillus buchneri (9/13)	LA 1151; LA 1152; LA 1154; LA 1155; LA 1156; LA 1157; LA 1158; LA 1159; LA 1160	R4: Anaerobic spoilage in FCS (0-6% NaCl) with Reduced NaCl source	MRS	30°C	4-6
Lactobacillus rapi (2/13)	LA 1165; LA 1169	R4: Anaerobic spoilage in FCS (0-6% NaCl) with Reduced NaCl source	MRS	30°C	4-6
Lactobacillus parafarraginis (2/13)	LA 1153; LA 1168	R4: Anaerobic spoilage in FCS (0-6% NaCl) with Reduced NaCl source	MRS	30°C	4-6
Lactobacillus brevis (4/22)	LA 1145	Commercial spoilage 2	MRS	30°C	2
Lactobacillus casei (7/22)	LA 1141	Commercial spoilage 2	MRS	30°C	2

Table 2.2 Continued

Lactobacillus harbinensis (2/22)	LA 1144	Commercial spoilage 2	MRS	30°C	2
Lactobacillus plantarum (5/22)	LA 1142	Commercial spoilage 2	MRS	30°C	2
Lactobacillus coryniformis (1/22)	LA 1143	Commercial spoilage 2	MRS	30°C	2
Pediococcus parvulus (1/22)	LA 1140	Commercial spoilage 2	MRS	30°C	2
Lactobacillus camelliae (1/22)	LA 1146	Commercial spoilage 2	MRS	30°C	2
Lactobacillus buchneri (5/6)	LA 1161; LA 1163; LA 1164; LA 1166; LA 1167	R4: Anaerobic spoilage in FCS (0-6% NaCl) with Commercial spoilage 2	MRS	30°C	4-6
Lactobacillus harbinensis/perolens (1/6)	LA 1162	R4: Anaerobic spoilage in FCS (0-6% NaCl) with Commercial spoilage 2	MRS	30°C	4-6

[1]USDA-ARS Food Science Research Unit, Raleigh, NC

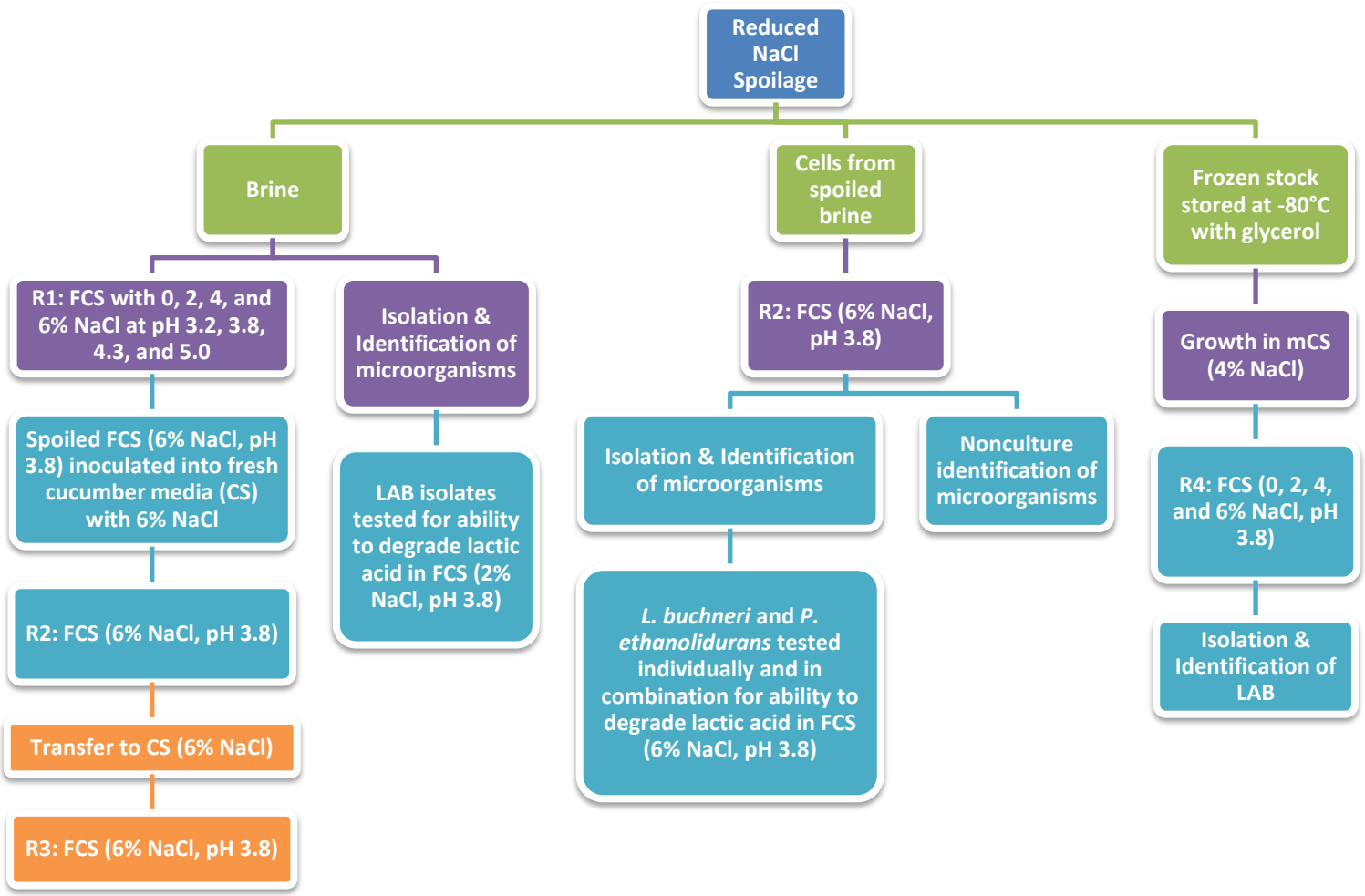

Figure 2.1 Experimental flow chart for reproduction of anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms from reduced NaCl fermented cucumbers

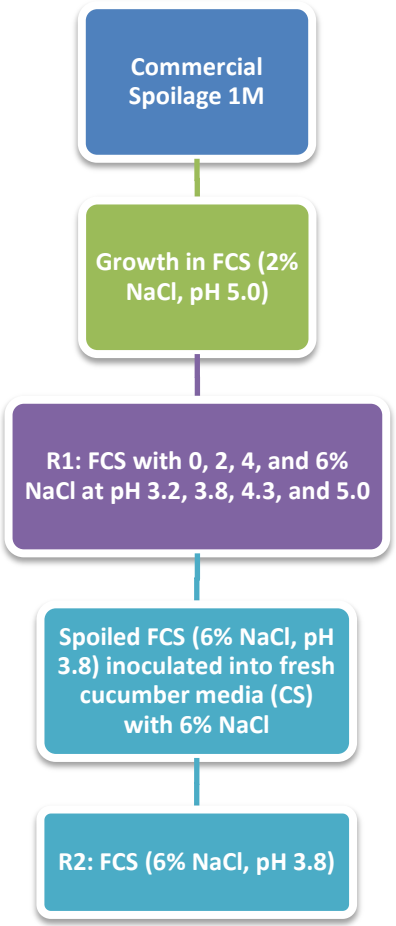

Figure 2.2 Experimental flow chart for reproduction of anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms from a commercial fermentation tank (1M)

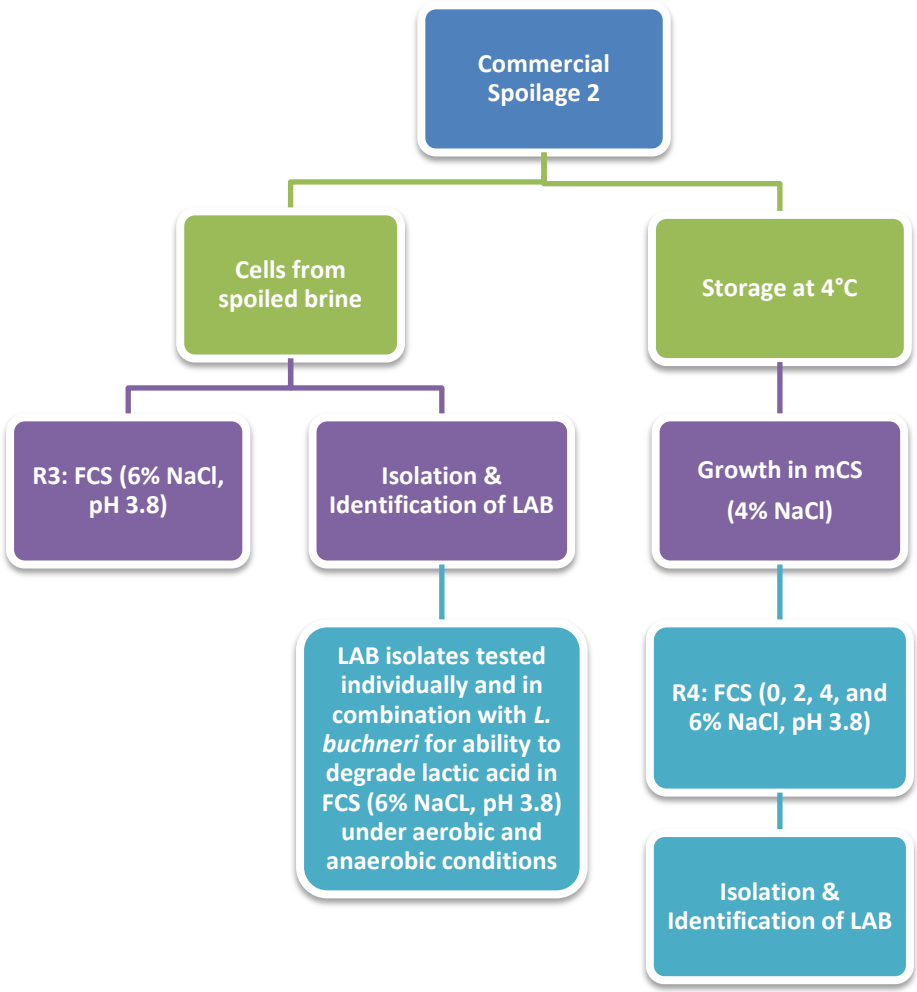

Figure 2.3 Experimental flow chart for reproduction of anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms from commercial spoiled fermentation brine

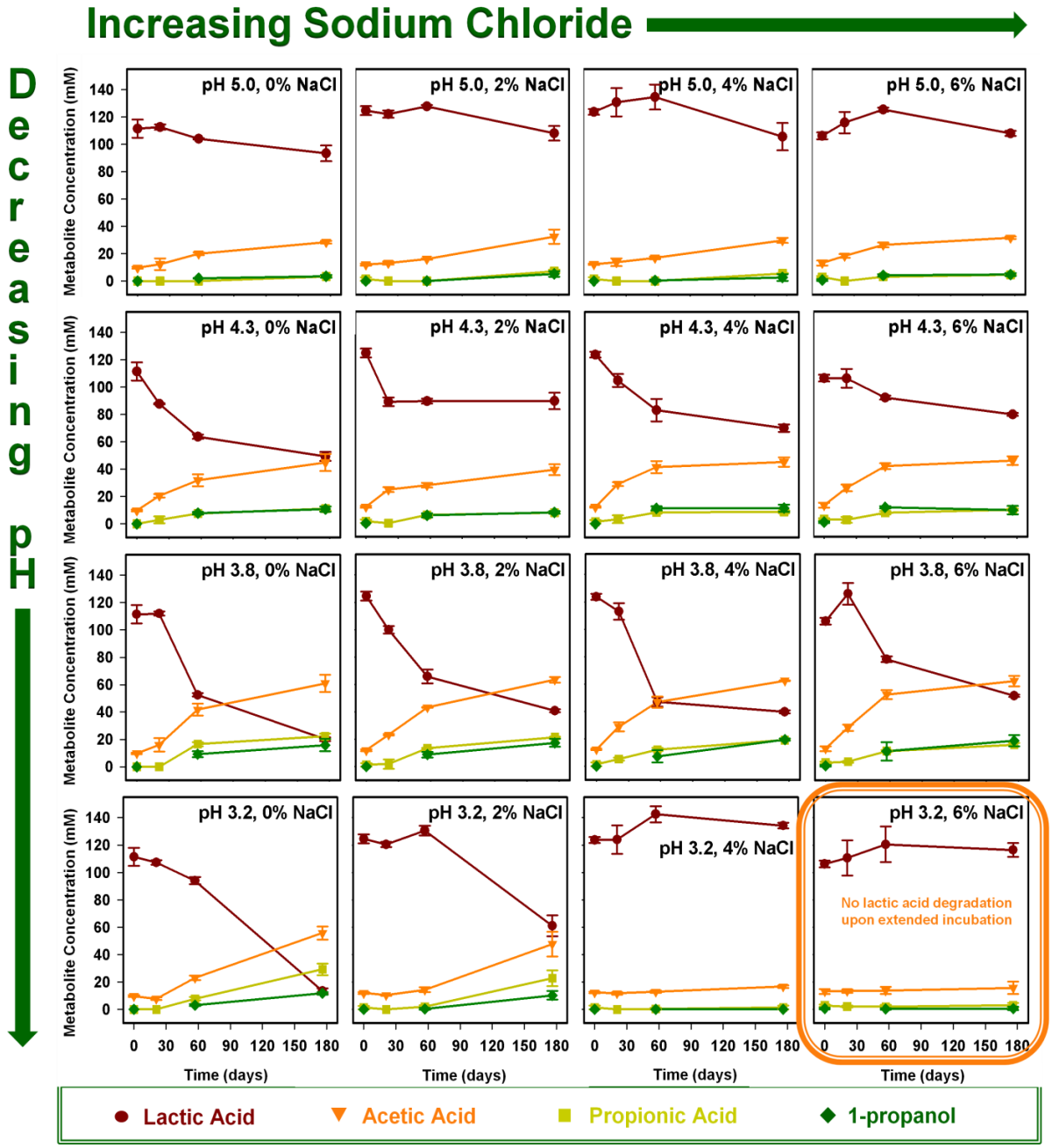

Figure 2.4 Effects of pH and NaCl concentration on anaerobic lactic acid utilization in fermented cucumber slurry by spoilage microorganisms from reduced NaCl cucumber fermentation

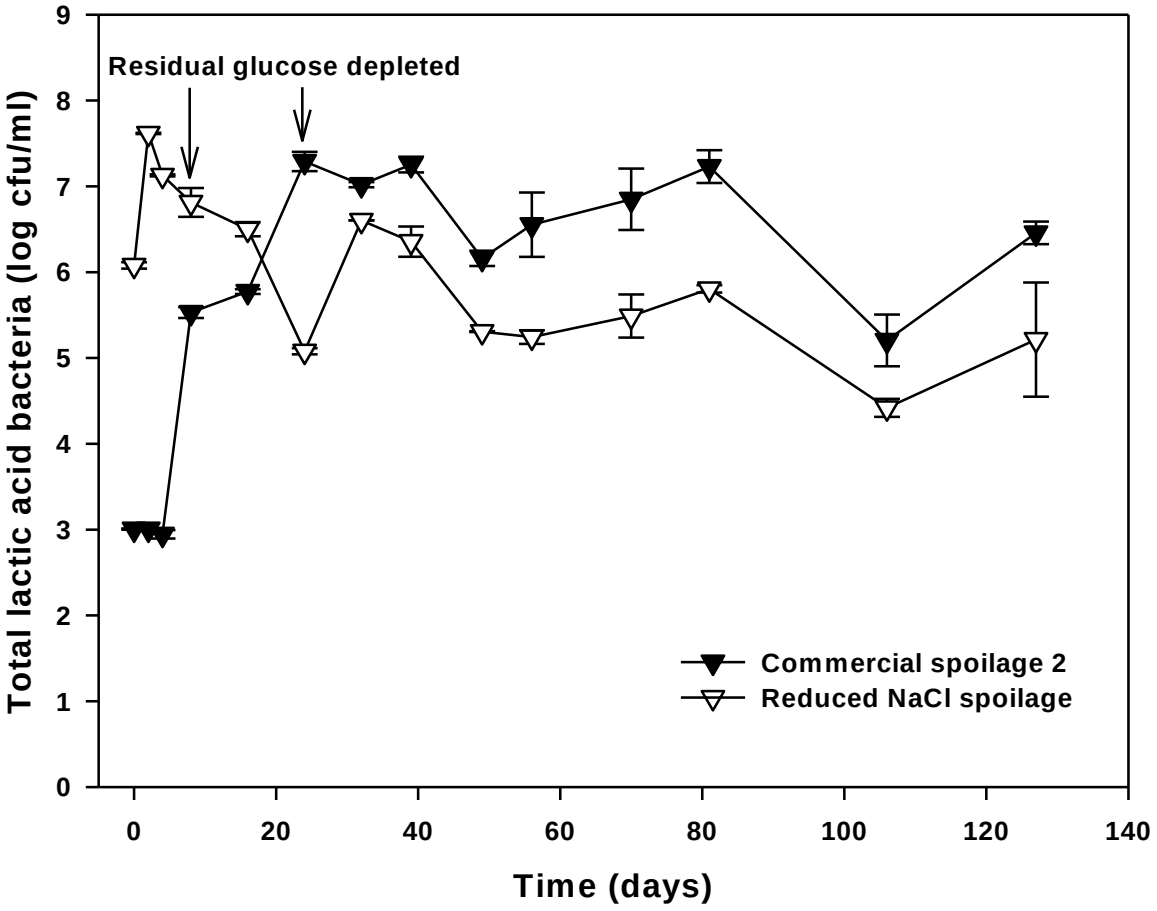

Figure 2.5 Presence of lactic acid bacteria during anaerobic spoilage of fermented cucumber slurry (6% NaCl, pH 3.8) inoculated with spoilage organisms from reduced NaCl and commercial spoilage 2 brines

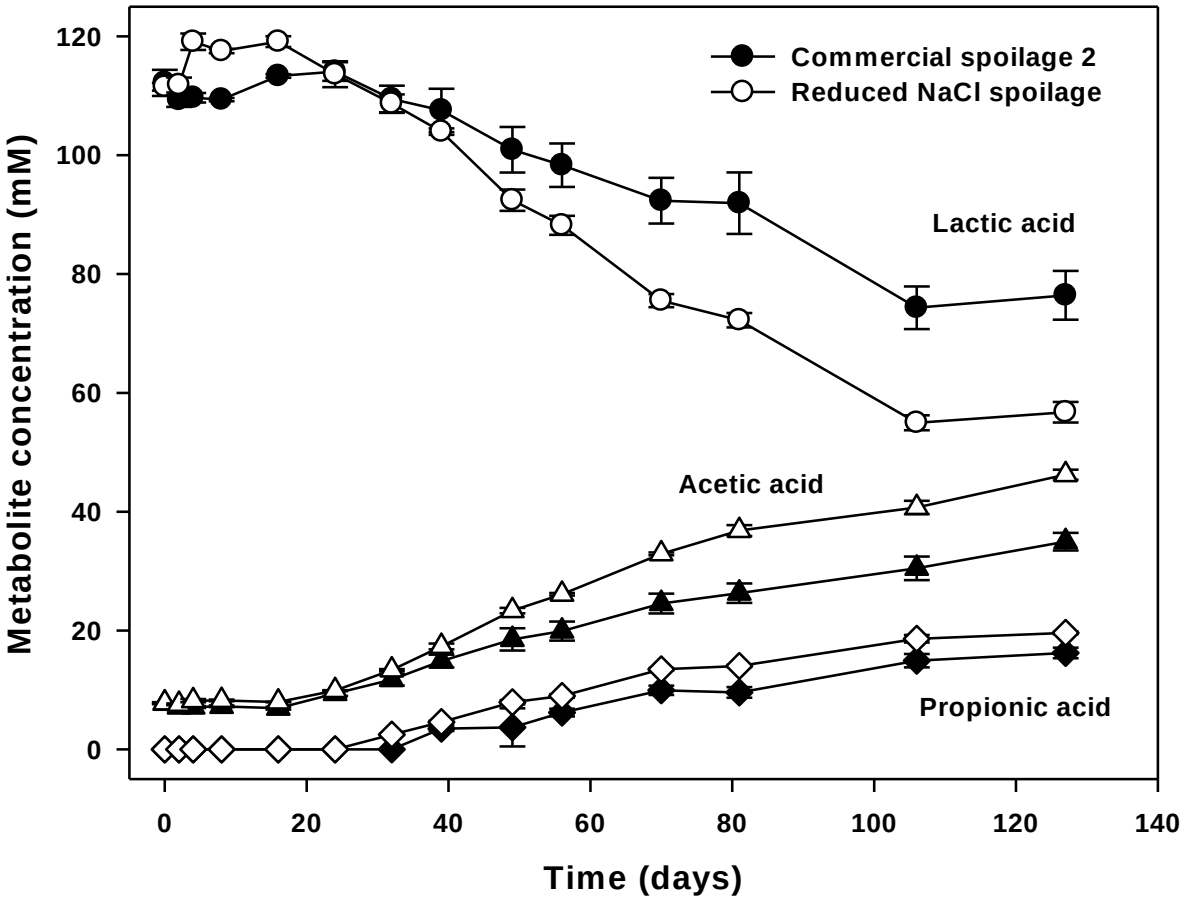

Figure 2.6 Metabolite changes during anaerobic spoilage of fermented cucumber slurry (6% NaCl, pH 3.8) inoculated with spoilage organisms from reduced NaCl and commercial spoilage 2 brines (Circles = lactic acid; triangles = acetic acid; and diamonds = propionic acid. Commercial spoilage represented by filled symbols. Reduced NaCl spoilage represented by open symbols.)

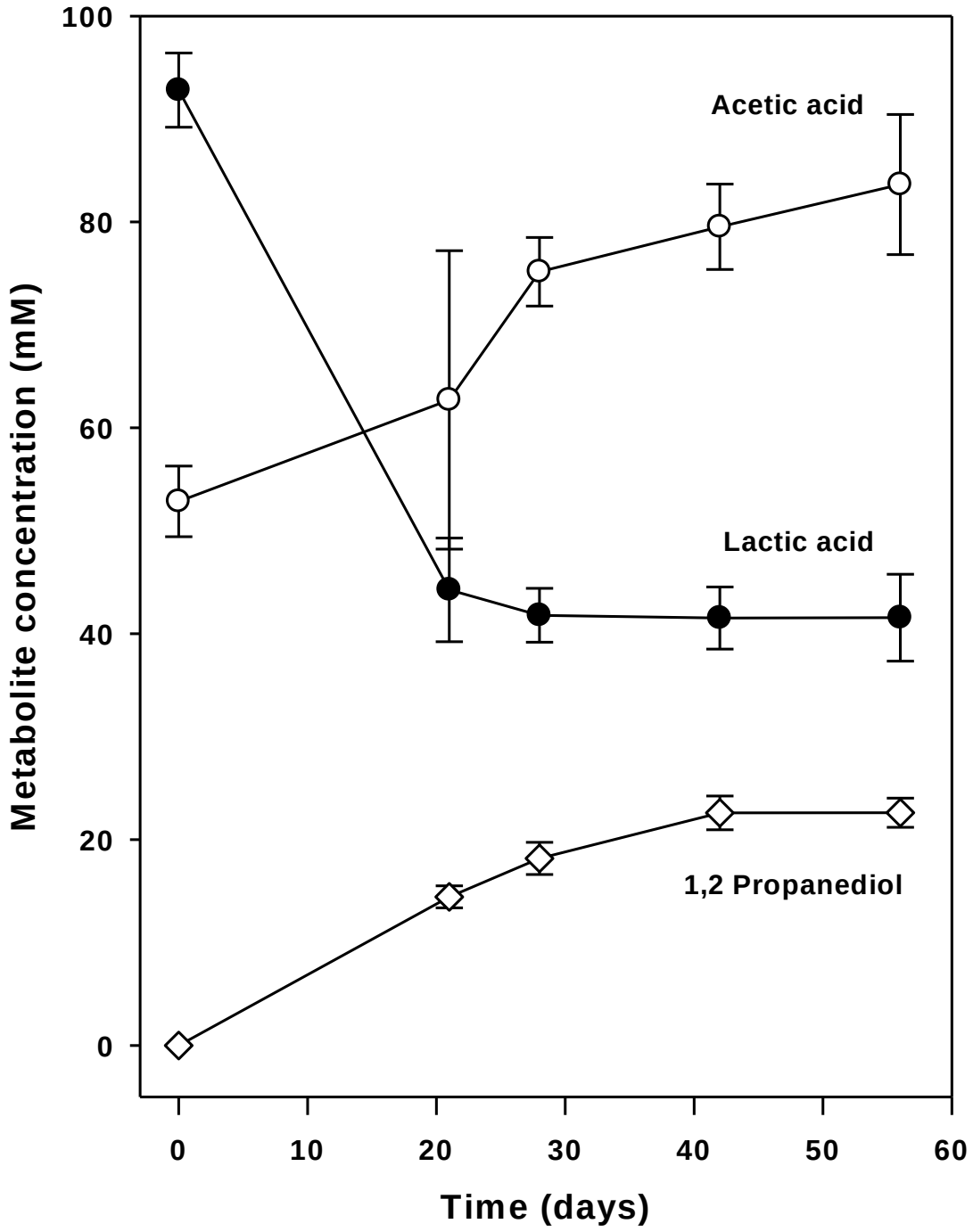

Figure 2.7 Anaerobic lactic acid utilization in fermented cucumber slurry (2% NaCl, pH 3.8) by a *Lactobacillus buchneri* strain isolated from spoiled fermented cucumbers

Chapter 3

Lactic acid utilization by *Lactobacillus buchneri*, a potential spoilage organism in fermented cucumbers

Abstract

Recent evidence suggests that *Lactobacillus buchneri* may play an important role in fermented cucumber spoilage. Environmental conditions such as pH, sodium chloride (NaCl) and presence of oxygen are known to influence the microbial stability of fermented cucumbers. Objectives were to evaluate these effects on lactic acid utilization by *L. buchneri*, and to compare the biochemical changes to those which occur during spoilage of fermented cucumbers. Effects of NaCl (0, 2, 4, and 6% w/w), pH (3.8 vs 5.0), and aerobic environment were investigated by appropriate NaCl and pH adjustments to fermented cucumber slurry (FCS), followed by filter-sterilization and inoculation with spoilage microorganisms. HPLC was used to measure expected substrates and products of metabolism. At an initial pH of 3.8, *L. buchneri* was able to degrade lactic acid in FCS with 0, 2, 4, and 6% NaCl. The highest rate and greatest extent of lactic acid utilization occurred in 2% NaCl FCS. Lactic acid utilization was nearly identical in aerobic and anaerobic atmospheres, indicating that oxygen does not influence lactate degradation by *L. buchneri*. Decreases in lactic acid were accompanied by increases in acetic acid and 1,2-propanediol as the primary end products. *L. rapi* isolated from fermented cucumber spoilage was able to convert 1,2-propanediol to propionic acid and propanol in FCS. *L. buchneri* was capable of initiating spoilage under a wide range of environmental conditions that may be present in commercial cucumber fermentations, and *L. rapi* may be acting syntrophically with *L. buchneri* to produce the spoilage metabolites that have been frequently observed.

Introduction

Recent evidence in the study of anaerobic fermented cucumber spoilage indicates the potential involvement of *Lactobacillus buchneri*. The chemical changes that have been observed during fermented cucumber spoilage (Fleming and others 1989; Fleming and others 2002; Kim and Breidt 2007) were similar to those demonstrated in silage inoculated with *L. buchneri* as a fermentation adjunct (Driehuis and others 1999). Furthermore, *L. buchneri* has been isolated repeatedly from fermented cucumbers that had undergone spoilage, resulting in decreased concentrations of lactic acid, increased pH, and increased concentrations of acetic and propionic acids (Johanningsmeier and others unpublished; Franco and others unpublished). Several other LAB were isolated from fermented cucumber spoilage, but only *L. buchneri* was demonstrated to initiate lactic acid utilization in fermented cucumber slurry (Johanningsmeier and others unpublished). Therefore, it was hypothesized that *L. buchneri* may play an important role in fermented cucumber spoilage.

In silage fermentation, lactic acid degradation by *L. buchneri* has been demonstrated to be a useful metabolic pathway that results in increased aerobic stability of the silage. Inoculation of silage with *L. buchneri* resulted in decreased lactic acid content and increased concentrations of acetic acid, propionic acid, and propanol (Driehuis and others 1999). The anaerobic degradation of lactic acid by *L. buchneri* in pure cultures in laboratory medium required induction by acidic conditions and did not require an external electron acceptor. Products of the degradation were acetic acid and 1,2-propanediol (Oude Elferink and others

2001). In contrast to other studies on lactate utilization by lactic acid bacteria (LAB) (Murphy and others 1985; Lindgren and others 1990; Bobillo and Marshall 1991; Viega-da-Cunha and Foster 1992), this particular metabolism in *L. buchneri* was not found to support cell growth. A new lactobacillus species, *L. dioliverans*, that was able to degrade 1,2-propanediol to propionic acid and propanol, was isolated from aerobically stable silages that had been inoculated with *L. buchneri* (Krooneman and others 2002). Accordingly, the use of *L. buchneri* to enhance silage preservation and the proposed metabolic pathway for lactic acid degradation has been reviewed (Holzer and others 2003). Subsequently, a combination of *L. buchneri* and *L. dioliverans* was shown to increase propionic acid concentrations during sourdough fermentation, resulting in increased antifungal activity in the bread products (Zhang and others 2010). It is not yet known if a similar syntrophic organism is working in concert with *L. buchneri* during fermented cucumber spoilage.

Environmental factors such as sodium chloride (NaCl) concentration and terminal pH have a significant impact on cucumber fermentation and microbial stability during storage in commercial tanks of up to 40,000 liter capacity. It has been shown that addition of hydrochloric acid to reduce the pH to 3.5 after fermentation can substantially slow the utilization of lactic acid in fermented cucumbers where the terminal pH was between 3.6 and 3.8 and NaCl concentration was 4.8 – 5.5% (Fleming and others 2002). However, at reduced NaCl concentrations, spoilage potential was demonstrated at pH 3.5 within three months of anaerobic incubation (Kim and Breidt 2007). Reduced salt fermented cucumbers

(0, 2, and 4% NaCl) with a terminal pH of 3.2 were also subject to spoilage under anaerobic conditions (Johanningsmeier and others unpublished). Kim and Breidt (2007) found that spoilage rate was increased at pH 5.0 and low NaCl (2% wt/vol) under anaerobic conditions and proposed using these conditions for studying spoilage in a model system. In contrast, Johanningsmeier and others (unpublished) found that the rate of lactic acid utilization was increased at pH 3.8 as compared to pH 5.0 in fermented cucumber slurry inoculated with spoilage microorganisms from one source, but exhibited similar behavior to the previously reported pH effect when inoculated with a spoilage culture that had been subcultured at pH 5.0 and 2% NaCl. Since most cucumber fermentations have a terminal pH between 3.2 and 3.8, pH 3.8 was chosen for investigating the ability of LAB isolated from spoiled cucumber fermentations to initiate lactic acid utilization in filter-sterilized fermented cucumber slurry. The individual effects of NaCl concentration and pH on anaerobic lactic acid utilization in fermented cucumbers by *L. buchneri* have not been studied.

Most investigations on lactic acid degradation in fermented cucumbers have been carried out in sealed fermentation jars or an anaerobic chamber due to evidence that spoilage can occur under anaerobic conditions (Fleming and others 1989). The translation of this research to commercial fermentation tanks where air purging is commonly used has been questioned, and the role that aerobic microorganisms may have in the initiation of spoilage is currently under investigation (Franco and others unpublished). A few species of LAB have been shown to metabolize lactic acid under either aerobic or anaerobic conditions

with a variety of co-factors and end products (Murphy and others 1985; Lindgren and others 1990; Bobillo and Marshall 1991; Veiga-Da-Cunha and Foster 1992; Oude Elferink and others 2001). However, the effect of an aerobic environment on the metabolism of lactic acid by *L. buchneri* has not been reported. The objectives of this study were to evaluate the effects of NaCl concentration, pH, and aerobic environment on lactic acid utilization by *L. buchneri*, and to compare the resulting biochemical changes to those observed during mixed culture spoilage of fermented cucumbers under these conditions.

Materials and Methods

Spoilage inocula sources. A *Lactobacillus buchneri* strain (Culture Collection ID LA1147, USDA-ARS Food Science Research Unit, Raleigh, NC) isolated from spoiled fermented cucumbers brined with 2% NaCl was tested for its ability to degrade lactic acid in fermented cucumber slurry (FCS) under a variety of environmental conditions. Brines containing a mixture of unidentified microorganisms from a reduced NaCl cucumber fermentation and a commercial cucumber fermentation that had undergone undesirable secondary fermentation were also used as spoilage inocula. The reduced NaCl spoilage source has been previously described (Johanningsmeier and McFeeters 2011). The commercial spoilage source was obtained from a commercial brining facility after it was observed that a tank of fermented cucumbers was undergoing post-fermentation spoilage. A complete description of these spoilage sources is available in Chapter 2.

Growth media preparation. Size 2B pickling cucumbers were obtained from a local processor, blended into slurry, and frozen at -10°C until needed. Fresh cucumber slurry (CS) was thawed, pressed through cheesecloth and centrifuged in 250-mL bottles at 23,400 x g for 15 min to remove particulate matter. After centrifugation, NaCl, yeast extract, peptone and water were added to yield final concentrations of 67% fresh cucumber, 4% NaCl, 1% yeast extract, and 1% peptone. The resulting modified cucumber slurry (mCS) growth media was sterile-filtered with a 0.2 µm bottle top filter (Nalgene FAST PES, 0.2 µm pore size, 90-mm-diameter membrane, Daigger, Vernon Hills, IL) and stored at 4°C until use.

Fermented cucumber media preparation. Size 2B cucumbers (32-38 mm in diameter) were washed, packed into 3.84-L glass jars, and covered with brine (55:45 cucumber:brine ratio) to equilibrate at 0.25% (w/w) calcium chloride ($CaCl_2$) and 0%, 2%, 4% and 6% (w/w) sodium chloride (NaCl). Brined cucumbers were inoculated with 10^6 CFU/g *Lactobacillus plantarum* MOP3 starter culture (Culture Collection ID LA0219, USDA-ARS Food Science Research Unit, Raleigh, NC). Jars were closed with lids that were heated in boiling water to soften the plastisol liner, and a rubber septum was inserted into the lid of each jar to allow sampling of the brine with a syringe. Three replicate jars were packed for each NaCl concentration and stored at ambient temperature (21-25^{o}C) for 11 mo. Fermentation progressed normally in all jars as indicated by decreases in pH and changes in organic acids as measured by high-performance liquid chromatography (HPLC) with ultraviolet light (UV) detection (McFeeters and Barish 2003). Sugars and alcohols were quantified in the same analysis using a

refractive index detector connected in series. Fermented cucumbers were cut into pieces and blended into slurry to prepare sterile, fermented cucumber slurry (FCS) as a medium for inoculation with spoilage microorganisms. The FCS was pressed through cheesecloth and centrifuged in 250-mL bottles at 23,400 x g for 15 min to remove particulate matter. The pH of the clarified slurry was raised from 3.2 to 3.8 by addition of 6 N NaOH to increase the likelihood that spoilage would occur (Fleming and others 2002; Kim and Breidt 2007). The pH-adjusted, clarified FCS was sterile-filtered with a Nalgene FAST PES 0.2 µm pore size, 90-mm-diameter membrane, bottle-top filter apparatus (Daigger, Vernon Hills, IL). Five mL of sterile-filtered FCS was then aseptically transferred into sterile 15-mL conical tubes. The loosely capped tubes were placed into an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) for 3 d prior to inoculation to remove dissolved oxygen from the media.

Effect of pH. Fermented cucumber slurry (2% NaCl) as described above was adjusted to 6% NaCl and pH 3.8 or 5.0 prior to sterile filtration. Twelve mL of sterile-filtered FCS was then aseptically transferred into sterile 15-mL conical tubes. The loosely capped tubes were placed into an anaerobic chamber for 3 d prior to inoculation with 10^6 cfu/ml *L. buchneri* strain LA1147. Samples were aseptically withdrawn at several times during anaerobic incubation at ambient temperature (~25°C). Lactic acid degradation was measured by HPLC.

Effect of aerobic environment. Reduced NaCl and commercial spoiled fermentation brines (1 mL each) were inoculated into 9 mL mCS and incubated anaerobically at 30°C for 2

weeks. *L. buchneri* strain LA 1147 was streaked onto de Man, Rogosa, Sharpe (MRS) agar and incubated anaerobically at 30°C for 4 days. Three isolated colonies were transferred to 9 mL mCS and incubated anaerobically at 30°C for 2 weeks. One milliliter of each of the three resulting spoilage cultures was transferred to 25 mL mCS and incubated anaerobically at 30°C for 6 days to prepare inocula for reproduction of spoilage in FCS. Spoilage inocula were centrifuged to pellet cells and the spent growth media was discarded. Cells were washed with 5 mL FCS, pelleted, and then resuspended in 15 mL FCS. Spoilage inocula were serially diluted and spiral plated onto MRS to quantify LAB. Conical centrifuge tubes containing 5 mL sterile-filtered FCS were inoculated in triplicate with 0.5 mL of each spoilage culture and incubated anaerobically at ambient temperature along with triplicate noninoculated FCS controls. A duplicate set of treatments was incubated under static aerobic conditions at 25°C. Samples were aseptically withdrawn at 9, 22, 51, 77, 96, and 146 days of incubation and stored at -80°C until analysis.

Effect of NaCl. Cucumbers were fermented without NaCl in the presence of 50 mM acetic acid, 25 mM $CaCl_2$, and 10^6 cfu/ml *L. plantarum* starter culture (Fleming and others 1995). Cucumbers were washed but not blanched prior to packing. Fermentation proceeded normally as determined by HPLC analysis of acids and sugars. Fermented cucumbers were blended with equal parts brine and stored frozen until needed. Slurry was processed as described above. After centrifugation, NaCl was added to individual aliquots of FCS to give NaCl concentrations of 0, 2, 4, and 6% (w/w). The pH was then adjusted to pH 3.8 and the

solutions sterile-filtered prior to inoculation with 10^6 cfu/mL *L. buchneri* and spoilage cultures in the anaerobic chamber at ambient temperature. For the three replicates in this experiment, growth of the inocula and inoculation of FCS was conducted on three separate days. Samples were aseptically withdrawn after 2, 4, 6, 8, 12, and 22 weeks anaerobic incubation and stored at -80°C until analysis.

Utilization of 1,2-propanediol by spoilage organisms. FCS was prepared as previously described, adjusted to 6% NaCl and pH 3.8, and supplemented with 30 mM 1,2-propanediol (Sigma-Aldrich, St. Louis, MO) prior to sterile-filtration. Sterile 1,2-propanediol supplemented FCS was dispensed into sterile conical centrifuge tubes and placed in the anaerobic chamber for 3 days prior to inoculation with 10^6 cfu/ml spoilage organisms. Spoilage organisms from reduced NaCl and commercial sources were grown as described above. Lactic acid bacteria spoilage isolates, *L. buchneri* strain LA1147, *Pediococcus ethanolidurans* strain LA1139, *Pediococcus parvulus* strain LA1140, *Lactobacillus rapi* strains LA1169 and LA1165 , *Lactobacillus parafarraginis* strain LA1153, and *Lactobacillus harbinensi/perolens* strain LA1162, (Culture Collection of the USDA-ARS Food Science Research Unit, Raleigh, NC) were grown anaerobically on MRS agar at 30°C Three isolated colonies were transferred to MRS broth and incubated anaerobically at 30°C for 5 days. An aliquot of each culture was transferred 1:10 into mCS broth and incubated anaerobically at 30°C for 5 days. Cultures were centrifuged to pellet cells and the spent growth media was discarded. Cells were washed with 1,2-propanediol-FCS, pelleted, and then resuspended in

1,2-propanediol-FCS. Tubes of 1,2-propanediol-FCS were inoculated in triplicate in the anaerobic chamber and incubated at ambient temperature (~25°C). Samples were aseptically withdrawn after 8, 24, 50, and 93 days and stored at -80°C until analysis.

High Performance Liquid Chromatography (HPLC). HPLC quantification of glucose, fructose, glycerol, ethanol, propanol, 1,2-propanediol, malic, succinic, lactic, acetic, propionic, and butyric acids was used to measure anaerobic lactic acid degradation and formation of possible spoilage metabolites. HPLC analysis was conducted with minor modification of the method published by McFeeters and Barish (2003). Briefly, components of samples were separated on an Aminex HPX-87H resin column (300 x 7.8 mm, Bio-Rad Laboratories, Hercules, CA) with 0.03 N H_2SO_4 eluent at a flow rate of 0.6 mL/min. The column temperature was held at 37°C to separate propionic acid, a potential spoilage metabolite, from an unknown component that frequently occurs in fermented cucumbers. Sugars and alcohols were quantified in the same analysis using a refractive index detector connected in series.

pH measurements. Measurement of pH was done at ambient temperature with an Accumet AR25 pH meter (Catalog #13-636-AR25A) equipped with a gel-filled combination pH electrode (Catalog #13-620-290, Fisher Scientific, Pittsburgh, PA) that was calibrated with certified standards of pH 2.00, 4.00, and 7.00 (Fisher Scientific).

Results and Discussion

Lactic acid utilization by *Lactobacillus buchneri* in fermented cucumber slurry (FCS) occurred under a variety of environmental conditions. At an initial pH of 3.8, *L. buchneri* was able to degrade lactic acid in FCS with all four NaCl concentrations tested (0, 2, 4, and 6%) and under both aerobic and anaerobic atmospheres. In all cases, decreases in lactic acid concentration were accompanied by increases in acetic acid and 1,2-propanediol as the primary end products with small increases in ethanol (Table 3.1). Across multiple independent replications and varying NaCl concentrations, we found that 1 mole of lactic acid was anaerobically metabolized by *L. buchneri* in fermented cucumber slurry to produce approximately 0.5 mole acetic acid, 0.37 mole 1,2-propanediol, and 0.13 mole ethanol. These products are consistent with the pathway proposed by Oude Elferink and others (2001). However, the ratio of end products differed somewhat in FCS from that reported in microbiological media. We found a slightly greater proportion of ethanol produced in FCS than in the aforementioned study where it was reported that 1 mole of lactic acid was converted to 0.48 mole acetic acid, 0.48 mole 1,2-propanediol, and 0.04 mole ethanol.

No lactic acid degradation was observed in FCS with 6% NaCl when the initial pH was 5.0 (Figure 3.1). Oude Elferink and others (2001) have shown that the metabolism of lactic acid to acetic acid and 1,2-propanediol by *L. buchneri* in laboratory media required acid induction. They observed that lactic acid was degraded upon incubation in MRS when cells were grown in chemostat cultures at pH 3.8, 4.0, and 4.3, but not at 5.8. Similar behavior

observed in FCS suggests that the low pH that is typical of fermented cucumbers may be favorable for induction of the lactic acid degrading activity of *L. buchneri*. Further research to determine the lower pH limit for lactic acid utilization by *L. buchneri* is needed.

There was a significant effect of NaCl concentration on lactic acid utilization by *L. buchneri* ($P < 0.05$). Although lactic acid was degraded by *L. buchneri* in FCS with 0, 2, 4, and 6% NaCl, the rate of lactic acid utilization was slowest in 6% NaCl (Figure 3.2). More lactic acid was used in 0 and 4% NaCl FCS, and the highest rate and greatest extent of lactic acid utilization occurred in 2% NaCl FCS. This may explain the increased susceptibility to spoilage of cucumbers fermented with reduced NaCl concentrations that was observed in pilot scale (4,000 liter) closed tank fermentations (Fleming and others 1989; Fleming and others 2002) and model systems (Kim and Breidt 2007; Johanningsmeier and others unpublished).

The rate of lactic acid utilization was nearly identical in both aerobic and anaerobic atmospheres (Figure 3.3), indicating that oxygen is not required, nor does it influence the lactate degrading ability of *L. buchneri*. Therefore, both air purged and anaerobic cucumber fermentations would be equally susceptible to spoilage by *L. buchneri.* Studies of lactate utilization by *L. plantarum* have shown differences in the rate of lactate utilization (Bobillo and Marshall 1991) and end products formed (Lindgren and others 1990) in the presence of oxygen. Oude-Elferink and others (2001) proposed that the anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol did not require external electron acceptors. We

have observed that there is also no enhancement of this reaction when oxygen is present, and that 1,2-propanediol is still formed.

Under anaerobic conditions, the rates of lactic acid degradation by *L. buchneri* and spoilage cultures from reduced NaCl and commercial sources were similar, and both D- and L- forms of lactic acid were used simultaneously (Figure 3.4). Although the end products were different, the rise in pH that was observed during anaerobic lactic acid utilization in fermented cucumbers was strongly correlated with the decrease in lactic acid concentration for both mixed spoilage cultures (R^2 = 0.912) and pure cultures of *L. buchneri* (R^2 = 0.975) (Figure 3.5). The pathway proposed for lactic acid degradation by *L. buchneri* includes decarboxylation of pyruvate and formation of acetyl-CoA by a pyruvate dehydrogenase complex (Oude-Elferink and others 2001). The reaction mechanism for this enzyme includes the uptake of one hydrogen ion per molecule of pyruvate that is decarboxylated (Combs 1998), which may contribute to the observed deacidification. This reaction and the production of less acidic end products would explain the rise in pH that was correlated with lactic acid utilization.

The major difference in lactic acid degradation between *L. buchneri* and the mixed spoilage cultures was the formation of 1,2-propanediol by *L. buchneri* and the production of propionic acid by the latter (Table 3.1). This difference in end products indicates that *L. buchneri* cannot be solely responsible for the fermented cucumber spoilage that has been observed. Krooneman and others (2002) showed that *L. dioliverans* isolated from silage

prepared with *L. buchneri* as a fermentation adjunct was able to metabolize 1,2-propanediol to propionic acid and propanol. Therefore, FCS (6% NaCl, pH 3.8) supplemented with 1,2-propanediol was used to test individual LAB isolates and spoilage cultures from two different sources for the ability to metabolize 1,2-propanediol to propionic acid and propanol. *L. buchneri* exhibited the same behavior in the presence of additional 1,2-propanediol as in FCS, resulting in a decrease in lactic acid and increases in acetic acid and 1,2-propanediol. Two pediococci that have been observed in fermented cucumber spoilage, but do not use lactic acid in FCS were evaluated for their ability to be syntrophic with *L. buchneri* in the production of the components found with mixed culture spoilage. Neither *Pediococcus ethanolidurans* nor *Pediococcus parvulus* used 1,2-propanediol that was added to FCS (Figure 3.6) or that which was naturally produced in co-culture with *L. buchneri* (data not shown). However, both reduced NaCl and commercial spoilage cultures contained organisms that were able to utilize 1,2-propanediol in FCS with 6% NaCl at pH 3.8 under anaerobic conditions (Figure 3.6). Decreases in 1,2-propanediol were observed as early as 8 days after inoculation, and added 1,2-propanediol was not detected after 24 days. Utilization of 1,2-propanediol by these unidentified spoilage microorganisms preceded lactic acid utilization, which may explain why this intermediate has not been detected in fermented cucumber spoilage. Either 1,2-propanediol is a preferred substrate or the organism(s) that use(s) 1,2-propanediol cannot metabolize lactic acid. Two strains of *Lactobacillus rapi*, newly isolated LAB from fermented cucumber spoilage, were able to

degrade 1,2-propanediol in FCS when inoculated in pure culture (Figure 3.6). Consistent with the mixed spoilage cultures, decreases in 1,2-propanediol accompanied increases in propionic acid and propanol (Table 3.2). Another newly isolated LAB, *Lactobacillus parafarraginis* behaved similarly to *L. buchneri* in 1,2-propanediol-FCS. Within 24 days of anaerobic incubation, a significant decrease in lactic acid with concurrent increases in acetic acid and 1,2-propanediol and rise in pH were observed (Table 3.2). More research is needed to determine the prevalence of this lactic acid degrading bacterium in fermented cucumbers. Based on these results, we hypothesize that *L. buchneri* or genetically related LAB such as *L. parafarraginis* can initiate lactic acid utilization in fermented cucumbers resulting in the production of acetic acid and 1,2-propanediol. The 1,2-propanediol is used by one or more syntrophic organisms, including *L. rapi*, at a rate that does not allow accumulation of 1,2-propanediol, resulting in propionic acid and propanol as the spoilage metabolites in fermented cucumber brine. Furthermore, the utilization of lactic acid by *L. buchneri* and *L. parafarraginis* results in a rise in pH that may allow other propionic and butyric acid producing organisms to contribute to spoilage of fermented cucumbers.

Conclusions

Lactic acid utilization by *L. buchneri* occurred under a variety of environmental conditions that may be encountered in commercial cucumber fermentations. At an initial pH of 3.8, *L. buchneri* was able to degrade lactic acid in FCS under both aerobic and anaerobic

atmospheres and in the presence of 0-6% sodium chloride. Regardless of environmental conditions, decreases in lactic acid concentration accompanied increases in acetic acid and 1,2-propanediol and a rise in pH. *L. rapi* and unidentified spoilage organisms were able to convert 1,2-propanediol to propionic acid and propanol, suggesting that one or more organisms work in concert with *L. buchneri* to produce the spoilage metabolites that have been observed in fermented cucumber brines.

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Table 3.1: Chemical changes in fermented cucumber slurry with varying NaCl concentrations after 84 days anaerobic incubation with *L. buchneri*

Treatment	NaCl (%)	pH	Lactic acid (mM)	Acetic acid (mM)	1,2-propanediol (mM)	Ethanol (mM)	Carbon Balance[1] (%)
Control	0	3.86 ± 0.01	112.07 ± 3.33	72.37 ± 1.77	ND	18.68 ± 2.89	NA
L. buchneri	0	4.32 ± 0.07	67.05 ± 7.62	96.55 ± 4.04	17.48 ± 2.73	23.65 ± 2.53	104.16 ± 8.75
Control	2	3.85 ± 0.02	112.82 ± 1.06	72.86 ± 0.53	ND	19.18 ± 4.03	NA
L. buchneri	2	4.60 ± 0.14	39.48 ± 9.52	106.02 ± 5.15	24.72 ± 3.38	31.09 ± 3.14	94.56 ± 6.14
Control	4	3.86 ± 0.02	110.45 ± 1.35	72.21 ± 0.35	ND	18.22 ± 4.12	NA
L. buchneri	4	4.35± 0.05	60.67 ±3.34	97.13 ± 2.68	18.39 ± 1.46	25.26 ± 2.90	100.63 ± 10.61
Control	6	3.86 ± 0.01	110.88 ± 0.84	72.22 ± 0.48	ND	18.69 ± 3.47	NA
L. buchneri	6	4.14 ± 0.07	83.17 ±7.50	86.55 ± 3.81	10.76 ± 2.65	19.52 ± 2.41	92.42 ± 11.51

[1] Carbon balance was calculated for each replicate as the molar sum of the products (increases in acetic acid, 1,2-propanediol, and ethanol) divided by the decrease in molar concentration of lactic acid substrate and multiplied by 100 to be expressed as a percent. It was assumed that for each mole of lactic acid converted to acetic acid, 1 mole of carbon dioxide was formed (CO_2 not quantified).

Table 3.2: Chemical changes in fermented cucumber slurry (6% NaCl, pH 3.8) after 77 days incubation with spoilage cultures

Environment	Treatment	Lactic acid (mM)	Acetic acid (mM)	Propionic acid (mM)	Glucose (mM)	1,2-propanediol (mM)
Aerobic	Control	115.53 ± 2.02	7.78 ± 0.16	ND	3.14 ± 0.23	ND
	L. buchneri	72.86 ± 3.72	42.82 ± 3.77	ND	ND	14.79 ± 1.57
	Commercial spoilage	48.91 ± 1.01	14.15 ± 12.26	12.21 ± 8.12	ND	ND
	Reduced NaCl spoilage	51.39 ± 4.01	68.93 ±3.04	16.69 ± 1.09	ND	ND
Anaerobic	Control	117.81 ± 1.20	7.90 ± 0.16	ND	3.34 ± 0.08	ND
	L. buchneri	70.22 ± 7.99	38.00 ± 3.46	ND	ND	20.30 ± 3.85
	Commercial spoilage	67.01 ± 3.39	40.61 ± 0.65	16.13 ± 0.25	ND	ND
	Reduced NaCl spoilage	74.32 ± 6.44	37.89 ± 3.43	17.09 ± 2.79	ND	ND

Table 3.3: Chemical changes in fermented cucumber slurry (6% NaCl, pH 3.8) supplemented with 1,2 propanediol after 93 days anaerobic incubation with spoilage cultures and lactic acid bacteria

Treatment	**pH**	**1,2-propanediol (mM)**	**Lactic acid (mM)**	**Acetic acid (mM)**	**Propionic acid (mM)**	**n-Propanol (mM)**
Control	3.77 ± 0.01	25.63 ± 0.15	113.20 ± 1.22	70.43 ± 0.44	ND	ND
L. buchneri	4.04 ± 0.01	37.17 ± 0.63	80.65 ±3.65	87.31 ± 1.54	ND	ND
P. ethanolidurans	3.75 ± 0.01	25.47 ± 0.26	112.16 ± 0.70	69.30 ± 0.34	ND	ND
P. parvulus	3.76 ± 0.01	25.32 ± 0.36	112.72 ± 0.80	69.42 ± 0.59	ND	ND
Commercial spoilage	3.77 ± 0.01	ND	110.96 ± 0.35	71.11 ± 0.14	16.93 ± 0.96	12.91 ± 0.61
Reduced NaCl spoilage	3.89 ± 0.01	ND	99.73 ± 0.84	78.52 ± 1.17	15.60 ± 0.51	17.88 ± 0.44
L. rapi strain LA1169[1]	3.82 ± 0.01	16.91 ± 1.31	109.48 ± 0.26	76.17 ± 0.62	ND	1.31 ± 0.07
L. rapi strain LA1165[1]	3.81 ± 0.01	7.30 ± 2.22	108.36 ± 1.32	75.82 ± 0.91	8.07 ± 0.69	4.71 ± 0.74
L. parafarraginis strain LA1153[1]	4.00 ± 0.08	34.34 ± 4.06	85.16 ± 8.35	88.45 ± 5.21	ND	ND

[1] Anaerobic incubation in 1,2-propanediol supplemented FCS (6% NaCl, pH 3.8) for 24 days

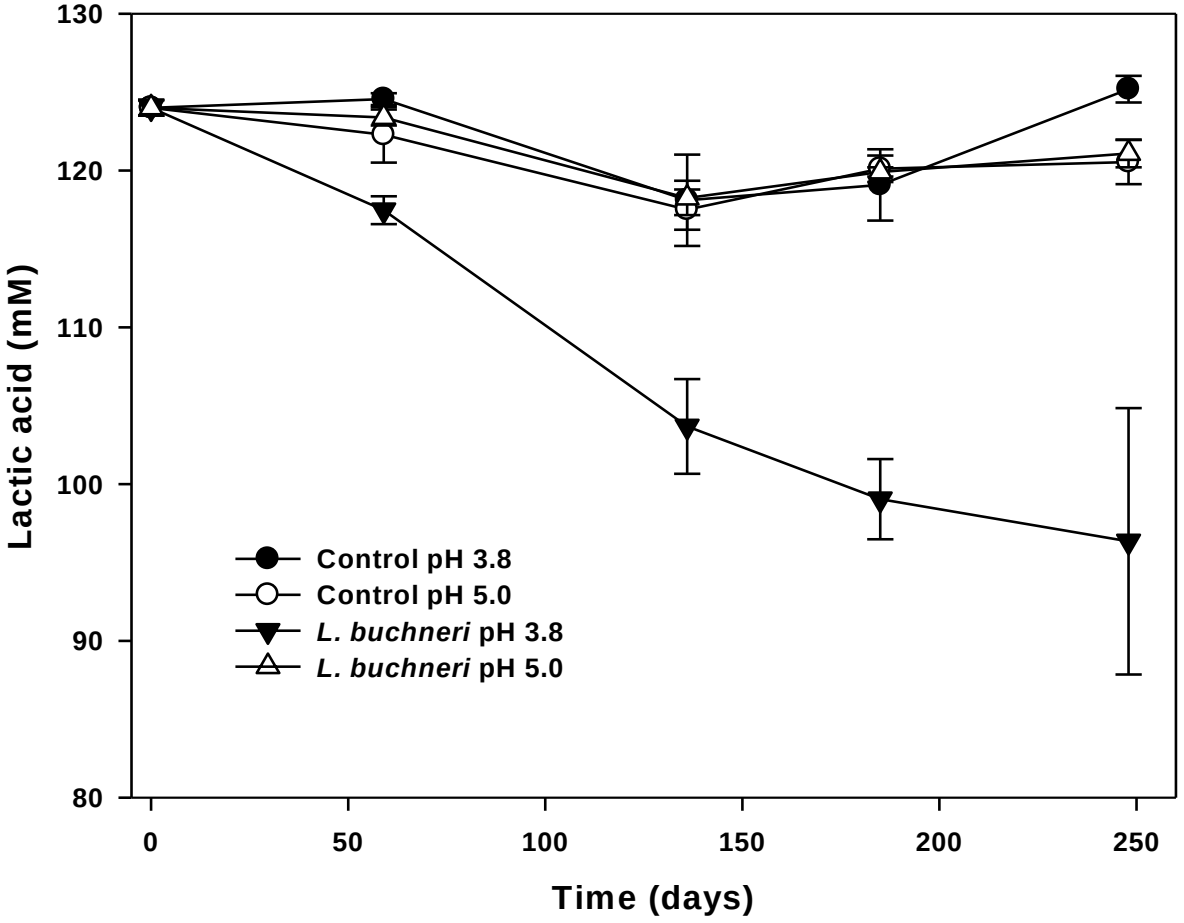

Figure 3.1: Effect of initial pH on lactic acid utilization by *L. buchneri* in fermented cucumber slurry (6% NaCl). Control samples were not inoculated.

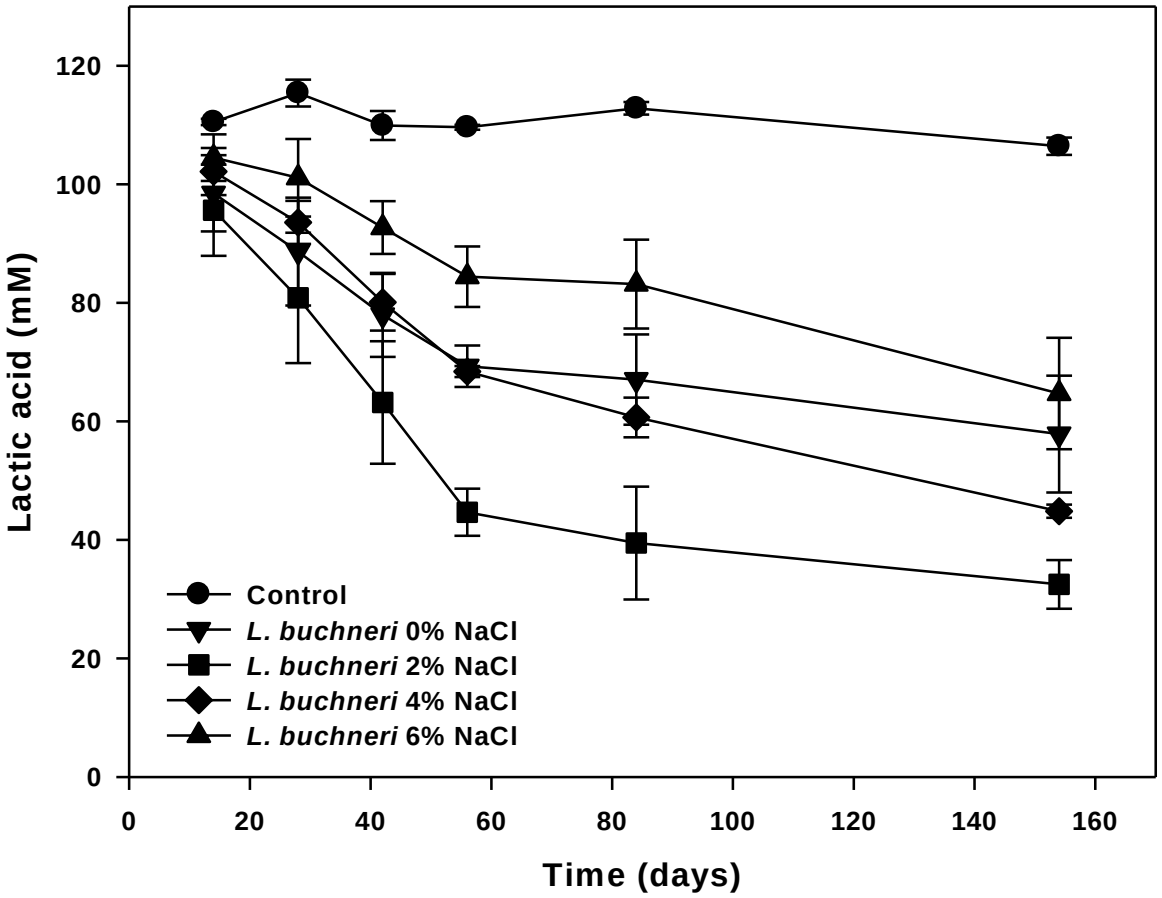

Figure 3.2: Lactic acid utilization by *L. buchneri* in fermented cucumber slurry (pH 3.8) at four NaCl concentrations. Control samples were not inoculated.

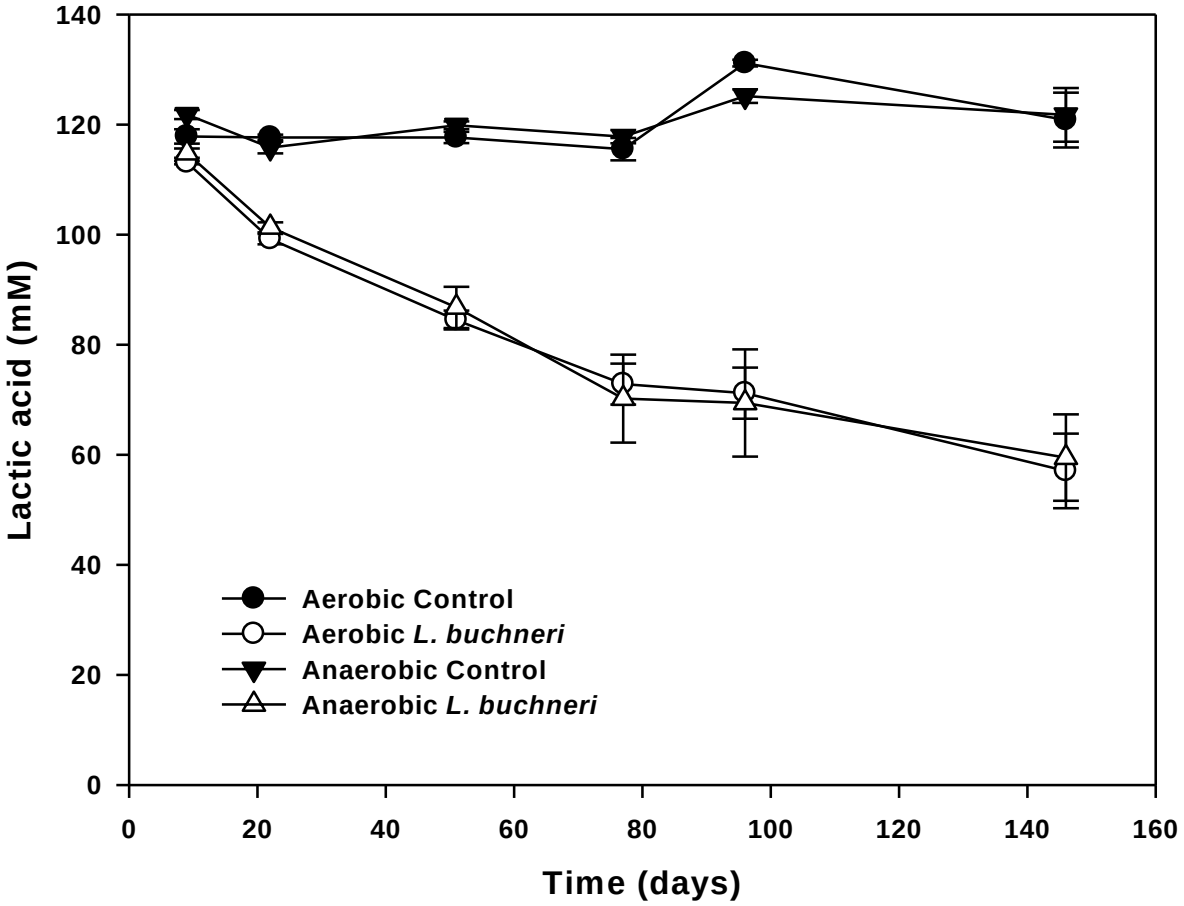

Figure 3.3: Aerobic and anaerobic utilization of lactic acid by *L. buchneri* in fermented cucumber slurry (6% NaCl, pH 3.8). Control samples were not inoculated.

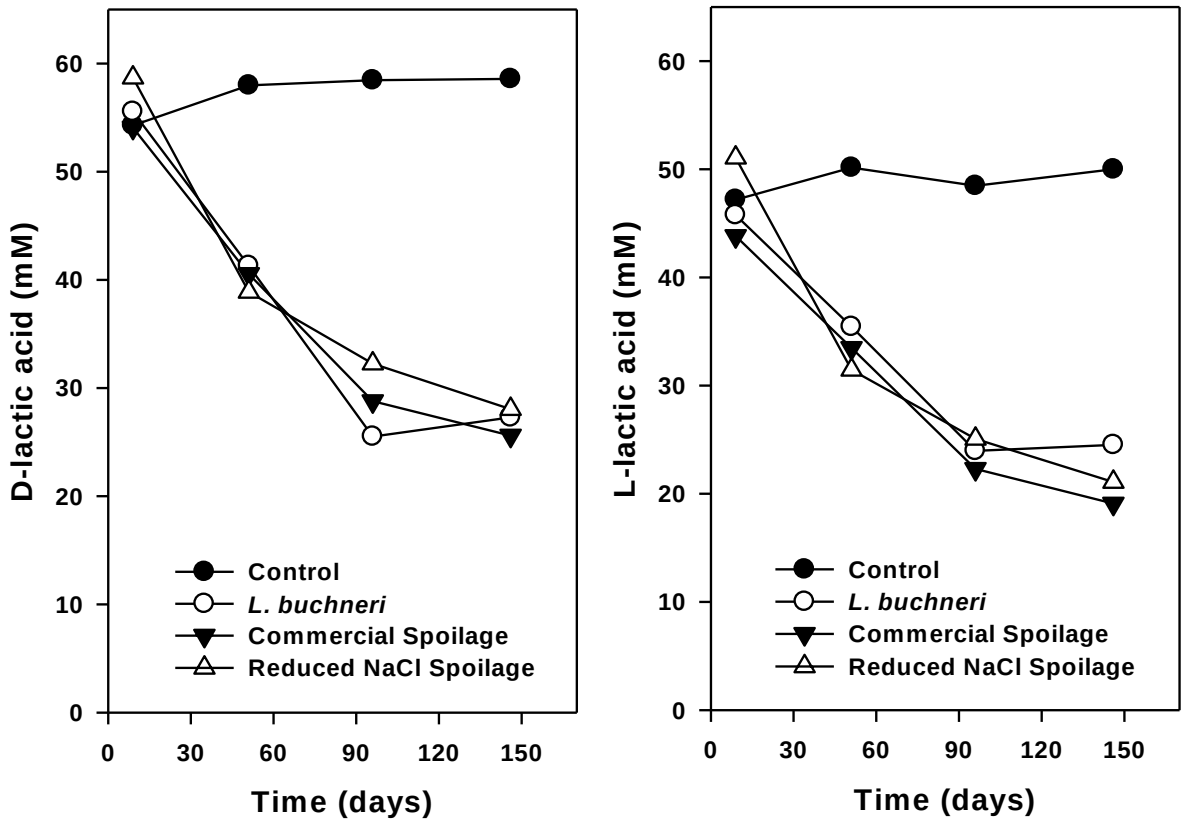

Figure 3.4: Utilization of D- and L-lactic acid in fermented cucumber slurry (6% NaCl, pH 3.8) by *L. buchneri* and spoilage cultures. Control samples were not inoculated.

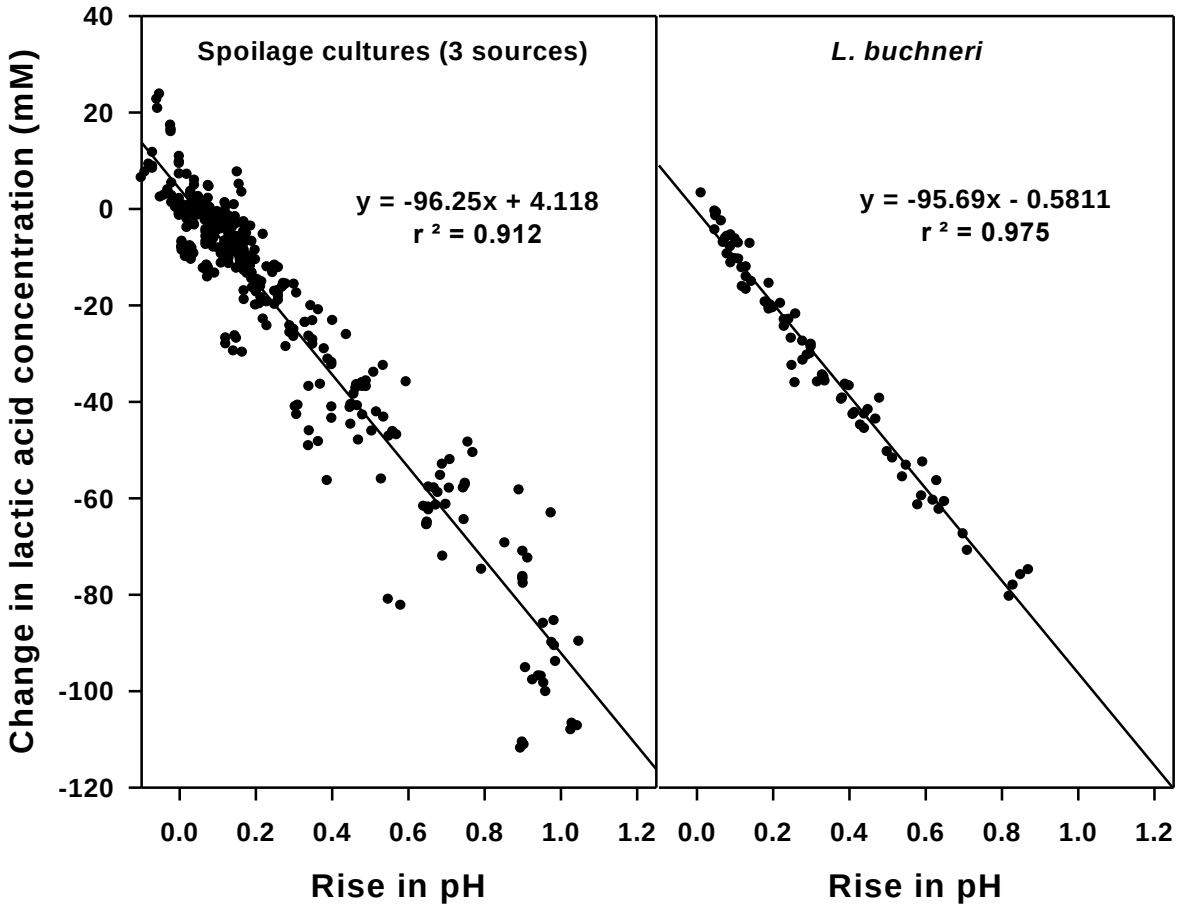

Figure 3.5: Rise in pH correlated with anaerobic lactic acid utilization in fermented cucumber slurries (initial pH 3.1 - 3.8) regardless of NaCl concentration (0 - 6%) by spoilage cultures from three sources (n = 366) and *L. buchneri* (n = 77).

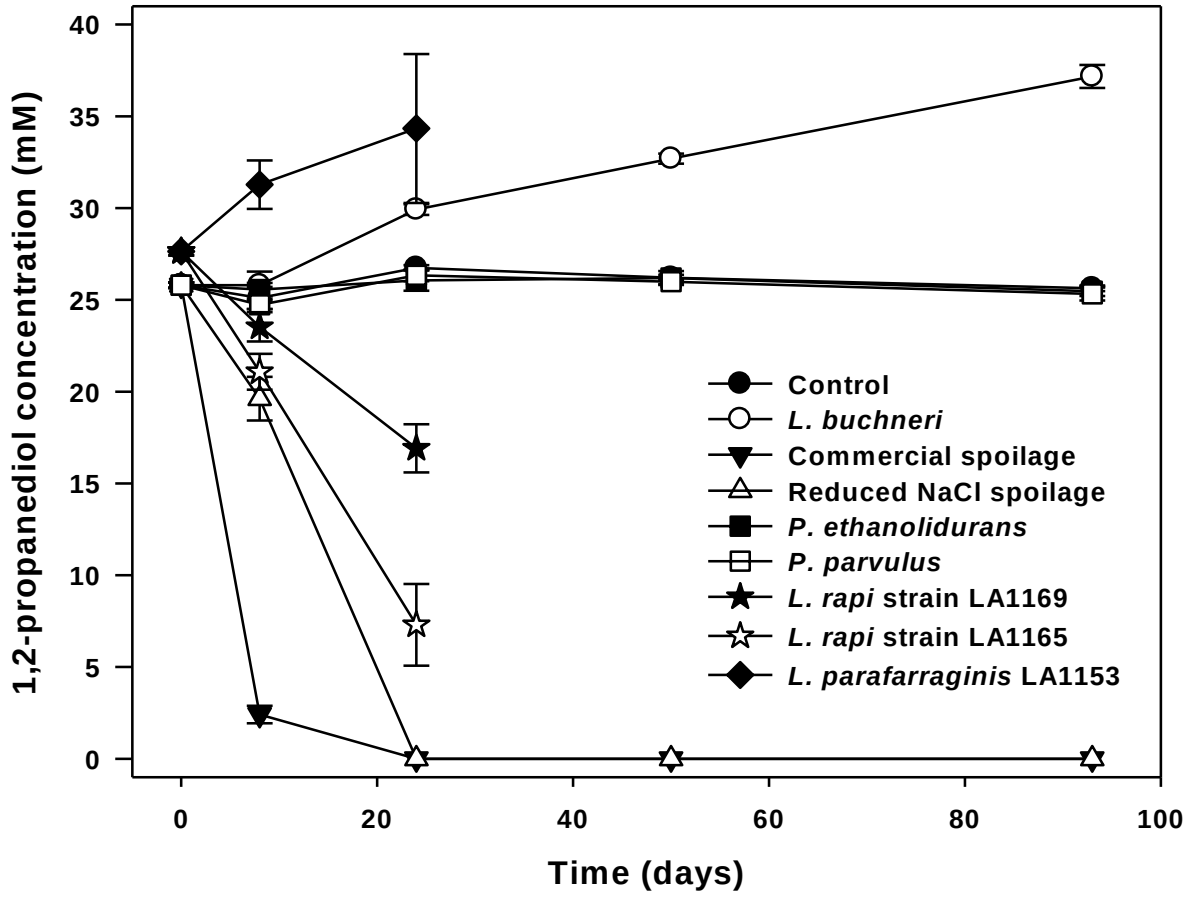

Figure 3.6: Utilization of 1,2-propanediol in fermented cucumber slurry (6% NaCl, pH 3.8) by spoilage cultures and *Lactobacillus rapi*. Control samples were not inoculated.

Chapter 4

Detection of volatile spoilage metabolites in fermented cucumbers using nontargeted comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS)

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Abstract

A nontargeted, comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS) method was developed for the analysis of fermented cucumber volatiles before and after anaerobic spoilage. Volatiles extracted by solid-phase microextraction were separated on a polyethylene glycol first-dimension column and 14% cyanopropylphenyl second-dimension column. Among 314 components detected in fermented cucumber brine, 199 had peak areas with coefficients of variation below 30%. Peak identifications established by mass spectral library matching were 92% accurate based on 63 authentic standards. Analysis of variance of analytes' log peak areas revealed 33 metabolites changed in concentration after spoilage ($P < 0.05$), including increases in acetic, propanoic, and butyric acids, n-propyl acetate, several alcohols, and a decrease in furfural. GCxGC-ToFMS with a nontargeted, semi-automated approach to data analysis made possible the separation, identification, and determination of differences in polar volatile components, facilitating the discovery of several metabolites related to fermented cucumber spoilage.

Keywords: GC x GC-TOFMS, Two-dimensional gas chromatography, fermented cucumber volatiles, metabolites, nontargeted data analysis, comprehensive 2D GC-MS

Introduction

Fermentation and storage in bulk tanks is used to preserve cucumbers for extended periods of time. The fermented cucumbers are then converted into a variety of processed pickle products, most notably hamburger dill chips. Fresh cucumbers of various sizes are typically brined in sodium chloride (NaCl) solutions so that the equilibrated concentration of NaCl is between 5-8% (w/w). This concentration of salt inhibits softening enzymes (Bell and Etchells 1961) and favors the growth of the naturally occurring lactic acid bacteria (Etchells and Jones 1943). Cucumber fruits contain approximately 2-3% fermentable sugars (Lu and others 2002), which are metabolized by lactic acid bacteria to predominantly lactic acid, thereby reducing the pH and the readily available energy sources for microbial growth. The combination of salt, acid pH, and lack of sugars results in a naturally preserved product that can typically be held for many months prior to final processing into pickle products.

One disadvantage of this fermentation process is the high concentration of NaCl in the waste stream. Efforts to reduce the NaCl used in fermentation and storage of cucumbers have resulted in the increased incidence of fermented cucumber spoilage. This spoilage has been characterized by an initial normal lactic acid fermentation followed by a gradual rise in pH and decrease in lactic acid concentration (Fleming and others 1989, 2002; Kim and Breidt 2007). The production of volatile compounds and increased pH compromise the quality of the product, often necessitating early processing of the tank or discarding the product if spoilage proceeds. If the pH rises above 4.6, clostridial spoilage may occur

(Fleming and others 1989), so the possibility of germination and growth of *Clostridium botulinum* spores cannot be ruled out. The currently unpredictable nature of this spoilage contributes to increased production costs for the pickling industry, mainly in the form of increased monitoring of fermentation tanks. In cases where the pH has risen beyond control, product losses and increased waste disposal costs are also incurred.

Given the potential diversity of chemical components in a food fermentation system, a discovery-based approach may provide new insight into the changes in volatile compounds that occur due to microbiological spoilage after the normal fermentation process has been completed. Advances in gas chromatography – mass spectrometry (GC-MS) systems and data collection capability provide the potential to carry out separations of volatile chemical components using 2 different separation mechanisms by connecting columns with different bonded phases in series. The benefits and challenges associated with this technology have been the subject of recent reviews (Marriott and Shellie 2002; Adahchour and others 2008; Mondello and others 2008; Cortes and others 2009). This comprehensive two-dimensional gas chromatography (GCxGC) methodology has been applied to the analysis of volatiles in a number of complex food matrices, including roasted coffee beans, butter, essential oils, grapes, roast beef, sugarcane spirits, honey, pepper, roasted barley, hazelnuts, olive oil, potato chips, basil, and Chinese liquor. Compared to chromatography with a single column, two-dimensional chromatography resulted in resolution of more components and in improved mass spectral matches when a time-of-

flight MS detector was used for the analysis of butter volatiles and grape monoterpenoids (Adahchour and others 2005; Rocha and others 2007). Analysis of lavender essential oil using an orthogonal 2D separation consisting of a nonpolar 1st dimension column followed by a polar 2nd dimension column resulted in a 25-fold increase in sensitivity and a 3-fold increase in the number of resolved components as compared to traditional GC analysis (Shellie and others 2001). Orthogonal 2D separation of Cheddar cheese volatiles using a comprehensive, two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS) showed that separation in the second dimension was necessary to resolve octane from hexanal and ethyl lactate from 3-octanol (Gogus and others 2006). Several other studies have reported separation of volatile compounds from complex food matrices with a nonpolar 1st dimension column followed by a polar 2nd dimension column (Cardeal and others 2006, 2008; Čajka and others 2007; Eyres and others 2007; Rocha and others 2007; Rochat and others 2007; Klimánková and others 2008; Cardeal and Marriott 2009; de Souza and others 2009; Lojzova and others 2009; Torres Vaz-Freire and others 2009). However, the reverse column combination as well as nonorthogonal polar-semipolar column combinations have also been demonstrated as viable alternatives for separation of volatile compounds in foods (Ryan and others 2004; Mondello and others 2004; Adahchour and others 2004, 2005; Bianchi and others 2007; Zhu and others 2007; Cordero and others 2008). Although the orthogonal, nonpolar-polar column combination was suitable for separating coffee bean volatiles, the reversed column combination showed a comparable

structured order of the components and yielded a volatile compound profile that utilized more of the available separation space (Ryan and others 2004). Adahchour and others (2004) found that improved peak shapes and retention behavior for acids and alcohols were obtained on a polar-semipolar column combination. In addition, a useful pattern of separation for homologous series of compounds with different functional groups was obtained (Adahchour and others 2004; Cordero and others 2008). Therefore, this 'reverse-type' GCxGC separation may have advantages for some analyses of food volatiles.

While GCxGC-ToFMS offers greatly increased capability for separating and detecting volatile components present in complex samples, the data sets generated are large and cumbersome. In metabolite profiling studies, target compounds are unknown and the goal is to identify a set of metabolites associated with a particular treatment or phenotype (also known as biological markers) among the hundreds to thousands of metabolites detected. The size and complexity of these types of data sets requires automation of the data analysis process. This study describes a nontargeted, comprehensive GCxGC-ToFMS method for separating and identifying volatile compounds in fermented cucumbers and detecting changes in volatile metabolites occurring as a result of fermented cucumber spoilage.

Materials and Methods

Cucumber fermentation. Size 2B cucumbers (32-38 mm in diameter) were washed, packed into three 3.84-L glass jars, and covered with brine (55:45 cucumber:brine ratio) containing

calcium chloride ($CaCl_2$) and sodium chloride (NaCl) so that the equilibrated concentrations were 0.25% and 6% (w/w), respectively. Brined cucumbers were inoculated with 10^6 CFU/g *Lactobacillus plantarum* MOP3 starter culture (Culture Collection ID LA0219, USDA-ARS Food Science Research Unit, Raleigh, NC). Jars were closed with lids that were heated in boiling water to soften the plastisol liner, and a rubber septum was inserted into the lid of each jar to allow sampling of the brine with a syringe. The jars were stored at ambient temperature (21-25^{o}C) for 11 mo. Fermentation progressed normally in all 3 jars as indicated by decreases in pH and changes in organic acids as measured by high-performance liquid chromatography (HPLC) with ultraviolet light (UV) detection (McFeeters and Barish 2003). Sugars and alcohols were quantified in the same analysis using a refractive index detector connected in series. Seven replicate samples of fermented cucumber brine from a single fermentation jar were analyzed in random order among 12 other fermented cucumber brine samples over the course of a 3-d run of the instrument to assess the analytical reproducibility for the nontargeted analysis of volatile components. Volatile compounds were also analyzed in triplicate for brine samples from the other 2 replicate fermentation jars.

Media preparation. Fermented cucumbers as described above were cut into pieces and blended into a slurry to prepare sterile, fermented cucumber slurry (FCS) as a medium for inoculation with spoilage microorganisms. The FCS was pressed through cheesecloth and centrifuged in 250-mL bottles at 23,400 g for 15 min to remove particulate matter. The pH

of the clarified slurry was raised from 3.1 to 3.8 by addition of 6 N NaOH to increase the rate at which spoilage occurred (Fleming and others 2002; Kim and Breidt 2007). The pH-adjusted, clarified FCS was sterile-filtered with a Nalgene FAST PES 0.2 µm pore size, 90-mm-diameter membrane, bottle-top filter apparatus (Daigger, Vernon Hills, IL). Twelve mL of sterile-filtered FCS was then aseptically transferred into sterile 15-mL conical tubes. The loosely capped tubes were placed into an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) for 3 days prior to inoculation to remove dissolved oxygen from the media.

Spoilage inoculum source. Brine from a laboratory cucumber fermentation that had undergone an undesirable secondary fermentation was used as spoilage inoculum. Two 5-gallon plastic pails with tightly fitting lids were packed with 9.5 kg size 2B cucumbers (38-44 mm in diameter) and covered with an equal volume of brine. One cover brine contained 4% NaCl, 36 mM $CaCl_2$, and 50 mM acetic acid from 20% vinegar to equilibrate at 2% NaCl, 18 mM $CaCl_2$, and 25 mM acetic acid during the fermentation. The second cover brine contained NaCl, $CaCl_2$, KCl, $MgCl_2 \cdot 6H_2O$, and acetic acid to equilibrate at 1.2% NaCl, 0.8% KCl, 30 mM $CaCl_2$, 20 mM $MgCl_2$, and 25 mM acetic acid (McFeeters and Fleming 1997). The pails were inoculated with *L. plantarum* starter culture and fermented normally as indicated by a decrease in pH to 3.2 and typical utilization of sugars and production of lactic acid determined by HPLC one month after initiating the fermentations. However, when the fermentations were sampled after 11 mo storage at ambient temperature, it was noted that

the lactic acid had decreased substantially and the pH had risen to 4.1 (Table 4.1), which is typical of the anaerobic cucumber spoilage described by Fleming and others (1989, 2002) and Kim and Breidt (2007). Brine from the spoiled fermented cucumbers (2% NaCl) was used as the inoculum to reproduce spoilage in filter-sterilized FCS (6% NaCl, pH 3.8). Additionally, solid-phase microextraction (SPME) GCxGC-ToFMS was carried out on frozen aliquots of these 1- and 11-mo brine samples that were thawed, diluted, and randomized for run order prior to analysis. Changes in volatile metabolites that occurred during spoilage were determined by comparison of the volatile profile of brine samples taken from the pails after the primary fermentation (1 mo storage) and after the lactic acid had decreased (11 mo storage).

Reproduction of spoilage. Conical centrifuge tubes containing 12 mL sterile-filtered FCS were inoculated in triplicate with 1 mL of spoilage brine and incubated anaerobically at ambient temperature along with triplicate noninoculated FCS controls. Samples were taken immediately after inoculation and after 3 wk, 2 mo, and 6 mo of incubation and stored at -80°C until analysis. A significant decrease in lactic acid concentration, as measured by HPLC, was used to indicate the appropriate samples to use for analysis of changes in the volatile components that occurred upon spoilage (Table 4.1). SPME-GCxGC-ToFMS was carried out on initial and 6-mo samples that were thawed, diluted and randomized for run order. Components that changed during anaerobic incubation of noninoculated FCS were presumed to have been formed as a result of chemical changes that occurred during the

extended incubation period and were excluded from the group of compounds that changed as a result of microbial spoilage.

Solid-phase microextraction (SPME) of volatile components. Fermented cucumber brines or spoilage samples (200 uL) were diluted 1:5 with deionized water (796 uL) and acidified with 3 N H_2SO_4 (4 uL) in 10 mL screw-cap headspace vials (Microliter Analytical Supplies, Inc., Suwanee, Ga., U.S.A.). Sodium chloride (NaCl, 0.40 g) was added to "salt out" volatile components from the samples. Spoilage samples were also analyzed at a 1:250 dilution to account for volatile components present in amounts that resulted in column overloading at the 1:5 dilution. Samples were randomized for analysis order (PROC PLAN, version 9.1.3 SAS® software, SAS Institute, Cary, NC) and placed into a refrigerated sample tray (2 °C). Automated sampling was performed using a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies, Carrboro, NC). Headspace vials containing the diluted samples were agitated at 500 rpm (5 s on and 2 s off) for 15 min at 40 °C prior to extraction. Volatile compounds were collected by insertion of a 1-cm, 50/30 µm DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco, Bellefonte, Pa., U.S.A.) into the headspace above the sample for 30 min at 40°C with 100 rpm agitation (5 s on and 2 s off). Extracted volatile compounds were desorbed from the SPME fiber into the GC inlet at 250°C for 15 min. A blank sample (1.0 mL deionized water containing 6 mM sulfuric acid and 0.4 g NaCl) was run between each fermented cucumber sample to reduce carry-over of components on the SPME fiber.

Comprehensive, two - dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS). A LECO® Pegasus III® two - dimensional gas chromatograph (GCxGC) time-of-flight mass spectrometer (TOFMS) instrument (Model# 614-100-700, Leco Corporation, St. Joseph, MI) included an Agilent GC (Model# 6890N, Agilent Technologies, Santa Clara, CA) fitted with a secondary oven and cryogenic modulator. The two - dimensional separation was achieved using a SolGel-Wax™, 30 m x 0.25 mm ID x 0.25 µm film thickness (SGE, Austin, TX), polyethylene glycol 1st dimension column in the primary oven and an RTX 17-01, 1.0 m x 0.1 mm ID x 0.1 µm film thickness (Restek, Bellefonte, PA), 14% cyanopropylphenyl – 86% dimethyl polysiloxane 2nd dimension column in the secondary oven. Columns were conditioned according to manufacturer recommendations prior to use. A 0.75-mm-ID Siltek deactivated SPME liner (Restek, Bellefonte, PA.) was used in the inlet. It was set at 250°C and operated in pulsed splitless mode with a pulse pressure of 37 psi for 1 min. The split vent was opened 2 min following injection, and the GC was operated in constant flow mode with 1.3 mL /min helium carrier gas. The primary oven temperature was maintained at 40°C for 2 min and then increased at 5°C/min to 140°C. The temperature ramp was then increased to 10 °C/min to 250°C and the temperature was held at 250°C for 3 min. The secondary oven followed the same temperature program except the temperature was maintained at 10°C higher than the main oven until the temperature reached a maximum of 250°C in the secondary oven. The transfer line temperature was maintained at 250 °C. The modulator offset was +30°C with a 1.5 sec 2nd dimension

separation time and 0.3sec hot pulse. Compressed air (35 psi) was used for the hot pulses, and liquid nitrogen-cooled nitrogen gas (18 psi) was used for the cold pulses.

The mass spectrometer was operated with -70eV and an ion source temperature of 200°C. The detector voltage was set at 1500 V and masses 25-500 were collected at 200 spectra per second. No solvent delay was employed.

Data processing and analysis. Data analysis involved a series of steps that made use of the instrument software, ChromaTOF® version 3.25 (Leco Corporation, St. Joseph, MI) for data processing, Excel® 2003 (Microsoft Corporation, Redmond, WA) for data compilation, and SAS® version 9.1.3 (SAS Institute, Cary, NC) for statistical analysis. At the time of data acquisition, user fields were created in the ChromaTOF® acquisition menu to include information that uniquely identified each sample injected as to treatment type, replicate number, time of sampling, and so on. This information was then accessible in the peak tables for every peak associated with that sample. Inclusion of this information at the acquisition step was a key element contributing to efficiency in subsequent review and statistical analysis of the peak table data.

ChromaTOF® software data processing methods were used to detect and quantify peaks based on unique masses as determined by the deconvolution algorithm. Data processing parameters are shown in Table 4.2. A library search of the NIST/EPA/NIH Mass Spectra Library (National Institute of Standards and Technology (NIST), Gaithersburg, MD, 2005) was utilized for tentative identification of deconvoluted chromatographic peaks.

Chemical names were assigned to peaks that had a minimum mass spectral similarity ≥ 800 (1000 is an exact match). The unique mass (U) for each peak, as assigned by the ChromaTOF® deconvolution algorithm, was used for peak area calculations. All samples were processed in comparison to a single run of a composite brine sample. The composite sample for each experiment was prepared by mixing equal volumes of samples from each treatment of the experiment. Therefore, the composite sample peak table should theoretically contain most components that are present in the experimental samples. In ChromaTOF®, a reference table was created using the composite sample peak table as a standard. Criteria for the reference table were set as detailed in Table 4.2, and peak tables for each sample were standardized against this reference using the compare function in the ChromaTOF® data processing method. The resulting standardized peak tables containing each peak associated with a quantification name and peak area, based on the respective unique mass, were copied into an Excel® spreadsheet for further analysis. Creation of a reference in ChromaTOF® was necessary to standardize the name assignment for a given peak (including unknowns which were named unknown 1, unknown 2, and so on) and to allow standardized quantification of the peak area with the same specific unique mass for each component in all chromatograms of an experiment. Even in replicate chromatograms of brine from a single sample, the ChromaTOF® algorithm may select different unique masses for quantification of the same analyte, resulting in the inability to make comparisons of peak areas among chromatograms for a given component. This

inconsistency is beyond the control of the instrument operator and has been noted by other researchers (O'Hagan and others 2007). In addition to stipulating a single mass per analyte for peak area quantification, employing the reference chromatogram for standardizing peak tables had the advantage of assigning the same unknown number to the matching components in all chromatograms. Therefore, it was possible to do peak area comparisons of unidentified metabolites that without standardization would have been variably numbered depending on the number of unknowns detected in each chromatogram.

Peaks not found in a sample chromatogram that were included in the reference table resulted in blank cells for the peak area value of that analyte. These missing values represented the absence of a component within the detection limits of the analytical method, referred to as left - censored data, and needed to be replaced prior to statistical analysis to avoid the loss of fundamental information. Substitution of left - censored data with a random number between zero and the detection limit has been shown to be an adequate statistical alternative in environmental data analysis where observations below the instrumental detection limit constituted less than 70% of the data (Antweiler and Taylor 2008). To obtain an estimate of the experiment-wide detection limit, the minimum reported peak area from all chromatograms within an experiment was located. For example, from the fermented cucumber spoilage experiment, this area was 196. Therefore, blank peak area cells for undetected analytes in the dataset were replaced with a random

number between 1 and 195 (<196) to provide substitution data that reflected possible responses below the method's detection limit for undetected components.

Peak areas of volatile components ranged from 196 to $>10^8$ in magnitude and peak area variability within replicate analyses increased as peak area increased. Since the standard deviation of peak areas was generally found to be proportional to the mean peak areas, log transformation was used to homogenize the variances prior to analysis of variance (ANOVA) (Steel and Torrie 1980). An ANOVA of log peak areas by quantification name was conducted to detect differences in volatile compounds among treatments (version 9.1.3 SAS® software, SAS Institute, Cary, NC). Significance was established at $P < 0.05$ after adjustment of p-values to control the false discovery rate using the method of Benjamini and Hochberg (1995).

Reference compounds. With the exception of the following, all chemicals were obtained from Sigma-Aldrich in their purest available form (Sigma-Aldrich, St. Louis, MO). Pentane, methyl propionate, 3-methyl-2-butanone, benzene, methyl isobutyl ketone, 3-penten-2-ol, 1-pentanol, and 3-hydroxy-2-butanone were acquired from Fluka (Sigma-Aldrich, St. Louis, MO). 3-octanol was sourced from Alfa Aesar (Ward Hill, MS), 3-pentanol was obtained from Riedel-de-Haen (Seelze, Germany), and 4-methyl-2-heptanol was purchased from ChemSampCo (Trenton, NJ).

Results and Discussion

Volatile components in fermented cucumbers. Approximately 477 peaks with S/N ≥ 250 were detected in the brine of cucumbers fermented with 6% NaCl (Figure 4.1). Of these, 314 peaks were attributed to the fermented cucumber brine based on manual inspection of the chromatograms and peak table data for brine samples compared to water blank chromatograms. The 163 artifact peaks included siloxanes, other system contaminants, and column bleed at the higher end of the temperature program. Fortunately, with the polar-semipolar column combination, these artifacts were well resolved from sample volatile components (Figure 4.1), making it possible to detect low- level volatile metabolites in the midst of system contaminants. The presence of contaminant compounds is not unusual and often creates a mass spectral background that can interfere with identification and quantification of sample analytes in one-dimensional GC chromatograms.

Of the 314 sample peaks detected in fermented cucumber brine, 214 (68%) were tentatively identified by ChromaTOF® data processing based on the best spectral match to the NIST05 library with similarity ≥ 800. To evaluate the quality of these tentative identifications, authentic standards of 63 compounds were individually chromatographed. The 63 test compounds were chosen from throughout the chromatographic run subject to commercial availability. Based upon retention time and mass spectral matches with components detected in the fermented cucumber brine samples, the best library match was a correct identification in 58 of the 63 cases (92%). The incorrect identification of acetic

acid was most likely due to column overload, which has been demonstrated to create problems with the ChromaTOF® deconvolution algorithm (Lisec and others 2006). Although it was incorrectly identified, the overloaded acetic acid peak would have interfered with detection of at least 3 other components in the 1st dimension. These components were clearly resolved in the 2nd dimension, enabling their detection and identification (Figure 4.1).

Among the 314 volatile components in fermented cucumber brine, 199 had <30% relative standard deviation (RSD) in their peak areas for 7 replicate analyses randomized among 12 other fermented cucumber brine samples over the course of a 3-d run of the instrument (Figure 4.2). This volatile compound profile was representative of fermented cucumbers in all 3 of the 6% NaCl fermentations analyzed. The 199 reproducibly detected volatile components in fermented cucumber brines included 40 unknowns and 159 tentatively identified compounds. Further manual inspection of the data and comparison with retention indices reported in the literature resulted in the identification of 137 volatile compounds in fermented cucumber brine (Table 4.3). Comparison of these metabolites with previously reported volatile compounds in fermented cucumber brines (Zhou and McFeeters 1998; Marsili and Miller 2000) indicated that this method may be more sensitive in the detection of plant terpenoids, esters, alcohols, highly volatile aldehydes, and light hydrocarbons as greater numbers of these compounds were found in the present study. Several volatile compounds previously identified in fermented cucumber brines using one-

dimensional GC-MS on nonpolar columns were also found in this study, including butanol, pentanol, hexanol, octanol, 2-pentanol, 2-heptanol, 2-ethyl-1-hexanol, eucalyptol, α-terpineol, phenylethyl alcohol, hexanal, nonanal, 2-heptenal, benzaldehyde, acetone, acetic acid, butanoic acid, hexanoic acid, linalool oxide, 2-methoxy-3-(1-methylethyl) pyrazine, and dimethyl disulfide (Zhou and McFeeters 1998; Marsili and Miller 2000).

Nontargeted detection of volatile metabolites associated with spoilage fermentations. Fermented cucumber slurries before and after spoilage with a mixed culture inoculum obtained from spoiled fermented cucumbers were subjected to the GCxGC-ToFMS analysis described. ANOVA of analyte log peak areas revealed 33 metabolites that changed significantly ($P < 0.05$) in concentration after spoilage (Table 4.4). The nontargeted data analysis approach narrowed the field of approximately 500 peaks per sample to 33 metabolites of interest without extensive manual inspection of the 2D peak table data and chromatograms. The only manual inspection required was review of representative chromatograms to be certain that an appropriate sample dilution was chosen for analysis. Since several compounds of interest were overloaded at the low dilution and many compounds were undetected at higher dilutions, it was necessary to run the fermented cucumber slurry samples at 2 dilutions and compile the results to avoid floor and ceiling effects. Noninoculated, sterile-filtered fermented cucumber slurry controls were used to exclude volatile compounds that may have changed due to chemical reactions during the extended incubation time. Changes in compounds tentatively identified as 1-(2,4-dimethyl-

furan-3-yl)-ethanone, 2-methyl-2-pentanol, and amylene hydrate were similar in magnitude and direction in both control and spoilage samples. Therefore, they were excluded from the list of potential spoilage metabolites.

Volatile metabolites that changed during spoilage (Table 4.4) included increases in acetic acid, propanoic acid, butanoic acid, n-propyl acetate, several alcohols, and a decrease in furfural. The observed increases in acetic, propanoic, and butanoic acids were in accordance with previously published studies that showed increases in these components in fermented cucumbers that had undergone secondary spoilage fermentation, as measured by HPLC and tentatively identified based on retention time match (Fleming and others 1989, 2002; Kim and Breidt 2007). In the present study, the increase in butanoic acid detected by GCxGC-ToFMS was below the detection limit of the HPLC. However, quantitative analysis of acetic and propanoic acids by HPLC confirmed the fold increases detected with this nontargeted volatile analysis method in the anaerobic reproduction of the spoilage in fermented cucumber slurry (Table 4.1). In addition to confirming these 2 primary spoilage metabolites, several other target spoilage metabolites were discovered. Comparison of these metabolite changes to the original brine samples from spoiled fermented cucumbers showed that 11 of the 33 metabolites that changed in cucumber slurries upon controlled reproduction of spoilage coincided with metabolites that were formed or utilized in the brines of spontaneously spoiled fermented cucumbers. In addition to acetic, propanoic, and butanoic acids, there were increases in n-propyl acetate, isoamyl acetate, and 1-

butanol. Decreases were observed in components identified as ethyl lactate, acetonitrile, methyl lactate, tetrahydrofuran, and 1-penten-3-ol. The other 22 metabolites observed to change when the spoilage was transferred to sterilized fermented cucumber slurry in an anaerobic chamber showed that the transferred bacteria were able to utilize or produce a number of metabolites differently than in the original spoilage. The significance of these metabolites in the overall spoilage process remains to be determined.

Conclusions

Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GCxGC-ToFMS) provides the analytical capability to resolve and identify many more volatile components from food samples than traditional GC-MS methods. However, complex data sets are generated for each sample such that standard approaches to data analysis are impractical when the target analytes are unknown. Optimization of the 2D separation combined with a semi-automated approach to data reduction using the instrument software and basic statistical analysis made it feasible to detect and identify many volatile components in fermented cucumbers. Fermented cucumber brines were found to contain 137 reproducibly detected and identified volatile compounds from a variety of chemical classes including hydrocarbons, aldehydes, ketones, alcohols, acids, esters, furans, and terpenoids. The nontargeted GCxGC-ToFMS method and data analysis made possible the separation, identification, and determination of differences in polar

volatile components, facilitating the discovery of several metabolites that were formed or utilized during anaerobic spoilage of fermented cucumbers. Further study of these metabolites will enhance our ability to understand and potentially control the metabolism of spoilage bacteria that can degrade lactic acid under the restrictive environmental conditions present in fermented cucumbers.

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Table 4.1: Changes in organic acids and pH as an indicator of spoilage

	Time (months)	pH	Lactic acid (mM)	Acetic acid (mM)	Propanoic acid (mM)
Fermented cucumber spoilage					
After primary fermentation	1	3.17 ± 0.01	116.8 ± 5.6	27.6 ± 0.9	None detected
After spoilage	11	4.08 ± 0.01	10.4 ± 1.0	80.0 ± 1.9	39.5 ± 1.8
Reproduction of spoilage in fermented cucumber slurry					
Noninoculated control	0	3.79 ± 0.00	125.1 ± 12.1	5.7 ± 1.1	None detected
Noninoculated control	6	3.80 ± 0.00	128.6 ± 0.7	5.9 ± 0.1	None detected
Inoculated with spoilage brine	0	3.82 ± 0.00	106.2 ± 2.5	13.2 ± 1.8	2.9 ± 0.8
Inoculated with spoilage brine	6	4.46 ± 0.01	51.8 ± 0.9	62.5 ± 3.8	16.1 ± 1.3

Table 4.2: Data processing parameters used to create standardized peak tables in ChromaTOF®

Data step	Parameter	Value
Peak detection	Baseline offset	0.8
	Number of points averaged for smoothing	3
	Peak width (sec)	0.1
	Signal to noise (S/N)	250
	Number of apexing masses	2
GC x GC parameters	Match required to combine	500
	Override the allowed retention time shift for combine (early and late) (sec)	0.1
	First dimension peak width (sec)	15
Library identification	Search mode	Normal, Forward
	Number of library hits to return	10
	Molecular weight range	40-1000
	Mass threshold	10
	Minimum similarity match before name is assigned	800
	Library	NIST mainlib
Quantification	Mass to use for area/height calculation	U (unique mass)

Table 4.2 Continued

Reference (Compare criteria)	Name, 1st dimension retention time (s), 2nd dimension retention time (s), and masses (unique mass in this case)	Fields populated from peak table of the composite sample
	R. T. deviation (s)	4.5
	Quantitate	Area
	Match threshold	500
	S/N threshold	5.0

Table 4.3: Volatile compounds in fermented cucumber brines detected using SPME GCxGC-ToFMS

Compound[1]	CAS[2] registry #	Method of identification[3]	Similarity	RI_{calc}[4]	RI_{lit}[5]	Unique mass[6]	Average area	RSD[7]
Hydrocarbons								
pentane	109-66-0	MS, RI, ST	934	500	500	41	45,602	17.6
hexane	110-54-3	MS, RI, ST	925	600	600	41	655,816	27.2
1,4-pentadiene	591-93-5	MS, ST	943	646	Nf	67	37,400	19.7
ethylcyclobutane	4806-61-5	MS	896	692	Nf	56	472,102	17.0
cyclohexane	110-82-7	MS, RI, ST	904	742	737	56	88,364	15.1
cis-5,5-dimethyl-2-hexene	39761-61-0	MS	901	757	Nf	41	29,255	16.7
benzene	71-43-2	MS, RI, ST	970	938	936	78	743,703	12.6
toluene	108-88-3	MS, RI, ST	911	1042	1040	91	909,212	9.1
m-xylene	108-38-3	MS, RI	909	1144	1132	91	17,265	16.5
Cardene	694-87-1	MS, RI	926	1272	1269	104	64,928	18.1

Table 4.3 Continued

Alcohols								
2-methyl-2-pentanol	590-36-3	MS, RI	885	1110	1101	59	200,446	11.0
3-pentanol	584-02-1	MS, RI, ST	938	1116	1112	59	81,102	17.0
2-pentanol*	6032-29-7	MS, RI, ST	922	1129	1142	45	223,939	14.4
2,4-dimethyl-2-pentanol	625-06-9	MS, ST	875	1147	Nf	59	40,578	18.0
butanol*	71-36-3	MS, RI, ST	876	1153	1152	56	794,956	13.7
2-methyl-3-pentanol	565-67-3	MS, RI	907	1167	1121	59	21,068	21.2
1-penten-3-ol	616-25-1	MS, RI, ST	891	1169	1176	57	318,459	4.6
3-penten-2-ol	1569-50-2	MS, RI, ST	841	1181	1182	71	11,164	27.6
2,4-dimethyl-4-penten-2-ol	19781-53-4	MS	866	1195	Nf	59	38,954	15.7
eucalyptol*	470-82-6	MS, RI	852	1223	1216	81	29,202	10.5
2-hexanol	52019-78-0	MS, RI, ST	908	1238	1238	45	49,865	17.0

Table 4.3 Continued

3-methyl-3-buten-1-ol	763-32-6	MS, RI	883	1264	1263	68	37,794	13.7
pentanol*	71-41-0	MS, RI, ST	920	1265	1256	42	477,300	12.8
2-methyl-2-heptanol	625-25-2	MS	812	1265	Nf	59	47,995	14.4
trans-2-penten-1-ol	1576-96-1	MS, RI, ST	929	1325	1335	57	22,334	4.9
2-methyl-2-buten-1-ol	4675-87-0	MS, RI	862	1333	1315	71	55,966	29.2
2-heptanol*	543-49-7	MS, RI, ST	942	1334	1334	45	299,671	12.8
2-methyl-2-propen-1-ol	513-42-8	MS	808	1337	Nf	72	11,391	12.4
hexanol*	111-27-3	MS, RI	891	1362	1354	43	2,564,937	9.5
4-methyl-2-heptanol	56298-90-9	MS, ST	928	1369	Nf	45	120,208	21.1
trans-3-hexen-1-ol	544-12-7	MS, RI	926	1371	1371	67	20,351	12.4
2,3-dimethyl-1-pentanol	10143-23-4	MS	834	1388	Nf	85	3,623	10.8
cis-3-hexen-1-ol	928-96-1	MS, RI, ST	951	1389	1388	67	335,213	11.4
2-methyl-2-octanol	628-44-4	MS, ST	893	1397	Nf	59	71,673	10.8

Table 4.3 Continued

3-octanol	589-98-0	MS, RI, ST	917	1399	1395	55	19,815	16.1
trans-2-hexen-1-ol	928-95-0	MS, RI	862	1408	1410	57	31,238	24.4
2-octanol	5978-70-1	MS, RI	904	1421	1430	45	29,309	13.6
1-octen-3-ol	3391-86-4	MS, RI, ST	932	1451	1456	57	248,344	13.4
Heptanol	53535-33-4	MS, RI, ST	900	1457	1460	56	106,479	10.2
2-ethyl-1-hexanol*	104-76-7	MS, RI	932	1495	1492	57	291,814	12.3
cis-3-hepten-1-ol	1708-81-2	MS, RI	877	1509	1491	81	18,911	15.5
2-nonanol	628-99-9	MS, RI, ST	840	1528	1528	45	34,665	17.4
octanol*	111-87-5	MS, RI	900	1568	1561	56	63,772	8.0
4-terpineol	562-74-3	MS, RI	827	1614	1617	93	4,391	9.1
Myrcenol	543-39-5	MS, RI	860	1622	1604	59	28,202	17.4
cis-2-octen-1-ol	26001-58-1	MS, RI	896	1626	1616	57	15,126	12.0
cis-ocimenol	5986-38-9	MS, RI	847	1662	1662	93	55,339	12.7

Table 4.3 Continued

trans-ocimenol	5986-38-9	MS, RI	837	1685	1688	93	72,282	11.9
α-terpineol*	98-55-5	MS, RI, ST	914	1703	1718	59	345,764	13.3
cis-6-nonen-1-ol	35854-86-5	MS, RI	941	1720	1711	67	19,277	11.8
benzyl alcohol	100-51-6	MS, RI, ST	900	1900	1874	79	48,419	9.6
phenylethyl alcohol*	60-12-8	MS, RI, ST	942	1939	1939	91	54,906	10.5
Aldehydes								
acetaldehyde	75-07-0	MS, RI, ST	928	727	727	44	7,442,303	12.2
pivaldehyde	630-19-3	MS, RI	872	807	809	41	146,801	11.0
2-methylbutanal	96-17-3	MS, RI, ST	878	914	914	57	90,940	17.4
3-methylbutanal	590-86-3	MS, RI, ST	864	918	917	41	431,295	5.8
hexanal*	66-25-1	MS, RI, ST	923	1084	1080	57	664,650	7.9
trans-2-methyl-2-butenal	497-03-0	MS, RI	912	1098	1094	84	29,451	26.2
2-pentenal	1576-87-0	MS, RI, ST	884	1137	1135	55	175,677	6.8

Table 4.3 Continued

2,4,4-trimethyl-2-pentenal	53907-61-2	MS	800	1254	Nf	55	17,749	23.2
cis-2-heptenal*	57266-86-1	MS, RI	932	1340	1331	41	540,286	14.7
nonanal*	124-19-6	MS, RI, ST	902	1402	1396	41	283,024	16.4
trans-2-octenal	2548-87-0	MS, RI, ST	873	1432	1432	55	317,319	19.4
Furfural	98-01-1	MS, RI, ST	892	1464	1474	96	2,442,243	4.5
2,4-heptadienal	5910-85-0	MS, RI	873	1469	1468	81	30,513	10.8
(E,E)-2,4-heptadienal	3/5/4313	MS, RI	873	1501	1497	81	156,003	12.0
benzaldehyde*	100-52-7	MS, RI	872	1530	1528	77	77,720	3.1
2-decenal	2497-25-8	MS, RI	926	1658	1652	41	77,800	23.2
3,5-dimethyl-benzaldehyde	5779-95-3	MS	912	1837	Nf	133	100,878	13.6

Table 4.3 Continued

Ketones								
acetone*	67-64-1	MS, RI, ST	922	814	814	58	1,363,820	8.2
3-methyl-2-butanone	563-80-4	MS, RI, ST	847	929	929	39	25,302	27.9
3,3-dimethyl-2-butanone	75-97-8	MS, RI, ST	870	949	978	57	32,611	10.4
2-methyl-3-pentanone	565-69-5	MS, RI	863	997	1003	57	37,274	7.6
2,4-dimethyl-3-pentanone	565-80-0	MS, RI, ST	880	1000	995	71	10,099	11.9
methyl isobutyl ketone	108-10-1	MS, RI, ST	926	1008	1008	43	524,065	14.1
3-methyl-2-pentanone	565-61-7	MS, RI, ST	902	1019	1016	43	70,287	6.4
1-penten-3-one	1629-58-9	MS, RI, ST	837	1024	1024	55	698,188	9.8
4,4-dimethyl-2-pentanone	590-50-1	MS	886	1025	Nf	43	144,329	10.2
3-hexanone	589-38-8	MS, RI, ST	914	1055	1052	57	65,095	12.7
2-methyl-1-penten-3-one	25044-01-3	MS, RI	907	1069	1069	69	40,626	10.4
trans-3-penten-2-one	3102-33-8	MS, RI, ST	844	1134	1123	69	29,502	17.1

Table 4.3 Continued

4-methyl-3-penten-2-one	141-79-7	MS, RI	886	1140	1131	98	8,040	14.0
4-methyl-2-heptanone	6137-06-0	MS, RI	902	1224	1206t	58	124,174	13.9
3-hydroxy-2-butanone	513-86-0	MS, RI, ST	863	1301	1289	45	983,577	11.4
1-octen-3-one	4312-99-6	MS, RI	905	1319	1299	55	210,558	16.0
6-methyl-5-hepten-2-one	110-93-0	MS, RI	838	1351	1340	43	112,007	22.2
2-hydroxy-2,4-dimethyl-3-pentanone	3212-67-7	MS	865	1376	Nf	59	7,580	9.9
acetophenone	98-86-2	MS, RI, ST	935	1660	1660	77	42,629	9.8
p-methylacetophenone	122-00-9	MS, RI	890	1789	1794	119	19,819	10.1
Acids								
acetic acid*	64-19-7	MS, RI, ST	927	1446	1450	60	9,802,352	17.5
propanoic acid	79-09-4	MS, RI, ST	938	1543	1534	45	230,741	7.3
pivalic acid	75-98-9	MS, RI	863	1586	1579	57	106,234	11.5

Table 4.3 Continued

butanoic acid*	107-92-6	MS, RI, ST	846	1636	1620	60	55,323	9.8
2-methyl-butanoic acid	116-53-0	MS, RI, ST	861	1677	1682	74	136,728	9.5
pentanoic acid	109-52-4	MS, RI, ST	913	1734	1734	60	81,854	8.9
hexanoic acid*	142-62-1	MS, RI, ST	864	1865	1841	60	721,279	6.4
octanoic Acid	124-07-2	MS, RI, ST	891	2071	2053	60	375,641	9.2
nonanoic acid	112-05-0	MS, RI, ST	892	2157	2157	60	501,018	17.3
decanoic acid	334-48-5	MS, RI	869	2219	2263	60	52,296	14.4
Esters								
methyl acetate	79-20-9	MS, RI, ST	882	825	828	74	1,012,974	13.7
methyl propionate	554-12-1	MS, RI, ST	815	905	911	57	57,501	14.0
ethyl propionate	105-37-3	MS, RI	880	956	957	57	81,551	11.7
ethyl nitrate	625-58-1	MS	934	969	Nf	76	4,708	14.7
isoamyl acetate	123-92-2	MS, RI, ST	851	1127	1127	43	33,883	28.2

Table 4.3 Continued

methyl lactate	2155-30-8	MS, RI, ST	948	1331	Nf	45	1,719,881	9.6
ethyl lactate	97-64-3	MS, RI, ST	949	1354	1353	45	7,808,306	7.4
isoamyl lactate	19329-89-6	MS, RI	852	1580	1583	45	27,084	22.9
trans-3-hexenyl butanoate	53398-84-8	MS, RI	826	1621	1602	71	42,111	20.8
2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl propanoate	74367-34-3	MS	894	1902	Nf	71	281,380	6.0
2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl propanoate	74367-33-2	MS	854	1921	Nf	71	185,822	5.4
Ethers								
Oxetane	503-30-0	MS, ST	897	790	Nf	58	305,941	5.2
tert-amyl methyl ether	994-05-8	MS	871	790	Nf	73	32,913	14.2
1,2-oxidolinalool	76985-29-0	MS	896	1446	Nf	59	379,361	8.0

Table 4.3 Continued

diphenyl ether	101-84-8	MS, RI	863	2038	2017	51	37,843	8.2
Furans								
2-methylfuran	534-22-5	MS, RI, ST	895	864	876	82	54,359	9.0
2-ethylfuran	3208-16-0	MS, RI, ST	900	950	945	81	60,954	8.8
trans-linalool oxide*	34995-77-2	MS, RI	886	1477	1484	59	112,235	7.9
2-acetylfuran	1192-62-7	MS, RI	911	1509	1511	95	19,327	12.7
butyrolactone	96-48-0	MS, RI	962	1637	1635	42	276,487	11.3
5-pentyl-γ-lactone	104-61-0	MS, RI	882	2056	2055	85	100,505	6.8
Pyrans								
linalool 3,7-oxide	7392-19-0	MS, RI	868	1111	1109	71	255,202	10.4
trans-rose oxide	876-18-6	MS, RI	822	1365	1341	139	7,646	13.9
nerol oxide	1786-08-9	MS, RI	831	1476	1466	83	10,567	13.3

Table 4.3 Continued

Phenols								
butylated hydroxytoluene	128-37-0	MS, RI	858	1946	1902	205	49,914	19.8
p-propylguaiacol	2785-87-7	MS, RI	919	2117	2103	137	11,239	11.8
3,5-di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	MS	834	> 2219	Nf	219	5,239	15.9
Nitrogenous compounds								
methyl isocyanide	593-75-9	MS	980	1002	Nf	41	302,897	9.9
3-methyl-butanenitrile	625-28-5	MS, RI	797	1132	1120	41	23,517	8.5
3,3-dimethyl-butanamide	926-04-5	MS	813	1205	Nf	59	134,717	14.0
5-methyl-isoxazole	5765-44-6	MS	878	1215	Nf	43	25,887	14.4
acetaldoxime	107-29-9	MS	941	1301	Nf	59	14,668	21.9
hexanenitrile	628-73-9	MS, RI, ST	872	1315	1303	54	58,876	21.4
4-O-acetyl-2,5-di-O-methyl-3,6-dideoxy-d-	N/A	MS	848	1335	Nf	129	4,639	13.5

Table 4.3 Continued

gluconitrile								
2-methoxy-3-isopropyl-pyrazine*	25773-40-4	MS, RI	863	1432	1443	137	51,619	8.8
Sulfur compounds								
dimethyl disulfide*	624-92-0	MS, RI, ST	981	1072	1075	94	128,995	27.9
3-methylthiophene	616-44-4	MS, RI, ST	920	1120	1120	97	14,082	17.8
dimethyl sulfoxide	67-68-5	MS, RI, ST	935	1576	1582	63	249,017	16.3

[1] Compounds reported previously in fermented cucumber brine are designated with an *

[2] Chemical Abstracts Service registry number

[3] MS: identification based on mass spectral match to the NIST 05 library with >800 similarity, RI: comparison with published retention indices on polyethylene glycol column phase, ST: mass spectral and retention index match to authentic standard

[4] Retention indices based on first dimension retention of components on a SOL-GEL-WAX (polyethylene glycol) column using SPME GCxGC-ToFMS

[5] Retention indices reported in the literature (nf = not found); References available at the NIST Chemistry WebBook database, http://webbook.nist.gov

Table 4.3 Continued

[6] Mass selected by ChromaTOF software during automated data processing to represent an interference free mass for each analyte; The unique mass for each component was used for calculation of peak area

[7] Relative standard deviation (n = 7)

Table 4.4: Changes in volatile metabolites associated with anaerobic spoilage of fermented cucumber slurry (pH 3.8, 6% NaCl)

Metabolite[1]	CAS[2] registry #	RI[3]	Fold change[4]	Direction of change
4-methyl-benzenemethanol	589-18-4	1960	579.4	Increase
unknown A	N/A	1371	342.2	Increase
4-methyl-2-heptanol	56298-90-9	1375	188.2	Increase
4-methyl-2-pentanol	108-11-2	1173	183.5	Increase
2-hexanol	626-93-7	1231	33.4	Increase
4-methyl-3-hepten-2-one [MS]	22319-25-1	1015	29.4	Increase
unknown B	N/A	1902	11.7	Increase
2-pentanol	6032-29-7	1126	11.4	Increase
3-methylene-2-pentanone [MS]	4359-77-7	1137	6.3	Increase
isoamyl acetate*	123-92-2	1127	6.0	Increase
n-propyl acetate*	109-60-4	961	6.0	Increase
propanoic acid*	79-09-4	1543	4.8	Increase
2-heptanol	543-49-7	1334	4.7	Increase
tert-butyl ethyl ether	637-92-3	710	4.6	Increase
acetic acid*	64-19-7	1449	4.5	Increase
unknown C	N/A	920	3.7	Increase
unknown D	N/A	962	3.3	Increase
butanoic acid	107-92-6	1636	3.0	Increase

Table 4.4 Continued

3-pentanol	584-02-1	1110	2.8	Increase
1-butanol*	71-36-3	1153	2.5	Increase
2-methyl-2-propanol	75-65-0	897	2.5	Increase
methyl acetate	79-20-9	825	2.3	Increase
unknown E		882	1.6	Increase
ethyl lactate*	97-64-3	1353	1.7	Decrease
methyl propionate	554-12-1	905	1.9	Decrease
acetonitrile*	75-05-8	988	2.3	Decrease
methyl lactate*	2155-30-8	1328	3.2	Decrease
tetrahydrofuran*	109-99-9	854	3.5	Decrease
1-penten-3-ol*	616-25-1	1166	4.5	Decrease
unknown F	N/A	1535	6.2	Decrease
furfural	98-01-1	1464	30.1	Decrease
unknown G	N/A	967	85.0	Decrease
2,3-butanedione	431-03-8	965	100.1	Decrease

[1] Identification based on mass spectral and retention index match to authentic standards except where noted. [MS] indicates a tentative identification based on mass spectral match to the NIST library. Metabolites marked with an * symbol indicate those compounds that were also found to increase or decrease in the brines from the original spoilage of fermented cucumbers.

[2] Chemical Abstracts Service registry number

Table 4.4 Continued

[3] Retention indices based on first dimension retention of components on a SOL-GEL-WAX (polyethylene glycol) column using SPME GCxGC-ToFMS

[4] Fold change based on ratio of unique mass peak area of a given metabolite in spoiled fermented cucumber slurry as compared to initial fermented cucumber slurry

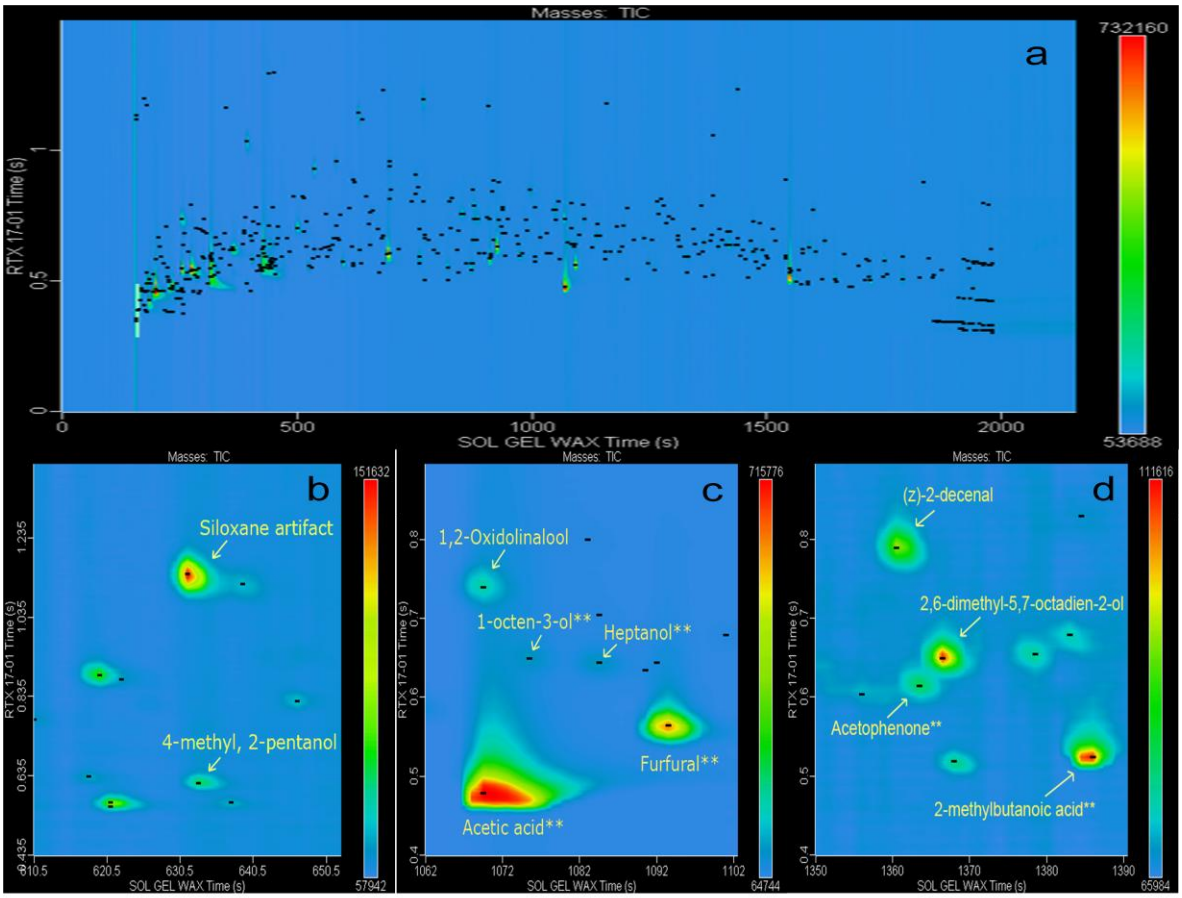

Figure 4.1: GCxGC-ToFMS total ion current (TIC) contour plot of volatile components in fermented cucumber brine (a). Three detail regions of the 2D separation of volatile components in fermented cucumber brine with a polar-semipolar column combination are shown, illustrating increased separation capacity (b), resolution of siloxane artifacts from metabolites of interest (c), and resolution of low intensity metabolite peaks in the second dimension from an overloaded acetic acid peak (d). Peaks detected with S/N ≥ 250 are indicated by black peak markers.

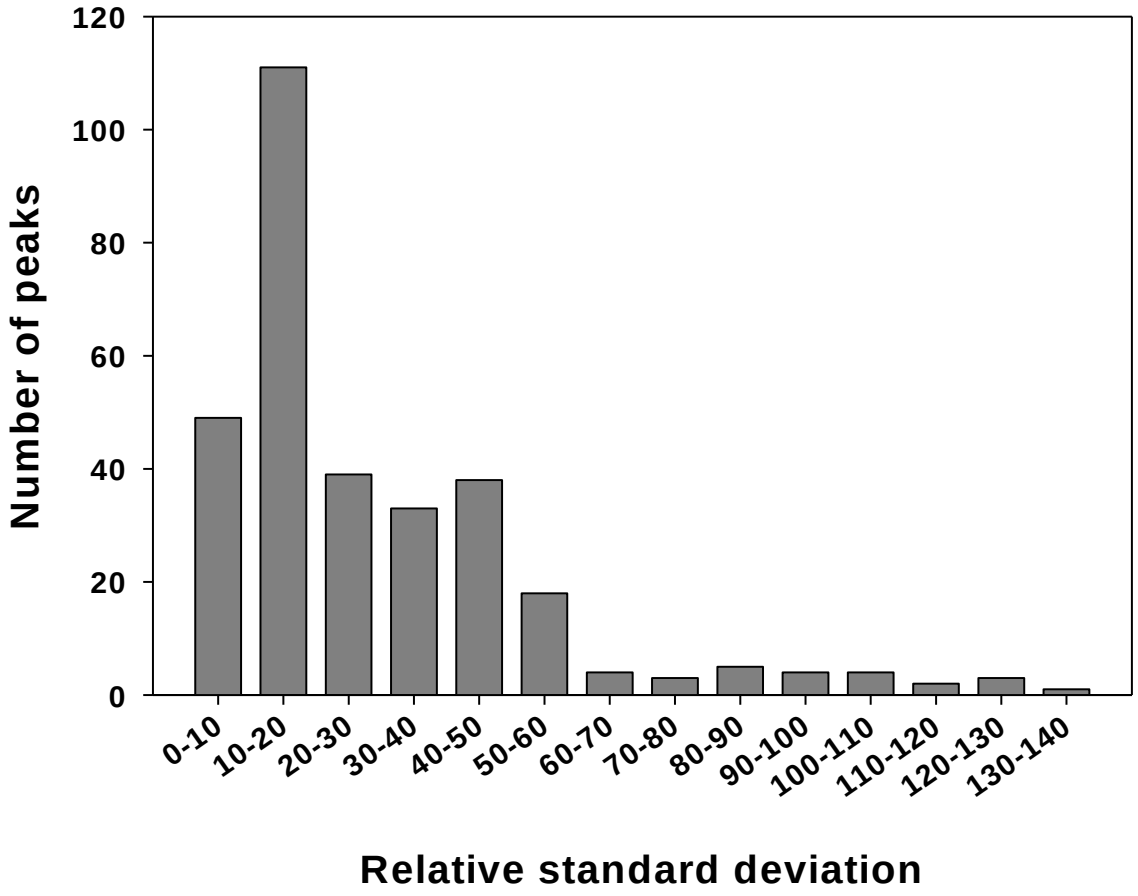

Figure 4.2: Peak area reproducibility (n = 7) for volatile components detected in fermented cucumber brine with SPME GCxGC-ToFMS

Chapter 5

Metabolomic characterization of fermented cucumber spoilage using nontargeted comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS)

Abstract

Our current knowledge of the biochemistry of fermented cucumber spoilage is limited to metabolites that can be measured using HPLC with ultraviolet and refractive index detection. Although this technology is excellent for targeted analysis of metabolites that change in millimolar concentrations, we have no knowledge of other components that may be related to spoilage. Complementary GCxGC-ToFMS metabolite profiling methods were developed and applied to analysis of volatile and nonvolatile compounds to determine changes that occurred during fermented cucumber spoilage. Volatiles extracted by solid phase microextraction and nonvolatiles derivatized with methoxylamine hydrochloride and BSTFA to form TMS derivatives were separated on polar/less polar and midpolar/nonpolar column combinations, respectively. Taken together, more than 5000 peaks were detected. However, many of these peaks were not identified by mass spectral library matching, especially in the TMS-metabolite mixtures, and a substantial number of the peaks were due to excess reagent and column bleed. Nonetheless, univariate analysis of variance combined with hierarchial clustering analysis revealed 92 metabolites that changed during spoilage ($P < 0.01$). Decreases were observed in sugars, amino acids, nucleosides, long chain fatty acids, aldehydes, and ketones, and increases were observed in several alcohols and butanoic and pentanoic acids. Most of the changes preceded lactic acid utilization, indicating that lactic acid is not a preferred substrate for spoilage organisms in fermented cucumbers.

Introduction

Our current knowledge of the biochemistry of fermented cucumber spoilage is limited to metabolites that can be measured using HPLC with ultraviolet and refractive index detection. Although this technology is excellent for targeted analysis of metabolites that change in millimolar concentrations, we have no knowledge of other components that may be related to spoilage. Given the potential diversity of chemical species in a food fermentation system, a discovery based approach may be useful for studying the chemistry of fermented cucumber spoilage where much is yet unknown. Metabolomics has been defined as the field of research that involves characterization, including identification and quantification, of the complete collection of small molecule metabolites in a biological system. In practice, metabolomic studies apply a nontargeted approach to the analytical chemistry and statistical analyses used to discover changes in metabolites that occur related to some criteria of interest (i.e. a disease state, changes in a food due to processing treatments, changes in human plasma related to consumption of a food, etc…). The criteria that were set forth for metabolomic studies stated that the sample preparation, analytical method, and data analysis must include all classes of compounds; have high recovery; be robust, sensitive, reproducible, matrix independent and universal; and have a plan for identifying unknowns (Fiehn 2001). Achieving these goals with a single technology is a significant challenge given the extraordinary diversity of chemical species that make up the metabolome. The technologies that are primarily being used for metabolomic

investigations include nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS). Each of these techniques has advantages and limitations, and a single analytical technique to comprehensively study the metabolome is not yet readily available (Wishart 2008). Nonetheless, rapid developments in analytical and data handling technologies have dramatically increased the ability to perform extensive metabolite profiling.

Despite the limitation of requiring a volatile metabolite or a volatile metabolite derivative, two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS) is a powerful tool for obtaining metabolite information. Advances in GC-MS systems and data collection capability provide the potential to carry out separations of volatile chemical components using two separation mechanisms by connecting columns of different bonded phases in series. The benefits and challenges associated with this technology have been the subject of recent reviews (Marriott and Shellie 2002; Adachour and others 2008). This GCxGC methodology has been widely applied to the analysis of food volatiles. An increased number of compounds were detected, and significantly higher mass spectral similarity values were obtained for butter volatiles and grape monoterpenoids, providing evidence of the increased separation efficiency of GCxGC-ToFMS as compared to 1D GC-MS (Adachour and others 2005; Rocha and others 2007). Analysis of lavender essential oil using a 2D separation consisting of a non-polar 1st dimension column followed by a polar 2nd dimension column, resulted in a 25 fold increase in sensitivity and 3 fold

increase in the number of resolved components as compared to traditional GC analysis (Shellie and others 2001). This technology has been further developed for application to nontargeted profiling of fermented cucumber volatiles (Johanningsmeier and McFeeters 2011). Similarly, GCxGC-ToFMS may be applied for the study of nonvolatile components that are first chemically derivatized with one or more trimethylsilyl (TMS) group(s) to make them volatile, allowing the analysis of a number of chemical classes including mono and disaccharides, sugar alcohols, sugar phosphates, organic acids, amino acids, and long chain fatty acids. One-dimensional GC-MS based metabolomic platforms have been developed and applied for metabolite profiling in plants (Roessner and others 2000; Gullberg and others 2004; Weckworth and others 2004; Lisec and others 2006; Zörb and others 2006; Rudell and others 2008), microorganisms (Barsch and others 2004; Strelkov and others 2004; Bölling and Fiehn 2005; O'Hagan and others 2005; Koek and others 2006; van der Werf and others 2008), and human fluids and tissues (Jiye and others 2005; O'Hagan and others 2005; Denkert and others 2008; Pasikanti and others 2008; Mal and others 2009; Begley and others 2009). Two-dimensional GC-ToFMS has been less extensively used for metabolomic investigations due to the challenges associated with the large volumes of data produced and the greater expense of the instrumentation itself. However, Welthagan and colleagues (2005) clearly demonstrated the advantages of GCxGC-ToFMS for biomarker discovery in spleen tissue extracts. GCxGC-ToFMS required approximately 10 fold less sample than GC-ToFMS, exhibited excellent peak resolution, and separated 7 fold more

peaks with S/N > 50 and high quality mass spectra. Similarly, a GcxGC-ToFMS metabolomic platform was applied to rice extracts in a study of phenotypic variation. Peak responses were double that observed with the corresponding one-dimensional GC method, and approximately 620 peaks were observed in each sample (Kusano and others 2007). Twenty-six metabolites that distinguished fermenting yeast cells from respiring yeast cells were identified from amongst more than 2500 peaks detected using nontargeted GCxGC-ToFMS metabolite profiling (Mohler and others 2006). Similar platforms have been developed for metabolomic investigations in rye grass (Hope and others 2005), microbial metabolomics (Guo and others 2008), and biomarker discovery for diabetes mellitus (Li and others 2009). Optimization of instrumental analytical parameters for a nonpolar/polar column combination resulted in the detection of more than 1800 metabolite peaks in human serum (O'Hagan and others 2007). Most of the previously mentioned 2D GC studies used a nonpolar/polar column combination. However, Koek and others (2008) found that a polar/nonpolar column combination resulted in better resolution of components and greater use of the separation space with similar separation efficiency. The aforementioned studies have clearly demonstrated the usefulness of GC-MS for nontargeted metabolite profiling in a variety of matrices. Combining the information obtained from both volatile and nonvolatile analyses using a nontargeted GCxGC-ToFMS platform will allow a much more comprehensive view of the biochemical changes occurring during spoilage of fermented cucumbers than has been possible with high performance liquid

chromatography (HPLC) coupled to ultraviolet and refractive index detectors. The objective of this research was to apply a GCxGC-ToFMS metabolomic platform to study biochemical changes that occur during fermented cucumber spoilage by *Lactobacillus buchneri* and unidentified spoilage organisms from reduced NaCl and commercial cucumber fermentations.

Materials and Methods

Spoilage inocula sources. Brines from a reduced NaCl cucumber fermentation and a commercial cucumber fermentation that had undergone undesirable secondary fermentation were used as spoilage inocula. The reduced NaCl spoilage source has been previously described (Johanningsmeier and McFeeters 2011). The commercial spoilage source was obtained from a commercial brining facility after it was observed that a tank of fermented cucumbers was undergoing post-fermentation spoilage. *Lactobacillus buchneri* (Culture Collection ID LA1147, USDA-ARS Food Science Research Unit, Raleigh, NC), a lactic acid degrading lactic acid bacteria isolated from the reduced NaCl spoilage source, was also used to produce spoilage in fermented cucumber slurry (FCS). A complete description of these spoilage sources is available in Chapters 2 and 3.

Growth media preparation. Size 2B pickling cucumbers (32-38 mm in diameter) were obtained from a local processor, blended into slurry, and frozen at -10°C until needed. Fresh cucumber slurry (CS) was thawed, pressed through cheesecloth and centrifuged in

250-mL bottles at 23,400 x g for 15 min to remove particulate matter. After centrifugation, NaCl, yeast extract, peptone and water were added to yield final concentrations of 67% fresh cucumber, 4% NaCl, 1% yeast extract, and 1% peptone. The resulting modified cucumber slurry (mCS) growth media was sterile-filtered with a 0.2 µm bottle top filter (Nalgene FAST PES, 0.2 µm pore size, 90-mm-diameter membrane, Daigger, Vernon Hills, IL) and stored at 4°C until use.

Fermented cucumber media preparation. Size 2B pickling cucumbers were washed, packed into 3.84-L glass jars, and covered with brine (55:45 cucumber:brine ratio) containing calcium chloride ($CaCl_2$) and sodium chloride (NaCl) so that the equilibrated concentrations were 0.25% and 0-6% (w/w), respectively. Brined cucumbers were inoculated with 10^6 CFU/g *Lactobacillus plantarum* MOP3 starter culture (Culture Collection ID LA0219, USDA-ARS Food Science Research Unit, Raleigh, NC). Jars were closed with lids that were heated in boiling water to soften the plastisol liner, and a rubber septum was inserted into the lid of each jar to allow sampling of the brine with a syringe. Three replicate jars were packed for each NaCl concentration and stored at ambient temperature (21-25^{o}C) for 11 mo. Fermentation progressed normally in all jars as indicated by decreases in pH and changes in organic acids as measured by high-performance liquid chromatography (HPLC) with ultraviolet light (UV) detection (McFeeters and Barish 2003). Sugars and alcohols were quantified in the same analysis using a refractive index detector in series. Fermented cucumbers (4% NaCl) were cut into pieces and blended into slurry to prepare FCS as a

medium for inoculation with spoilage microorganisms. The FCS was pressed through cheesecloth and centrifuged in 250-mL bottles at 23,400 x g for 15 min to remove particulate matter. The pH of the clarified slurry was raised from 3.1 to 3.8 by addition of 6 N NaOH to increase the likelihood that spoilage would occur (Fleming and others 2002; Kim and Breidt 2007), and NaCl was added to bring the equilibrated concentration to 6% w/w. The pH-adjusted, clarified FCS was sterile-filtered with a Nalgene FAST PES 0.2 µm pore size, 90-mm-diameter membrane, bottle-top filter apparatus (Daigger, Vernon Hills, IL). Twelve mL of sterile-filtered FCS was then aseptically transferred into sterile 15-mL conical tubes. The loosely capped tubes were placed into an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) for 3 days prior to inoculation to remove dissolved oxygen from the media.

Reproduction of spoilage. Reduced NaCl and commercial spoiled fermentation brines (1 mL each) were inoculated into 9 mL mCS and incubated anaerobically at 30°C for 2 weeks. *L. buchneri* strain LA 1147 was streaked onto MRS and incubated anaerobically at 30°C for 4 days. Three isolated colonies were transferred to 9 mL mCS and incubated anaerobically at 30°C for 2 weeks. One milliliter of each of the three resulting spoilage cultures was transferred to 25 mL mCS and incubated anaerobically at 30°C for 6 days to prepare inocula for reproduction of spoilage in FCS. Spoilage inocula were centrifuged to pellet cells and the spent growth media was discarded. Cells were washed with 5 mL FCS, pelleted, and then resuspended in 15 mL FCS. Spoilage inocula were serially diluted and spiral plated onto

MRS to count LAB. Conical centrifuge tubes containing 5 mL sterile-filtered FCS were inoculated in triplicate with 0.5 mL of each spoilage culture and incubated anaerobically at ambient temperature along with triplicate noninoculated FCS controls. Samples were aseptically withdrawn after 9, 22, 51, 77, 96, and 146 days of incubation and stored at -80°C until analysis. Lactic acid utilization by spoilage inocula was quantified by HPLC. Nontargeted analysis of volatile and nonvolatile components was conducted using SPME-GCxGC-ToFMS and TMS-GCxGC-ToFMS, respectively.

Volatile compound analysis by solid-phase microextraction-comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (SPME-GCxGC-ToFMS). Nontargeted volatile compound analysis was performed with slight modification of the analytical method described by Johanningsmeier and McFeeters (2011). Briefly, spoilage samples (100 µL) were diluted 1:10 with deionized water (896 µL) and acidified with 3 N H_2SO_4 (4 µL) in 10 mL screw-cap headspace vials (Microliter Analytical Supplies, Inc., Suwanee, GA). Sodium chloride (NaCl, 0.40 g) was added to "salt out" volatile components from the samples. Samples were blocked by replicate, randomized for analysis order (PROC PLAN, version 9.1.3 SAS® software, SAS Institute, Cary, NC) and placed into a refrigerated sample tray (2°C). Automated sampling was performed using a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies, Carrboro, NC). Headspace vials containing the diluted samples were agitated at 500 rpm (5 sec on and 2 sec off) for 15 min at 40°C prior to extraction. Volatile compounds were collected by

insertion of a 1-cm, 50/30 µm DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco, Bellefonte, PA) into the headspace above the sample for 30 min at 40°C with 100 rpm agitation (5 sec on and 2 sec off). Extracted volatile compounds were desorbed from the SPME fiber into the GC inlet at 250°C for 15 min. A blank sample (1.0 mL deionized water containing 6 mM sulfuric acid and 0.4 g NaCl) was included in order to determine which detected components were system artifacts.

A LECO® Pegasus III® two - dimensional gas chromatograph (GCxGC) time-of-flight mass spectrometer (ToFMS) instrument (Model# 614-100-700, Leco Corporation, St. Joseph, MI) included an Agilent GC (Model# 6890N, Agilent Technologies, Santa Clara, CA) fitted with a secondary oven and cryogenic modulator. The two - dimensional separation was achieved using a SolGel-Wax™, 30 m x 0.25 mm ID x 0.25 µm film thickness (SGE, Austin, TX), polyethylene glycol 1st dimension column in the primary oven and an RTX 17-01, 1.0 m x 0.1 mm ID x 0.1 µm film thickness (Restek, Bellefonte, PA), 14% cyanopropylphenyl – 86% dimethyl polysiloxane 2nd dimension column in the secondary oven. Columns were conditioned according to manufacturer recommendations prior to use. A 0.75-mm-ID Siltek deactivated SPME liner (Restek, Bellefonte, PA) was used in the inlet. The inlet temperature was set at 250°C and operated in pulsed splitless mode with a pulse pressure of 37 psi for 1 min. The split vent was opened 2 min following injection, and the GC was operated in constant flow mode with 1.3 mL /min helium carrier gas. The primary oven temperature was maintained at 40°C for 2 min and then increased at 5°C/min to 140°C. The temperature

ramp was then increased to 10°C/min to 250°C and the temperature was held at 250°C for 3 min. The secondary oven followed the same temperature program except the temperature was maintained at 10°C higher than the main oven until the temperature reached a maximum of 250°C in the secondary oven. The transfer line temperature was maintained at 250°C. The modulator offset was +30°C with a 2.75 sec 2nd dimension separation time and 0.55 sec hot pulse. Compressed air (35 psi) was used for the hot pulses, and liquid nitrogen-cooled nitrogen gas (18 psi) was used for the cold pulses.

The mass spectrometer was operated with -70 eV and an ion source temperature of 200°C. The detector voltage was set at 1500 V and masses 25-500 were collected at 200 spectra per second. No solvent delay was employed.

ChromaTOF® software version 4.33 data processing methods were used to detect and quantify peaks based on unique masses as determined by the deconvolution algorithm. A library search of the NIST/EPA/NIH Mass Spectral Library (National Institute of Standards and Technology (NIST), Gaithersburg, MD, 2005) was utilized for tentative identification of deconvoluted chromatographic peaks. Chemical names were assigned to peaks that had a minimum mass spectral similarity ≥ 800 (1000 is an exact match). The unique mass (U) for each peak, as assigned by the ChromaTOF® deconvolution algorithm, was used for peak area calculations. Alignment of chromatograms was accomplished using the StatCompare® feature in ChromaTOF®. Aligned peak information was exported for statistical analysis.

Nonvolatile compound analysis by trimethylsilyl derivatization-comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (TMS-GCxGC-ToFMS). Spoilage samples were thawed, briefly vortexed, and centrifuged at 16,000 x g for 7.5 min (Marathon 16KM microcentrifuge, Fisher Scientific). A 40 µL aliquot of supernatant was transferred to a microcentrifuge tube, and 10 µL internal standard stock solution containing 2 nmol ribitol (Sigma-Aldrich, St. Louis, MO), 4 nmol deuterated valine (Icon Isotopes, Summit, NJ), and 4 nmol C^{13}-labeled salicylic acid (Icon Isotopes, Summit, NJ) was added. Sample pH was adjusted to neutral by addition of 0.05 M Na_2CO_3 to each sample as indicated by titration with 1% bromothymol blue indicator. Samples were dried by centrifugal evaporation under vacuum (Savant SpeedVac model SVC100D, Instruments, Inc., Farmingdale, NY). Metabolites with aldehyde and keto groups were oximated by addition of 98 µL of 20 mg/ml *O*-methoxylamine HCl in pyridine to the dried samples, which were heated for 90 min at 70°C. Formation of the trimethylsilyl derivatives was done by heating the oximated samples for 30 min at 70^{o} C after addition of 98 µL of N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA). After the 30 min incubation, sample tubes were placed on ice for 5 min, centrifuged to pellet reaction precipitate, and transferred to an autosampler vial. Samples were blocked by replicate for derivatization, randomized for run order and placed into a refrigerated sample tray (2°C). Automated injection was performed using a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies, Carrboro, NC). A 1 µL hot needle injection was performed to avoid peak

discrimination. Three pre and post washes of the syringe with ethyl acetate and hexane and 3 sample prewashes were employed to avoid carryover. A LECO® Pegasus III® two - dimensional gas chromatograph (GCxGC) time-of-flight mass spectrometer (ToFMS) instrument (Model# 614-100-700, Leco Corporation, St. Joseph, MI) included an Agilent GC (Model# 6890N, Agilent Technologies, Santa Clara, CA) fitted with a secondary oven and cryogenic modulator. The two - dimensional separation was achieved using a BPX50, 30 m x 0.25 mm ID x 0.25 µm film thickness (SGE, Austin, TX), 50% phenyl polysilphenylene-siloxane 1^{st} dimension column in the primary oven and a BPX5, 1.0 m x 0.15 mm ID x 0.15 µm film thickness (SGE, Austin, TX), 5% phenyl polysilphenylene-siloxane 2^{nd} dimension column in the secondary oven. Columns were conditioned according to manufacturer recommendations prior to use. A 4-mm-ID deactivated tapered splitless injection liner (Agilent Technologies, Santa Clara, CA) was used in the inlet. The inlet temperature was set at 260°C and operated in pulsed splitless mode with a pulse pressure of 24 psi for 1 min. The split vent was opened 1 min following injection, and the GC was operated in constant flow mode with 1.0 mL /min helium carrier gas. The primary oven temperature was maintained at 70°C for 4 min and then increased at 5°C/min to 230°C. The temperature ramp was then increased to 15 °C/min to 290°C and the temperature was held at 290°C for 15 min. The secondary oven followed the same temperature program except the temperature was maintained at 15°C higher than the main oven. The transfer line temperature was maintained at 280°C. The modulator offset was +30°C with a 2.75 sec 2^{nd}

dimension separation time and 0.55 sec hot pulse. Compressed air (35 psi) was used for the hot pulses, and liquid nitrogen-cooled nitrogen gas (18 psi) was used for the cold pulses.

The mass spectrometer was operated at -70 eV with an ion source temperature of 250°C. The detector voltage was set at 1550 V and masses 35-800 were collected at 200 spectra per second. A solvent delay of 7 min was employed to reduce wear on the detector. ChromaTOF® software version 4.33 data processing methods were used to detect and quantify peaks based on unique masses as determined by the deconvolution algorithm. A library search of the LECO-Fiehn Rtx5 Mass Spectral Library (Leco Corporation, St. Joseph, MI) was utilized for tentative identification of deconvoluted chromatographic peaks. Masses 85-800 were used for library matching in order to remove the influence of the abundant mass 73 that is encountered with TMS-derivatives. Chemical names were assigned to peaks that had a minimum mass spectral similarity ≥ 800 (1000 is an exact match). The unique mass (U) for each peak, as assigned by the ChromaTOF® deconvolution algorithm, was used for peak area calculations. Alignment of chromatograms was accomplished using the StatCompare® feature in ChromaTOF®. Aligned peak information was exported for statistical analysis.

Statistical analysis. Analysis of variance (ANOVA) and hierarchial cluster analysis (HCA) were performed in JMP Genomics version 4.1 (SAS Institute, Cary, NC). Aligned peak information exported from ChromaTOF® was reformatted using Excel (Microsoft Corporation, Redmond, WA). A column was added in order to give each peak a unique

numerical identifier for statistical analysis. Missing value replacement was done to provide substitution data that reflected possible responses below the method's detection limit for undetected components, and log2 transformation of peak areas was performed prior to statistical analysis. An ANOVA of log2 peak areas was conducted to detect differences in volatile and nonvolatile compounds among treatments. Significance was established at $P < 0.01$ after adjustment of p-values to control the false discovery rate using the method of Benjamini and Hochberg (1995). Metabolite peaks that were found to be significantly different among treatments were subjected to hierarchial cluster analysis (HCA) that used the Fast Ward method as the default clustering process. The resulting heat maps were visually examined for biologically relevant trends. Clusters of metabolites that represented increases or decreases during spoilage for all spoilage inocula were selected for further examination. Components that changed during anaerobic incubation of noninoculated FCS were presumed to have been formed as a result of chemical changes that occurred during the extended incubation period and were manually excluded when necessary.

Results and Discussion

Characterization of both volatile and nonvolatile spoilage metabolites was conducted to determine biochemical changes that occurred during fermented cucumber spoilage other than those that were detected by HPLC. Interestingly, hundreds of metabolite peaks were found to change as a result of anaerobic metabolic activity of spoilage organisms. Although

lactic acid was utilized at a similar rate by *L. buchneri* and the reduced NaCl and commercial spoilage cultures (Figure 5.1), the three spoilages differed somewhat in their metabolite profiles. Approximately 692 volatile compounds were detected at S/N ≥ 250 in FCS inoculated with *L. buchneri* and spoilage cultures. Of these, 195 were significantly different among treatments ($P < 0.01$) after 146 days anaerobic incubation. These differential metabolites were subjected to hierarchial cluster analysis (HCA) resulting in 11 distinct clusters (Figure 5.2). The clusters contained groups of metabolites that represented several biologically significant trends. From top to bottom, the red (1), olive green (2), yellow (3), and turquoise (8) clusters represent volatile metabolites present in FCS (6% NaCl, pH 3.8) that were used differentially by the three spoilage inocula. The metabolites that grouped into the largest cluster, denoted in purple (4), were present in FCS and decreased substantially by *L. buchneri*, commercial and reduced NaCl spoilage organisms. Since lactic acid was utilized in FCS to a similar degree by all three spoilage inocula (Figure 5.1), this group of 46 metabolites was selected for manual inspection. The resulting 32 metabolites that decreased significantly as a result of spoilage by *L. buchneri*, commercial and reduced NaCl organisms are presented in Table 5.1. These compounds were mostly aldehydes and ketones and a few alcohols, hydrocarbons, and unidentified components. Clusters 5, 6, 7, 10, and 11 (grass green, sea green, teal, pink, and burnt orange clusters) represent groups of metabolites not present or in low abundance in FCS that were produced differentially by *L. buchneri*, commercial and reduced NaCl spoilage inocula. The blue cluster (9) was

comprised of 33 metabolite peaks that were increased similarly in FCS by all three spoilage inocula. Three artifact peaks were removed by manual inspection, and the 30 volatile metabolites that increased in fermented cucumber spoilage are presented in Table 5.2. There were increases in a number of alcohols and butanoic and pentanoic acids. The decreases in aldehydes and ketones combined with increases in alcohols may suggest alcohol dehydrogenase activity by spoilage microorganisms. NAD dependent alcohol dehydrogenases (E.C. 1.1.1.1), aryl alcohol dehydrogenases (E.C. 1.1.1.90) and NADP dependent alcohol dehydrogenases (E.C. 1.1.1.2) are widely distributed in nature, and have been annotated in *L. buchneri* by gene sequence homology (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/). The conversion of an aldehyde or ketone to an alcohol by these types of enzymes would generate $NAD(P)^+$ and therefore may act collectively as electron acceptors.

The volatile metabolites that were increased or decreased by all three spoilage inocula were further analyzed over the time course of lactic acid utilization to determine which components may be related to this activity by spoilage bacteria in fermented cucumbers. Several of the changes in volatile compounds occurred within the first nine days of anaerobic incubation ($P < 0.001$), which was prior to the onset of lactic acid degradation. These changes included decreases in acetone, butanal, 3-methylbutanal, 3,3-dimethyl-2-butanone, 2-methyl-2-butenal, 2-ethyl-2-butenal, 3-methyl-2-butenal, 2-pentenal, furfural, benzaldehyde, and 4-methylbenzaldehyde. Increases were observed in

2-butanol, 3-methyl-2-butanol, 3-pentanol, 2-propen-1-ol, 2-pentanol, 3,3-dimethylbutan-2-ol, 2-methyl-3-pentanol, 4-penten-1-yl acetate, 4-methyl-2-pentanol, 3-ethyl-1-pentyn-3-ol, butanoic and pentanoic acids, alpha-methylbenzyl alcohol, and 4-methylbenzyl alcohol. Only a few of the changes occurred during the time that lactic acid was being degraded ($P < 0.001$). These included decreases in acetaldehyde, methanol, acetonitrile, propanenitrile, and hexanal and increases in cyclopentanone, 3,3-dimethyl-1-butanol, and 2-methylbenzyl alcohol.

Nontargeted detection of nonvolatile spoilage metabolites was accomplished by chemical derivatization of samples to form volatile trimethylsilyl (TMS)-metabolites. TMS-metabolites were separated and detected using comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS) that used a midpolar first dimension separation followed by a nonpolar second dimension separation. The extreme complexity of the TMS-derivatized samples is illustrated in Figure 5.3 panel A. Despite the complexity, the derivatization reagent artifacts were clearly separated from metabolites in the second dimension, and separation of metabolite peaks was achieved with a relatively short modulation time of 2.75 seconds. The yellow box highlighted in panel A is expanded in view B to demonstrate the effectiveness of the BPX-50/BPX-5 column combination in resolving TMS-metabolites. Panel C is a one dimensional representation of the TMS-glycerol peak that was captured in 13 slices. Although the peak shape of the TMS-glycerol in one dimension suggests a well-separated single component, panel D illustrates

the separation of at least 8 components by the second dimension column at a single first dimension elution time. Further separation of peaks based on individual masses was achieved by the ChromaTOF® software deconvolution algorithm applied during data processing. Of the 4,888 aligned peaks per sample, only 1033 peaks were tentatively identified by mass spectral match with >800 similarity to the Fiehn metabolomics library, indicating the need to expand mass spectral libraries for TMS-metabolites. Identified metabolites were from a wide variety of chemical classes, including amino acids, organic acids, sugars, sugar alcohols, nucleotides, amines, disaccharides and fatty acids.

The 666 metabolite peaks (approximately 14% of total metabolite peaks) that changed significantly with spoilage ($P < 0.01$) after 146 days of anaerobic incubation were subjected to HCA (Figure 5.4). Similar trends were observed in the clustering of nonvolatile metabolites as previously described for the volatile spoilage metabolites. However, very few nonvolatile metabolites increased during spoilage. Of those that did, none were identified by mass spectral library match. The metabolites that decreased in FCS as a result of spoilage by both *L. buchneri* and mixed spoilage cultures are presented in Table 5.3. Similar to what was observed in the volatile composition changes in FCS, the majority of the nonvolatile metabolite changes preceded lactic acid utilization. The HCA of TMS-metabolite peaks revealed that sample treatments were clustered by their metabolite profiles according to the inoculum source that was used rather than the time course of lactic acid utilization (Figure 5.5). The most numerous metabolite changes occurred in the first 9 days

of anaerobic incubation, prior to lactic acid utilization, as illustrated by the large and distinct cluster at the bottom of Figure 5.5. The several columns on the left side of the figure represent the uninoculated control samples for days 9, 22, 51, 77, 96, and 146. The dark red bars in the dendogram indicate the presence of these metabolites in FCS, and the clear change to blue for all columns to the right indicate the degradation of these compounds by all three spoilage inocula prior to the onset of lactic acid utilization. Several of these metabolites were carbohydrates. These mono- and disaccharides may be serving as energy sources for the growth of spoilage organisms, which would account for the small increase in cell density that is usually observed during this early period of spoilage.

Conclusions

Nontargeted profiling of volatile and nonvolatile biochemical components during fermented cucumber spoilage revealed a number of changes that were previously unknown. In total, 92 compounds were found to change during spoilage by *L. buchneri*, reduced NaCl and commercial spoilage organisms from among more than 5000 peaks detected. Generally, decreases were observed in sugars, amino acids, nucleosides, long chain fatty acids, aldehydes, and ketones, and increases were observed in alcohols and short chain fatty acids. The majority of these changes preceded lactic acid utilization, indicating that lactic acid is not a preferred substrate for spoilage organisms in fermented cucumbers. The ability to detect biochemical changes that precede lactate utilization may provide an

opportunity to identify indicator compounds for the initiation of spoilage prior to any significant product degradation.

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Table 5.1 Volatile metabolites that decreased significantly ($P < 0.01$) during anaerobic spoilage of fermented cucumber slurry by *L. buchneri* and spoilage cultures from reduced NaCl and commercial fermentation sources

Metabolite ID	CAS[1] registry #	Method of Identification[2]	Similarity	Reverse	Prob	RI[3]	t_{r1} (sec)	t_{r2} (sec)	Mass
cis-1,2-dimethyl-cyclopropane	930-18-7	MS	909	910	1773	508	165.00	0.645	55
Acetaldehyde	75-07-0	MS, RI, ST	944	945	9193	702	181.50	0.615	29
Acetone	67-64-1	MS, RI, ST	902	903	6644	815	220.00	0.695	43
Butanal	123-72-8	MS, RI	900	900	8303	874	255.75	0.790	72
Methanol	67-56-1	MS, RI	921	936	9174	897	269.50	0.635	31
3-methylbutanal	590-86-3	MS, RI, ST	840	840	8454	915	286.00	0.890	41
ethyloxirane	106-88-7	MS	842	862	7098	934	305.25	0.785	72
2,4,5-trimethyl-1,3-dioxolane	3299-32-9	MS	836	885	9052	940	310.75	0.970	101
3,3-dimethyl-2-butanone	75-97-8	MS, RI, ST	858	870	5026	945	316.25	1.000	57

Table 5.1 Continued

Acetonitrile	75-05-8	MS, RI, ST	834	915	5965	1003	374.00	0.690	41
methyl isobutyl ketone	108-10-1	MS, RI, ST	900	900	5639	1007	379.50	1.035	43
Propanenitrile	107-12-0	MS, RI	884	918	9115	1025	404.25	0.775	54
Hexanal	66-25-1	MS, RI, ST	905	906	8095	1078	475.75	1.085	41
5-hexen-2-one	109-49-9	MS, RI	820	864	5169	1084	484.00	0.970	43
trans-2-methyl-2-butenal	497-03-0	MS, RI	888	919	6232	1095	497.75	0.940	84
3-methyl-2-hexanone	2550-21-2	MS, RI	839	874	5141	1103	508.75	1.215	72
1-cyclopropylethanone	765-43-5	MS	871	881	3464	1126	544.50	0.910	69
trans-2-pentenal	1576-87-0	MS, RI, ST	887	887	4466	1129	550.00	0.950	57
trans-2-ethyl- 2-butenal	63883-69-2	MS, RI	903	906	5826	1156	591.25	1.070	41
4-methyl-4-penten-2-ol	2004-67-3	MS	873	883	6556	1178	624.25	1.155	56

Table 5.1 Continued

5-methylisoxazole	5765-44-6	MS	854	904	9107	1199	657.25	0.820	83
3-methyl-2-butenal	107-86-8	MS, RI	922	935	5715	1199	657.25	0.940	59
1-chloro-2-propanone	78-95-5	MS	915	916	9447	1209	673.75	0.780	49
carbonic acid, ethyl isobutyl ester	0-00-0	MS	838	863	7776	1213	679.25	1.045	56
analyte 458	--	--	--	--	--	1322	852.50	0.870	43
analyte 537	--	--	--	--	--	1430	1020.25	1.115	73
Furfural	98-01-1	MS, RI, ST	851	879	7237	1460	1064.25	0.780	95
1,1-dimethyl-3-chloropropanol	1985-88-2	MS	874	933	8508	1471	1080.75	0.835	59
2-acetylfuran	1192-62-7	MS, RI	635	858	6023	1501	1124.75	0.825	95
Benzaldehyde	100-52-7	MS, RI	884	886	6061	1520	1152.25	0.870	77

Table 5.1 Continued

4-methyl-benzaldehyde	104-87-0	MS, RI	935	935	3796	1645	1328.25	0.940	91
neodecanoic acid	26896-20-8	MS	804	811	4468	2038	1691.25	0.695	88

[1] Chemical Abstracts Service registry number

[2] MS: identification based on mass spectral match to the NIST 05 library with >800 similarity, RI: comparison with published retention indices on polyethylene glycol column phase, ST: mass spectral and retention index match to authentic standard

[3] Retention indices based on first dimension retention of components on a SOL-GEL-WAX (polyethylene glycol) column using SPME GCxGC-ToFMS

[4] Mass selected by ChromaTOF software during automated data processing to represent an interference free mass for each analyte; The unique mass for each component was used for calculation of peak area

Table 5.2 Volatile metabolites that increased significantly (*P* < 0.01) during anaerobic spoilage of fermented cucumber slurry by *L. buchneri* and spoilage cultures from reduced NaCl and commercial fermentation sources

Metabolite ID	CAS[1] registry #	Method of Identification[2]	Similarity	Reverse	Probability	RI[3]	t_{r1} (sec)	t_{r2} (sec)	Mass[4]
2-butanol	78-92-2	MS, RI	920	921	4783	1021	398.75	0.800	45
3-methyl-2-butanol	598-75-4	MS, RI	917	919	6239	1092	495.00	0.825	45
3-pentanol	584-02-1	MS, RI, ST	906	916	6628	1106	514.25	0.830	59
2-propen-1-ol	107-18-6	MS, RI	855	875	7402	1111	522.50	0.690	57
2-pentanol	6032-29-7	MS, RI	918	921	5523	1118	533.50	0.825	45
3,3-dimethylbutane-2-ol	464-07-3	MS, RI	831	845	5275	1122	539.00	0.875	57
analyte 307	--	--	--	--	--	1124	541.75	1.385	97
2-methyl-3-pentanol	565-67-3	MS, RI	931	932	8508	1152	585.75	0.905	59
Cyclopentanone	120-92-3	MS, RI	883	907	8047	1179	627.00	0.980	55

Table 5.2 Continued

4-penten-1-yl acetate	1576-85-8	MS, RI	816	817	6838	1190	643.50	1.065	43
3-hexanol	623-37-0	MS, RI	797	856	5901	1192	646.25	0.900	55
analyte 377	--	--	--	--	--	1213	679.25	0.895	71
4-methyl-2-pentanol	108-11-2	MS, RI, ST	905	905	3802	1216	684.75	0.885	45
3,3-dimethyl-1-butanol	624-95-3	MS, RI	814	839	3967	1252	742.50	0.850	69
analyte 410	--	--	--	--	--	1263	759.00	0.905	82
2-heptanol	543-49-7	MS, RI, ST	941	942	6955	1315	841.50	0.950	45
3-hexen-1-ol	544-12-7	MS, RI	902	921	6325	1358	910.25	0.830	67
3-methyl-1-hexanol	13231-81-7	MS, RI	881	895	3781	1414	995.50	0.890	70
butylcyclopropane	930-57-4	MS	869	870	1741	1454	1056.00	0.895	56
3-ethyl-1-pentyn-3-ol	6/9/6285	MS	821	831	4002	1478	1091.75	0.815	57

Table 5.2 Continued

analyte 611	--	--	--	--	--	1536	1177.00	0.740	49
1-octanol	111-87-5	MS, RI	800	902	2082	1557	1207.25	0.930	56
butanoic acid	107-92-6	MS, RI, ST	869	870	8859	1622	1298.00	0.680	60
pentanoic acid	109-52-4	MS, RI, ST	910	910	8007	1730	1430.00	0.665	60
à-methylbenzyl alcohol	98-85-1	MS, RI	857	860	3018	1804	1507.00	0.720	79
benzyl alcohol	100-51-6	MS, RI	910	911	7213	1869	1562.00	0.675	79
4-methylbenzyl alcohol	589-18-4	MS, RI, ST	903	904	4285	1957	1630.75	0.695	79
2-methylbenzyl alcohol	89-95-2	MS, RI	850	879	7041	1986	1652.75	0.685	104
analyte 793	--	--	--	--	--	2001	1663.75	0.685	74
analyte 796	--	--	--	--	--	2008	1669.25	0.680	74

[1] Chemical Abstracts Service registry number

Table 5.2 Continued

[2] MS: identification based on mass spectral match to the NIST 05 library with >800 similarity, RI: comparison with published retention indices on polyethylene glycol column phase, ST: mass spectral and retention index match to authentic standard

[3] Retention indices based on first dimension retention of components on a SOL-GEL-WAX (polyethylene glycol) column using SPME GCxGC-ToFMS

[4] Mass selected by ChromaTOF software during automated data processing to represent an interference free mass for each analyte; The unique mass for each component was used for calculation of peak area

Table 5.3 Nonvolatile metabolites that decreased significantly ($P < 0.01$) during anaerobic spoilage of fermented cucumber slurry in *L. buchneri* and spoilage cultures from reduced NaCl and commercial fermentation sources

Metabolite ID	CAS[1] registry #	Method of Identification[2]	Similarity	Reverse	Probability	RI[3]	t_{r1} (sec)	t_{r2} (sec)	Mass[4]
D-alanyl-D-alanine	923-16-0	MS	844	844	7299	1065	483.25	0.820	116
lactic acid	50-21-5	MS	921	939	9796	1180	653.57	0.990	43
Isoleucine	73-32-5	MS, ST	876	880	7564	1296	833.71	1.420	45
Uracil	66-22-8	MS	923	938	9832	1461	1088.25	1.110	99
Lyxose	1114-34-7	MS	963	963	4741	1617	1312.86	1.590	103
Xylose	6763-34-4	MS, ST	871	871	3946	1625	1324.66	1.570	103
Synephrine	94-07-5	MS	780	823	6227	1720	1455.96	1.190	116

Table 5.3 Continued

3,6-Anhydro-D-galactose	14122-18-0	MS	748	754	9740	1767	1514.23	1.410	231
Fructose	57-48-7	MS, ST	968	970	4528	1784	1534.88	1.750	103
3-methylamino-1,2-propanediol	40137-22-2	MS	723	774	7556	1808	1564.55	1.230	116
3,6-Anhydro-D-galactose	14122-18-0	MS	792	802	9082	1814	1576.15	1.390	95
Allose	2595-97-3	MS	871	874	2023	1828	1589.58	1.760	157
unidentified carbohydrate	--	--	--	--	--	1830	1600.61	1.520	204
Sorbose	3615-56-3	MS	840	848	2672	1832	1594.70	1.550	103
citrulline 1	372-75-8	MS	934	950	9768	1879	1656.09	1.350	157
citrulline 2	372-75-8	MS	841	865	9451	1890	1672.04	1.170	70

Table 5.3 Continued

Glucose	50-99-7	MS, ST	918	918	2419	1892	1576.25	1.790	206
2-Amino-1-phenylethanol	7568-93-6	MS	816	848	3284	2016	1819.60	1.340	174
unidentified amino acid	--	--	--	--	--	2028	1835.46	1.340	158
palmitic acid	57-10-3	MS	955	973	9035	2104	1919.09	1.420	87
unidentified carbohydrate	--	--	--	--	--	2204	2030.02	1.720	204
unidentified carbohydrate	--	--	--	--	--	2498	2284.72	1.420	204
Cellobiose	528-50-7	MS, ST	893	893	2150	2518	2295.73	1.390	204
Trehalose	99-20-7	MS, ST	895	898	4800	2558	2317.74	1.350	361
unidentified disaccharide	--	--	--	--	--	2572	2325.61	1.340	204

Table 5.3 Continued

unidentified disaccharide	--	--	--	--	--	2638	2357.83	1.280	204
Gentiobiose	554-91-6	MS, ST	929	929	3903	2662	2369.06	1.250	204
stearic acid	57-11-4	MS	914	936	8447	2742	2404.98	0.680	117
Adenosine	58-61-7	MS	770	775	9566	2776	2417.77	0.990	236
Galactinol	3687-64-7	MS	839	849	4327	2965	2504.89	1.670	204
5'-methylthioadenosine	2457-80-9	MS	864	870	9815	3067	2559.81	1.190	236

[1] Chemical Abstracts Service registry number

[2] MS: identification based on mass spectral match to the LECO-Fiehn Rtx05 library with >800 similarity, ST: mass spectral and retention index match to authentic standard

[3] Retention indices of the trimethylsilylated metabolite based on first dimension retention of components on a BPX50 (50% phenyl 50% polydimethylsiloxane) column using GCxGC-ToFMS

[4] Mass selected by ChromaTOF software during automated data processing to represent an interference free mass for each analyte; The unique mass for each component was used for calculation of peak area

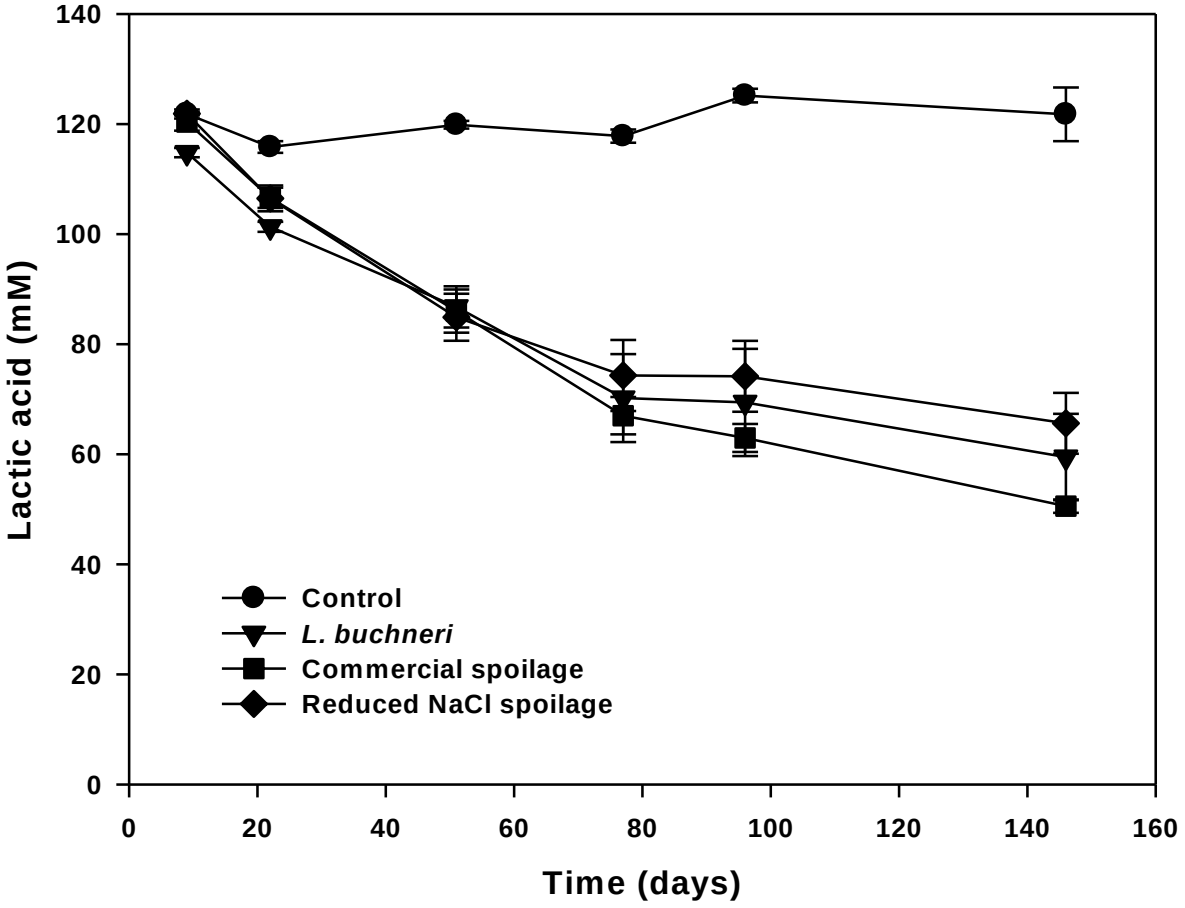


Figure 5.1 Lactic acid utilization in fermented cucumber slurry (pH 3.8, 6% NaCl) by *L. buchneri* and spoilage organisms from reduced NaCl and commercial fermentation sources

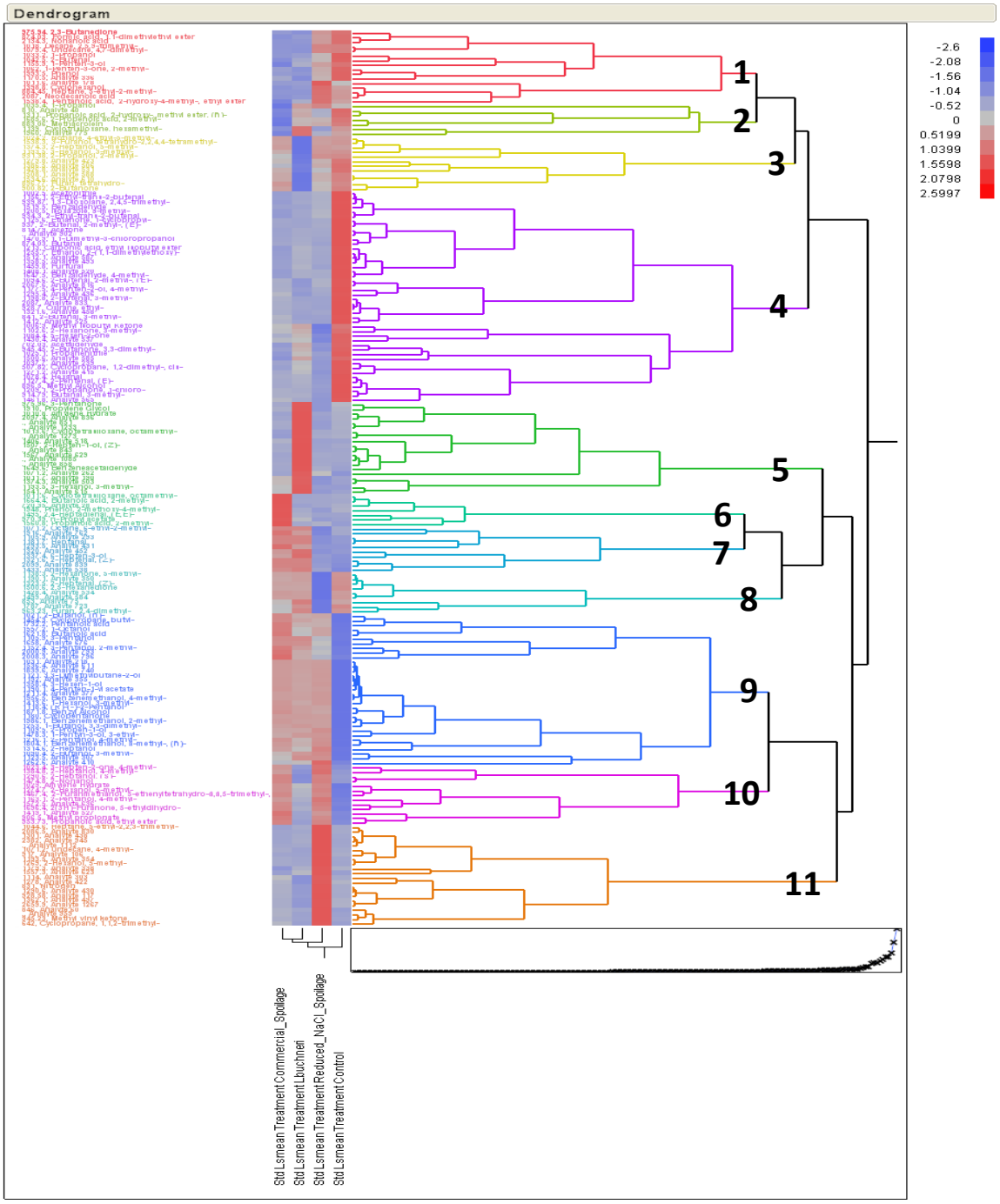


Figure 5.2 Hierarchial clustering of volatile metabolites that changed significantly (*P* < 0.01) in fermented cucumber slurry after 146 days anaerobic incubation with spoilage organisms

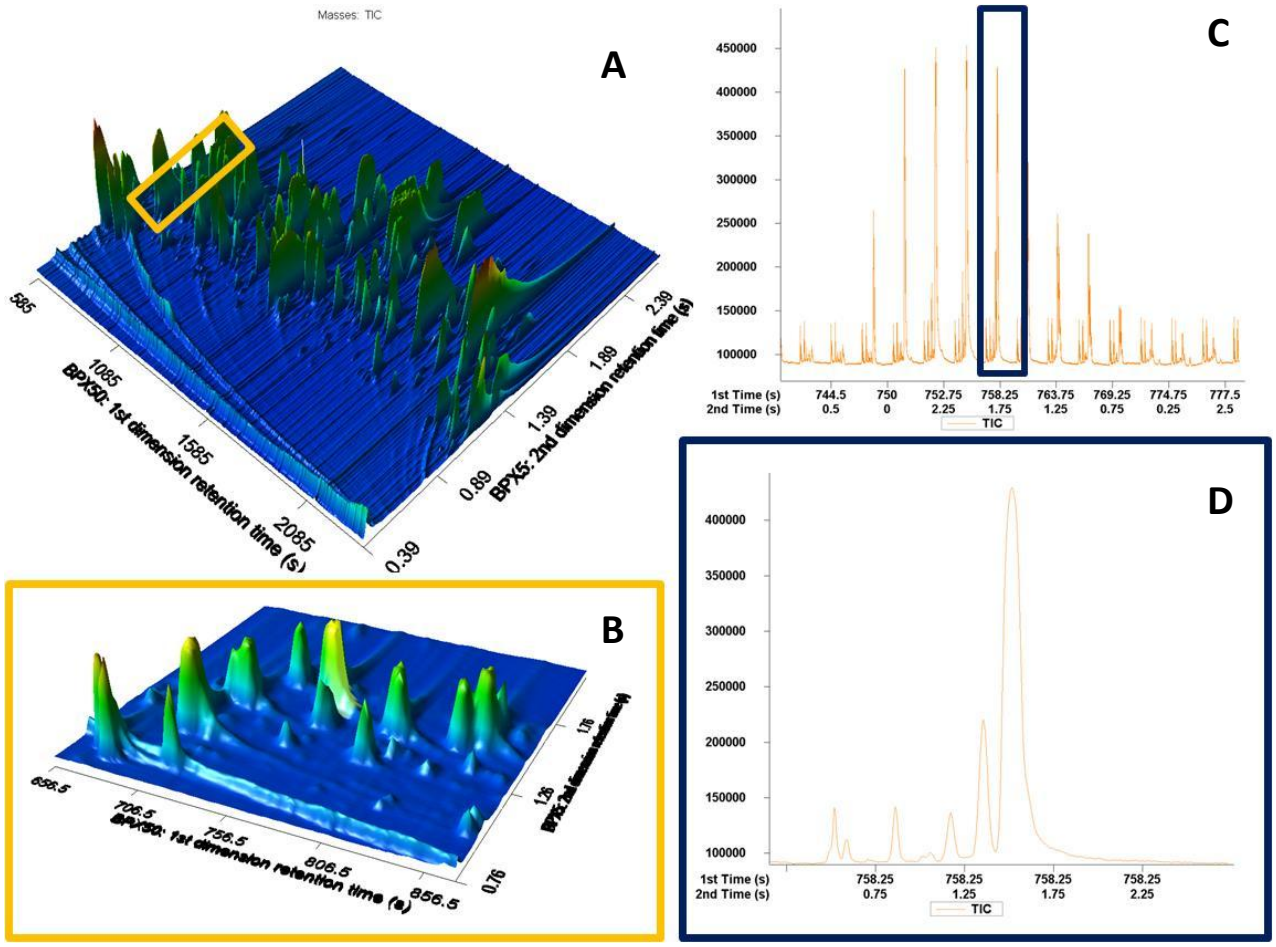


Figure 5.3 Two-dimensional separation of TMS-metabolites of fermented cucumber slurry by GCxGC-ToFMS

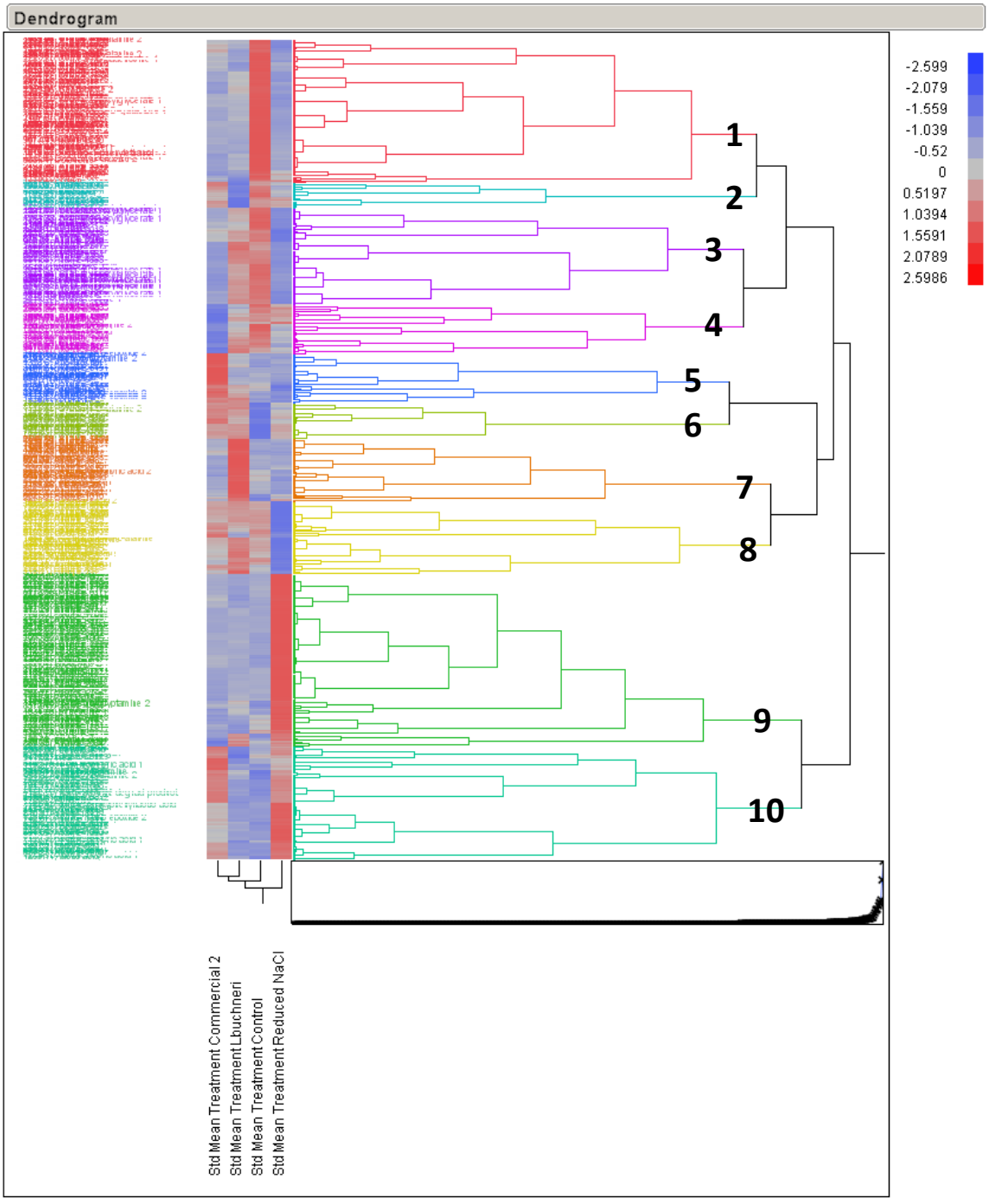


Figure 5.4 Hierarchial clustering of TMS-metabolite peaks that changed significantly (*P* <0.01) in fermented cucumber slurry after 146 days anaerobic incubation with spoilage organisms

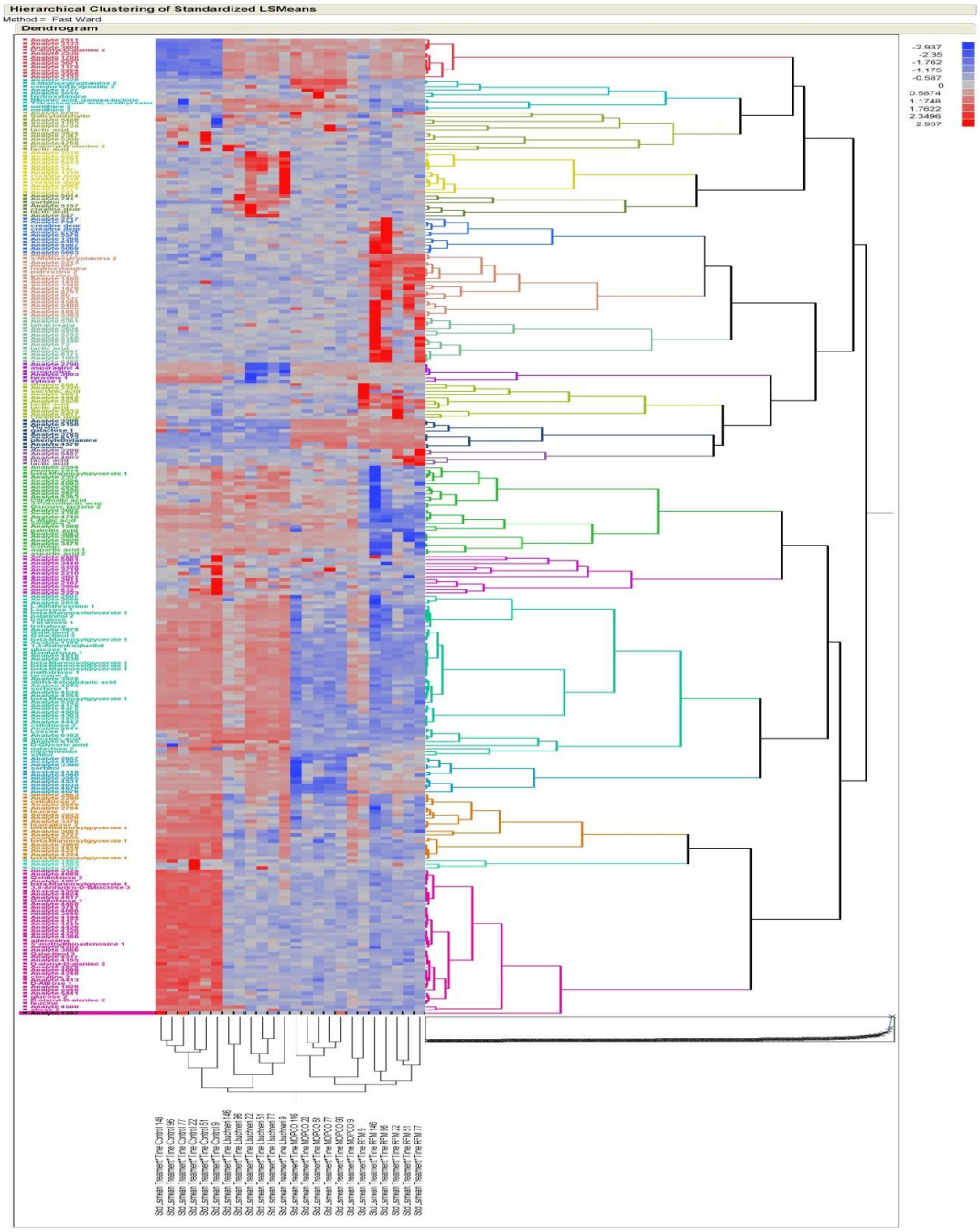


Figure 5.5 Hierarchial clustering of TMS-metabolite peaks that changed significantly (*P* < 0.0001) in fermented cucumber slurry during anaerobic incubation with spoilage organisms

Chapter 6

Conclusions

Fermentation of cucumbers with 6% sodium chloride to a terminal pH of 3.2 prevented lactic acid utilization during long term anaerobic incubation with spoilage organisms. However, lactic acid was degraded by spoilage microorganisms in cucumbers fermented with 0, 2, and 4% NaCl, even with a terminal pH as low as 3.2. *Lactobacillus buchneri* was isolated from spoiled fermented cucumbers and able to initiate lactic acid utilization at pH 3.8 under both aerobic and anaerobic conditions. Lactic acid was converted to acetic acid and 1,2-propanediol, resulting in a rise in pH that may create an opportunity for other bacterial species to become metabolically active and contribute to spoilage. 1,2-propanediol added to FCS was converted to propionic acid and propanol by *Lactobacillus rapi* at a rate that would not allow accumulation of 1,2-propanediol in fermentation brine. The spoilage biochemistry that has been observed in fermented cucumbers is consistent with the sum of the activities of these organisms: decreased lactic acid and increased pH, acetic acid, propionic acid, and propanol. However, these organisms would not account for the butyric acid that is frequently present in spoiled commercial fermentations, and we believe that butyric acid may be produced by *Clostridia* sp that are able to become active after the pH has risen (Fleming and others 1989). *Lactobacillus parafarraginis*, a close relative of *L. buchneri*, was also isolated from spoiled cucumber fermentations and demonstrated the ability to convert lactic acid to acetic acid and 1,2-propanediol. As such, this organism may also contribute to fermented cucumber spoilage. Several other species of LAB were present in spoiled fermented cucumber brines, including *L. plantarum*, *L.*

brevis, *L. casei*, *L. coryniformis*, *L. camelliae*, *L. harbinensis/perolens*, *P. ethanolidurans*, and *P. parvulus*, but these organisms did not exhibit lactic acid or 1,2-propanediol degrading ability in FCS. Nonetheless, there may be other lactate or 1,2-propanediol degrading organisms present in fermented cucumbers that have yet to be isolated and identified.

Lactic acid utilization by *L. buchneri* occurred under a variety of environmental conditions that may be encountered in commercial cucumber fermentations. Lactic acid was utilized at a faster rate and to a greater extent in 2% NaCl FCS than in 0, 4, or 6% NaCl FCS. Ongoing efforts to reduce sodium in commercial brining operations will need to include measures for controlling the increased susceptibility to spoilage of cucumbers fermented and stored with lower NaCl concentrations. *L. buchneri* may be an appropriate target for development of such control measures.

Nontargeted profiling of volatile and nonvolatile biochemical components during fermented cucumber spoilage revealed a number of changes that were previously unknown. In total, 92 additional compounds were found to change during spoilage by *L. buchneri*, reduced NaCl and commercial spoilage organisms. Generally, decreases were observed in sugars, amino acids, nucleotides, long chain fatty acids, aldehydes, and ketones, and increases were observed in alcohols and short chain fatty acids. The majority of these changes preceded lactic acid utilization, which may provide an opportunity to identify indicator compounds for the initiation of spoilage prior to any significant product degradation.

Appendices

Appendix 1

Supplemental data to Chapter 2: Environmental and microbial factors affecting anaerobic lactic acid utilization during spoilage of fermented cucumbers

Supplement 1: Chemical changes in sterile-filtered fermented cucumber slurry during spoilage reproductions R1, R2, and R4 as outlined in Figures 2.1, 2.2 and 2.3.

Table A1.1 Changes in organic acids during anaerobic lactic acid degradation in cucumbers fermented with 0, 2, 4, or 6% NaCl adjusted to pH 3.8, 4.3, or 5.0 (spoilage R1)

NaCl[1] (%)	pH[2]	Spoilage Source[3]	Time (days)	Lactic acid (mM)	Acetic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	3.2	Reduced NaCl	0	111.5 ± 6.7	9.8 ± 0.6	ND[4]	ND
			21	107.35 ± 1.6	7.9 ± 1	ND	ND
			57	94.1 ± 2.6	23.1 ± 1.7	7.8 ± 1.2	ND
			176	13.5 ± 2.0	55.8 ± 4.7	29.3 ± 4.2	ND
			374	8.9 ± 0.7	69.0 ± 0.2	36.5 ± 1.5	ND
0	3.2	Commercial 1M	0	119.7 ± 0.2	8.7 ± 1.0	ND	ND
			21	120.8 ± 1.5	8.5 ± 1.1	ND	ND
			57	113.6 ± 8.5	22.1 ± 0.3	ND	ND
			176	56.3 ± 5.3	52.9 ± 3.0	1.1 ± 1.9	ND
			374	14.4 ± 3.7	70.3 ± 0.9	2.1 ± 3.7	ND
2	3.2	Reduced NaCl	0	124.6 ± 3.3	12.0 ± 0.7	ND	ND
			21	120.5 ± 1.9	10.4 ± 0.3	ND	ND
			57	130.5 ± 3.6	14.5 ± 1.8	2.1 ± 1	ND
			176	61.0 ± 7.6	47.7 ± 9	22.8 ± 5.7	ND
			374	14.0 ± 0.2	71.1 ± 0.4	35.1 ± 1.5	ND
2	3.2	Commercial 1M	0	133.0 ± 1.0	9.5 ± 0.5	ND	ND
			21	128.8 ± 3.2	9.0 ± 0.8	ND	ND
			57	135.4 ± 1.6	14.0 ± 1.0	ND	ND
			176	41.5 ± 1.6	67.7 ± 2.0	ND	ND
			374	18.4 ± 0.8	72.7 ± 0.3	ND	ND

Table A1.1 Continued

4	3.2	Reduced NaCl	0	123.7 ± 2.1	12.3 ± 0.6	ND	ND
			21	124.1 ± 10.4	11.8 ± 0.9	ND	ND
			57	142.5 ± 5.9	12.9 ± 0.8	0.4 ± 0.7	ND
			176	134.2 ± 2.1	16.7 ± 1	1.5 ± 1.3	ND
			374	100.2 ± 4.6	19.5 ± 0.3	2.1 ± 0.1	ND
4	3.2	Commercial 1M	0	132.0 ± 15.8	10.6 ± 1.4	ND	ND
			21	143.6 ± 1.0	12.1 ± 0.1	ND	ND
			57	149.1 ± 10.2	11.7 ± 1.3	ND	ND
			176	37.1 ± 1.9	68.2 ± 3.5	ND	ND
			374	20.5 ± 0.6	65.5 ± 0.6	ND	ND
6	3.2	Reduced NaCl	0	106.2 ± 2.5	13.2 ± 1.8	2.9 ± 0.8	ND
			21	110.6 ± 12.8	13.5 ± 1.3	2.2 ± 0.7	ND
			57	120.5 ± 13.1	13.9 ± 2.6	2.1 ± 1.2	ND
			176	116.5 ± 5.1	15.8 ± 4.6	3.2 ± 1.6	ND
			374	91.7 ± 5.4	14.4 ± 5.3	3.5 ± 2.2	ND
6	3.2	Commercial 1M	0	115.4 ± 0.5	10.7 ± 0.2	ND	ND
			21	121.6 ± 9.3	11.6 ± 0.9	ND	ND
			57	121.5 ± 0.5	9.5 ± 0.5	ND	ND
			176	130.7 ± 2.4	14.1 ± 0.6	ND	ND
			374	99.7 ± 0.5	25.8 ± 0.3	1.3 ± 1.1	ND
0	3.8	Reduced NaCl	0	111.5 ± 6.7	9.8 ± 0.6	ND	ND
			21	112 ± 1.6	16.0 ± 4.9	ND	ND
			57	52.5 ± 1.2	41.8 ± 4.4	16.5 ± 1.5	ND
			176	20.1 ± 1.4	61.0 ± 6.2	22.2 ± 2.1	ND
			374	18.2 ± 0.3	65.6 ± 0.2	24.6 ± 0.6	ND

Table A1.1 Continued

0	3.8	Commercial 1M	0	119.7 ± 0.2	8.7 ± 1.0	ND	ND
			21	132.6 ± 5.7	11.6 ± 1.0	ND	ND
			57	107.6 ± 3.2	21.4 ± 3.5	ND	ND
			176	75.1 ± 1.9	46.5 ± 1.3	11.7 ± 3.3	ND
			374	36.9 ± 4.2	59.2 ± 3.0	16.5 ± 3.4	ND
2	3.8	Reduced NaCl	0	124.6 ± 3.3	12 ± 0.7	1.4 ± 1.2	ND
			21	100 ± 2.7	23.3 ± 0.5	1.9 ± 3.4	ND
			57	65.9 ± 5.0	43.3 ± 0.3	13.5 ± 0.3	ND
			176	40.9 ± 1.0	63.4 ± 2.2	21.5 ± 0.5	ND
			374	21.5 ± 18.6	67.8 ± 5.1	35.3 ± 21.8	ND
2	3.8	Commercial 1M	0	133.0 ± 1.0	9.5 ± 0.5	ND	ND
			21	137.2 ± 0.5	17.8 ± 2.3	ND	ND
			57	103.5 ± 10.7	29.2 ± 2.9	3.8 ± 1.0	ND
			176	73.7 ± 4.1	50.7 ± 7.8	8.6 ± 0.9	ND
			374	29.1 ± 25.2	54.9 ± 12.9	14.8 ± 0.3	11.7 ± 20.3
4	3.8	Reduced NaCl	0	123.7 ± 2.1	12.3 ± 0.6	1.5 ± 1.3	ND
			21	113.1 ± 6.1	28.8 ± 3.2	5.3 ± 1.1	ND
			57	47.1 ± 3.5	46.9 ± 4.2	12.2 ± 1.3	ND
			176	39.7 ± 1.1	62.5 ± 0.2	19.2 ± 0.5	ND
			374	22.2 ± 19.2	62.4 ± 3.5	32.0 ± 23.1	ND
4	3.8	Commercial 1M	0	132.0 ± 15.8	10.6 ± 1.4	ND	ND
			21	144.4 ± 14.5	17.3 ± 3.6	ND	ND
			57	119.1 ± 11.6	30.2 ± 3.0	3.6 ± 0.9	ND
			176	68.4 ± 2.7	57.1 ± 1.2	15.3 ± 1.7	ND
			374	47.6 ± 2.1	56.5 ± 0.6	15.8 ± 1.8	ND

Table A1.1 Continued

6	3.8	Reduced NaCl	0	106.2 ± 2.5	13.2 ± 1.8	2.9 ± 0.8	ND
			21	126.3 ± 7.9	28.5 ± 2.4	3.6 ± 0.6	ND
			57	78.5 ± 2	52.6 ± 3.2	11.4 ± 0.7	ND
			176	51.8 ± 0.9	62.5 ± 3.8	16.1 ± 1.3	ND
			374	38.4 ± 0.2	57.0 ± 0.3	15.4 ± 0.1	ND
6	3.8	Commercial 1M	0	115.4 ± 0.5	10.7 ± 0.2	ND	ND
			21	168.1 ± 11.7	16.3 ± 0.9	ND	ND
			57	160.8 ± 3.5	17.4 ± 0.7	ND	ND
			176	85.4 ± 0.4	48.6 ± 2.3	19.9 ± 0.2	ND
			374	51.2 ± 3.8	52.3 ± 2.0	23.4 ± 1.7	ND
0	4.3	Reduced NaCl	0	111.5 ± 6.7	9.8 ± 0.6	ND	ND
			21	87.7 ± 0.4	20.5 ± 1.3	2.9 ± 2.5	ND
			57	63.7 ± 1.6	31.8 ± 4.4	7.6 ± 0.9	ND
			176	49.3 ± 3.3	44.8 ± 6.3	11.1 ± 1.4	ND
			374	43.9 ± 0.8	54.0 ± 0.3	13.9 ± 0.1	ND
0	4.3	Commercial 1M	0	119.7 ± 0.2	8.7 ± 1.0	ND	ND
			21	117.7 ± 1.8	11.8 ± 2.6	ND	ND
			57	109.2 ± 1.3	17.3 ± 2.9	ND	ND
			176	7.4 ± 12.9	57.7 ± 1.4	72.5 ± 10.2	ND
			374	ND	63.7 ± 0.2	83.8 ± 1.9	ND
2	4.3	Reduced NaCl	0	124.6 ± 3.3	12 ± 0.7	1.4 ± 1.2	ND
			21	88.9 ± 3.2	24.6 ± 1.8	ND	ND
			57	89.4 ± 1.8	27.8 ± 1.7	6.0 ± 0.5	ND
			176	89.5 ± 6	39.2 ± 4	7.7 ± 0.9	ND
			374	62.1 ± 4.3	51.3 ± 2.1	13.0 ± 0.9	ND

Table A1.1 Continued

2	4.3	Commercial 1M	0	133.0 ± 1.0	9.5 ± 0.5	ND	ND
			21	128.3 ± 0.6	13.6 ± 2.1	ND	ND
			57	122.7 ± 1.5	18.8 ± 2.1	ND	ND
			176	ND	45.9 ± 13.8	75.3 ± 10.5	20.4 ± 17.9
			374	ND	46.3 ± 17.4	77.9 ± 18.3	19.6 ± 17.1
4	4.3	Reduced NaCl	0	123.7 ± 2.1	12.3 ± 0.6	1.5 ± 1.3	ND
			21	104.8 ± 4.7	29.1 ± 1.4	3.3 ± 2.9	ND
			57	83.2 ± 8.2	41.5 ± 4.4	8.3 ± 0.8	ND
			176	70.0 ± 2.7	45.2 ± 3.4	8.8 ± 1.0	ND
			374	60.3 ± 1.4	47.1 ± 1.0	10.8 ± 0.2	ND
4	4.3	Commercial 1M	0	132.0 ± 15.8	10.6 ± 1.4	ND	ND
			21	141.6 ±11.6	13.7 ± 1.5	ND	ND
			57	134.2 ± 12.2	26.1 ± 3.6	1.2 ± 1.1	ND
			176	ND	65.8 ± 5.1	93.4 ± 5.8	ND
			374	ND	65.2 ± 1.1	94.4 ± 3.3	ND
6	4.3	Reduced NaCl	0	106.2 ± 2.5	13.2 ± 1.8	2.9 ± 0.8	ND
			21	106.0 ± 6.9	25.8 ± 2.5	2.4 ± 2.1	ND
			57	92.0 ± 1.4	41.8 ± 2.1	7.6 ± 1.2	ND
			176	79.7 ± 1.1	45.8 ± 3.2	9.8 ± 0.7	ND
			374	61.5 ± 2.8	42.6 ± 2.2	9.3 ± 0.6	ND
6	4.3	Commercial 1M	0	115.4 ± 0.5	10.7 ± 0.2	ND	ND
			21	146.9 ±11.0	12.7 ± 1.3	ND	ND
			57	152.2 ± 10.6	16.6 ± 0.8	ND	ND
			176	104.9 ± 1.6	41.1 ± 1.3	11.1 ± 0.8	ND
			374	4.2 ± 7.3	62.4 ± 2.1	82.1 ± 7.6	ND

Table A1.1 Continued

0	5.0	Reduced NaCl	0	111.5 ± 6.7	9.8 ± 0.6	ND	ND
			21	112.6 ± 1.9	12.3 ± 4.2	ND	ND
			57	104.1 ± 0.5	20 ± 1	ND	ND
			176	93.4 ± 5.8	28.4 ± 1	3.3 ± 0.2	ND
			374	82.3 ± 1.4	33.9 ± 0.1	5.6 ± 0.1	ND
0	5.0	Commercial 1M	0	119.7 ± 0.2	8.7 ± 1.0	ND	ND
			21	35.1 ± 7.9	40.7 ± 4.7	ND	ND
			57	ND	48.3 ± 15.6	73.4 ± 23.3	ND
			176	ND	56.1 ± 5.7	85.6 ± 9.9	ND
			374	ND	66.4 ± 0.4	102.6 ± 0.5	ND
2	5.0	Reduced NaCl	0	124.6 ± 3.3	12 ± 0.7	1.4 ± 1.2	ND
			21	122.1 ± 2.7	13.2 ± 1.2	ND	ND
			57	127.7 ± 1	16.1 ± 0.5	ND	ND
			176	108.1 ± 5.4	32.5 ± 5.2	7.2 ± 1.2	ND
			374	84.9 ± 0.6	39.4 ± 0.4	10.3 ± 0.4	ND
2	5.0	Commercial 1M	0	133.0 ± 1.0	9.5 ± 0.5	ND	ND
			21	6.4 ± 10.0	38.1 ± 14.0	ND	11.6 ± 10.0
			57	ND	41.6 ± 10.2	76.0 ± 9.9	13.6 ± 11.9
			176	ND	49.3 ± 12.0	86.3 ± 12.3	17.0 ± 11.9
			374	ND	49.2 ± 11.8	89.4 ± 11.1	18.1 ± 12.1
4	5.0	Reduced NaCl	0	123.7 ± 2.1	12.3 ± 0.6	1.5 ± 1.3	ND
			21	130.8 ± 10.4	13.7 ± 2.8	ND	ND
			57	134.6 ± 9.2	17 ± 1	ND	ND
			176	105.6 ± 10	29.7 ± 1.9	5.5 ± 0.3	ND
			374	73.9 ± 0.8	39.6 ± 0.1	12.7 ± 0.4	ND

Table A1.1 Continued

4	5.0	Commercial 1M	0	132.0 ± 15.8	10.6 ± 1.4	ND	ND
			21	121.1 ± 16.4	16.6 ± 1.6	5.4 ± 5.8	ND
			57	ND	49.4 ± 3.7	85.8 ± 5.4	6.5 ± 5.9
			176	ND	54.4 ± 0.9	93.1 ± 5.2	7.9 ± 7.4
			374	ND	56.1 ± 6.0	96.5 ± 4.8	7.8 ± 7.1
6	5.0	Reduced NaCl	0	106.2 ± 2.5	13.2 ± 1.8	2.9 ± 0.8	ND
			21	115.9 ± 7.8	18.4 ± 1.4	--	ND
			57	125.4 ± 1.4	26.4 ± 1.8	3.1 ± 0.3	ND
			176	108 ± 1.9	31.6 ± 0.9	4.6 ± 0.1	ND
			374	75.3 ± 4.3	35.4 ± 2.2	8.2 ± 0.9	ND
6	5.0	Commercial 1M	0	115.4 ± 0.5	10.7 ± 0.2	ND	ND
			21	140.3 ± 10.0	13.5 ± 1.2	ND	ND
			57	143.3 ± 10.3	16.4 ± 2.2	ND	ND
			176	14.4 ± 14.2	63.3 ± 3.5	96.1 ± 8.5	ND
			374	ND	62.1 ± 0.1	100.5 ± 0.4	ND

[1] Equilibrated concentration of NaCl used for fermentation in % (w/w)

[2] pH of fermented cucumber slurry at day 0

[3] Spoilage sources are described in Table 2.1

[4] ND=not detected

Table A1.2 Biochemical changes during anaerobic lactic acid utilization in FCS (6% NaCl, pH 3.8) by *L. buchneri* and spoilage organisms from reduced NaCl and commercial cucumber fermentations (spoilage R2)

Spoilage Source[1]	Time (mo)	Δ pH[2]	Δ Lactic acid[2] (mM)	Δ Acetic acid[2] (mM)	Δ 1,2-propanediol[2] (mM)	Δ Propionic acid[2] (mM)
Reduced NaCl cells	2	0.58	-33.2	21.7	ND	12.4
Reduced NaCl cells	6	0.65	-43.3	33.3	ND	21.8
Reduced NaCl-2	2	0.62	-35.6	26.1	ND	8.5
Reduced NaCl-2	6	0.81	-52.2	44.9	ND	17.8
Commercial 1M-2	2	0.22	-10.5	12.4	ND	3.2
Commercial 1M-2	6	0.57	-40.9	33.4	ND	14.2
L. buchneri	2	0.20	-6.0	7.1	5.8	ND
L. buchneri	6	0.56	-36.9	24.0	14.2	ND

[1] Spoilage sources are described in Table 2.1. Reduced NaCl-2 and Commercial 1M-2 were produced from serial reproduction of spoilage in FCS (6% NaCl, pH 3.8) with growth of the spoilage organsims in filter-sterilized, fresh cucumber slurry containing 6% NaCl.

[2] Δ = the difference between the FCS inoculated with spoilage organisms and the non-inoculated control for each of the biochemical changes measured. All changes were significant at $P < 0.0001$ (n=7).

[3] ND=not detected.

Table A1.3 Biochemical changes in FCS (pH 3.8) with varying NaCl concentrations after 22 weeks anaerobic incubation with spoilage organisms (spoilage R4)

NaCl[1] (%)	Spoilage Source[2]	pH	Lactic acid (mM)	Acetic acid (mM)	Propionic acid (mM)	Propanol (mM)
0	Control	3.85 ± 0.03	105.8 ± 4.1	68.1 ± 1.9	ND[3]	ND
0	Reduced NaCl	4.17 ± 0.08	82.0 ± 4.5	81.4 ± 2.9	10.7 ± 2.4	4.5 ± 1.0
0	Commercial 2	4.02 ± 0.03	95.1 ± 3.8	72.7 ± 1.2	6.5 ± 1.1	1.7 ± 1.5
2	Control	3.83 ± 0.03	106.4 ± 1.5	68.0 ± 0.7	ND	ND
2	Reduced NaCl	4.28 ± 0.07	60.7 ± 8.5	88.7 ± 6.3	17.8 ± 2.7	7.7 ± 2.2
2	Commercial 2	3.99 ± 0.03	93.8 ± 3.3	74.8 ± 0.0	6.8 ± 0.3	2.7 ± 0.4
4	Control	3.83 ± 0.02	104.1 ± 2.2	67.5 ± 0.1	ND	ND
4	Reduced NaCl	4.26 ± 0.02	59.9 ± 3.2	87.5 ± 2.6	18.5 ± 1.3	7.1 ± 1.0
4	Commercial 2	3.99 ± 0.04	88.6 ± 7.1	76.5 ± 1.9	8.6 ± 1.9	1.9 ± 1.8
6	Control	3.84 ± 0.03	103.3 ± 2.2	66.8 ± 0.9	ND	ND
6	Reduced NaCl	4.08 ± 0.02	81.7 ± 4.5	77.7 ± 0.6	10.4 ± 1.5	3.4 ± 0.5
6	Commercial 2	3.95 ± 0.07	93.2 ± 9.3	73.5 ± 5.4	5.4 ± 5.4	2.1 ± 2.5

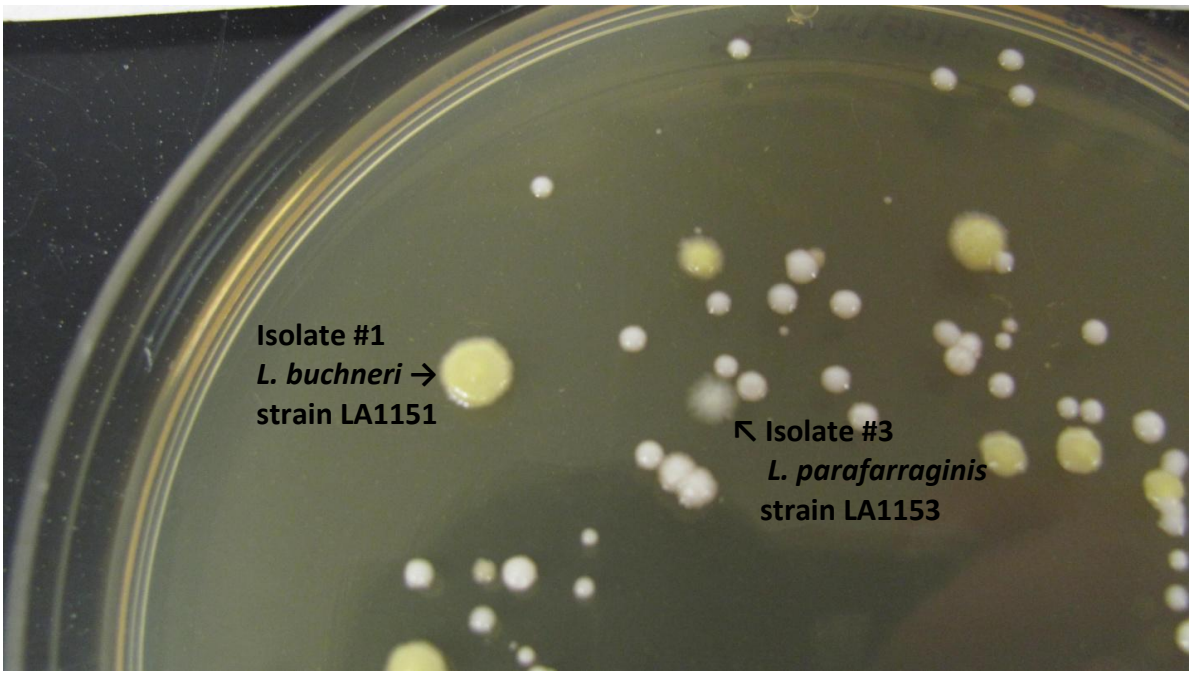
[1] Equilibrated NaCl concentration added to fermented cucumber slurry after fermentation in a no salt cover solution

[2] Spoilage sources are described in Table 2.1

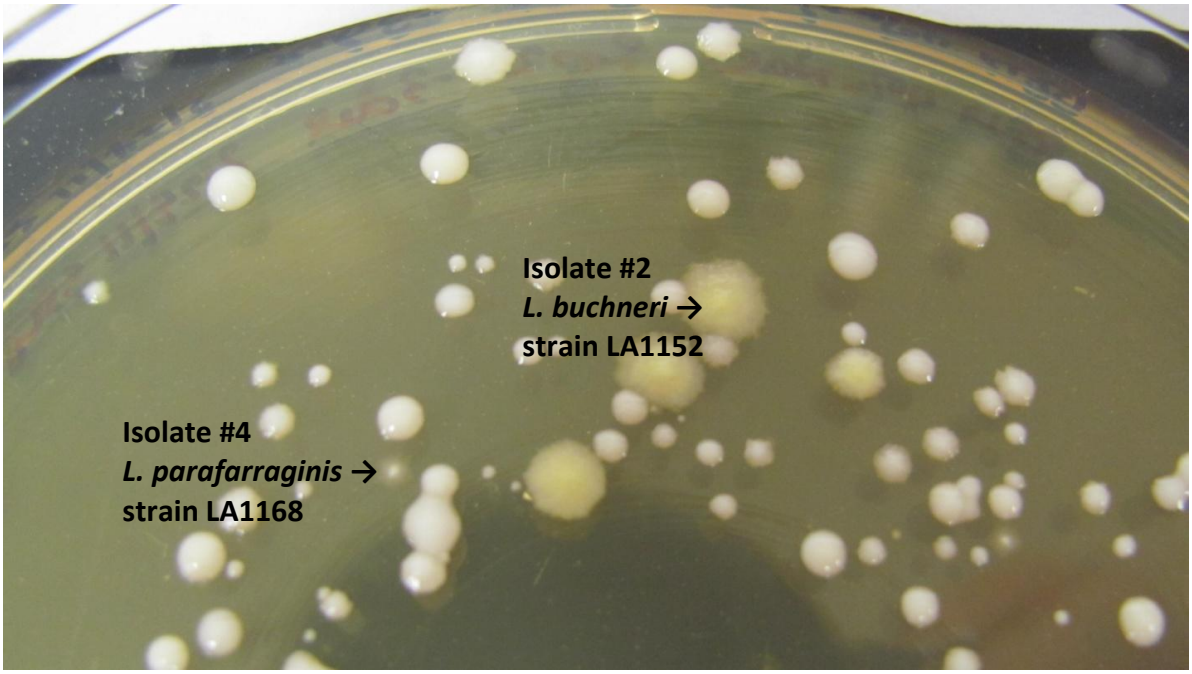
[3] ND=not detected

Supplement 2: Photographs of colonies of lactic acid bacteria isolated from spoiled fermented cucumber slurry that had been inoculated with reduced NaCl and commercial spoilage cultures. Samples that had demonstrated lactic acid degradation after 22 weeks of anaerobic incubation were spiral plated on MRS and incubated for 5 days at 30°C.

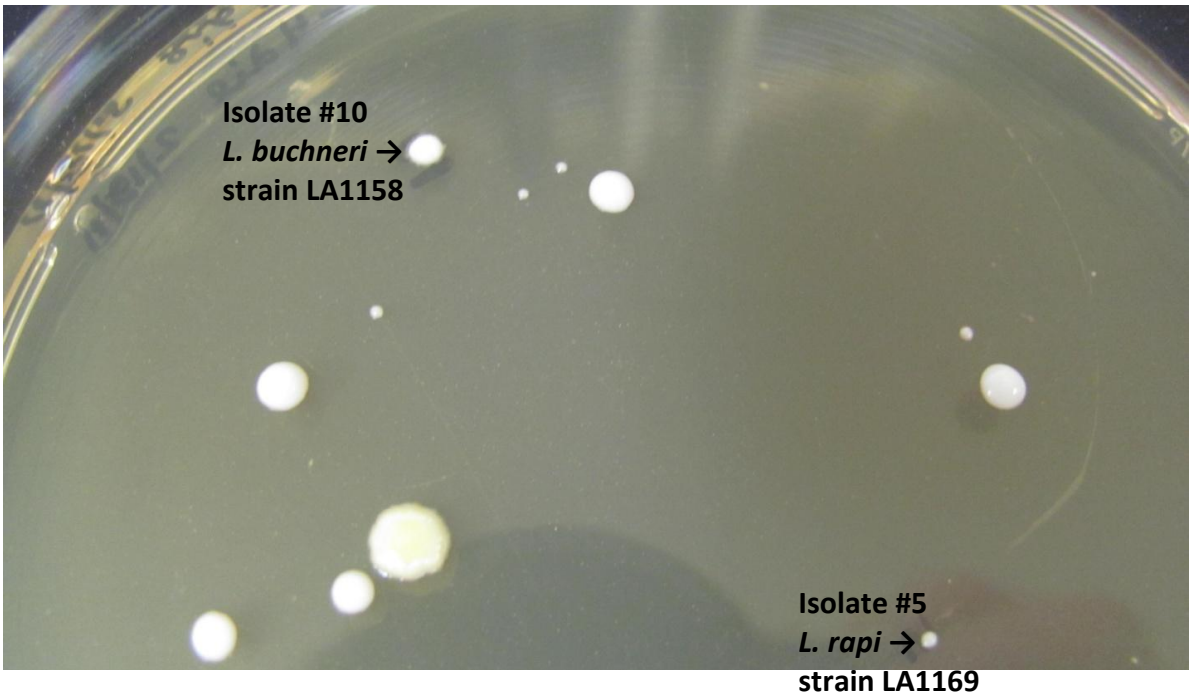
Reduced NaCl spoilage in FCS (4% NaCl, pH 3.8)



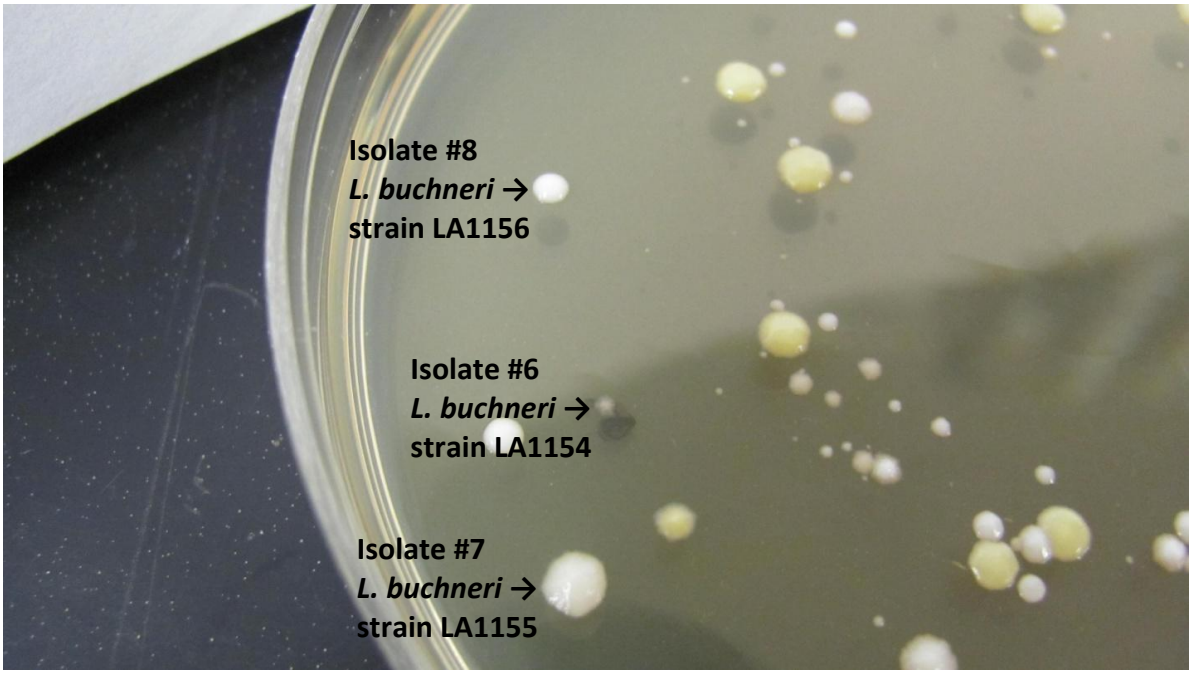
Reduced NaCl spoilage in FCS (4% NaCl, pH 3.8)



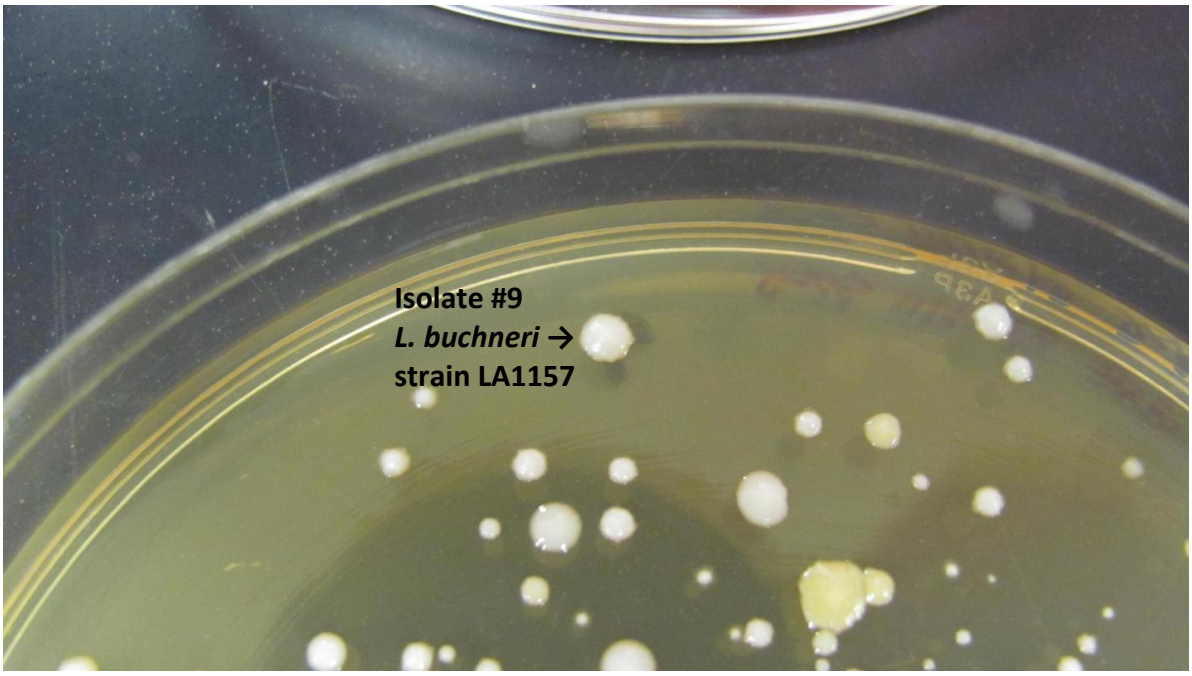
Reduced NaCl spoilage in FCS (4% NaCl, pH 3.8)



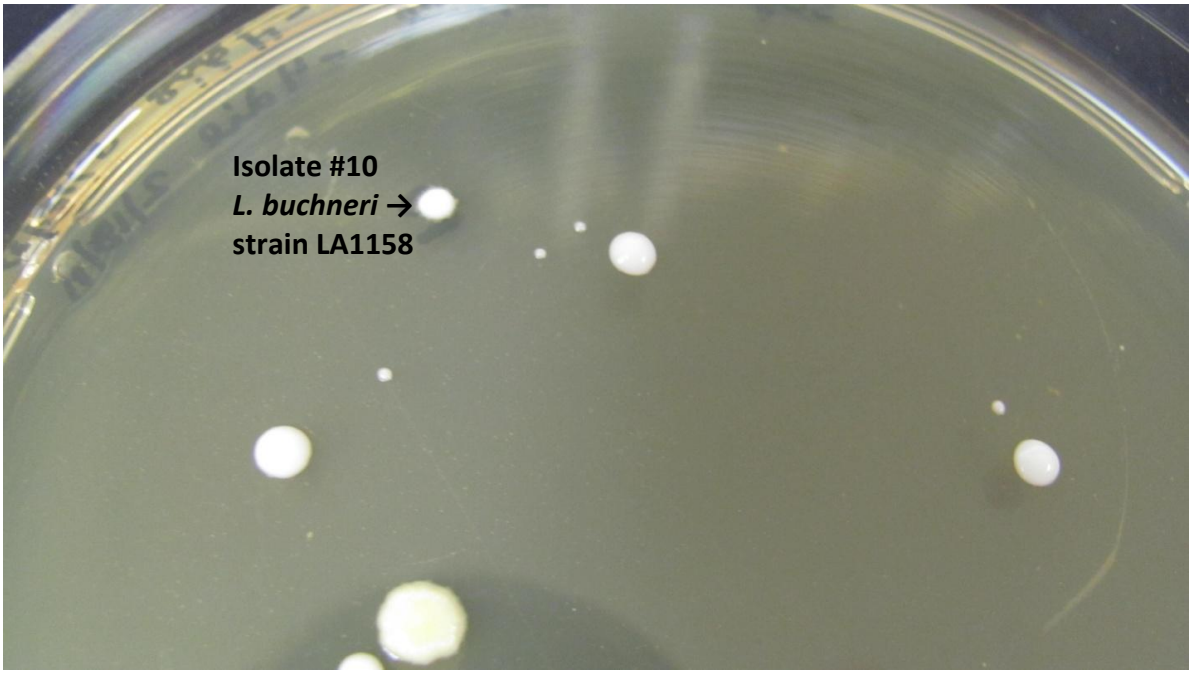
Reduced NaCl spoilage in FCS (2% NaCl, pH 3.8)



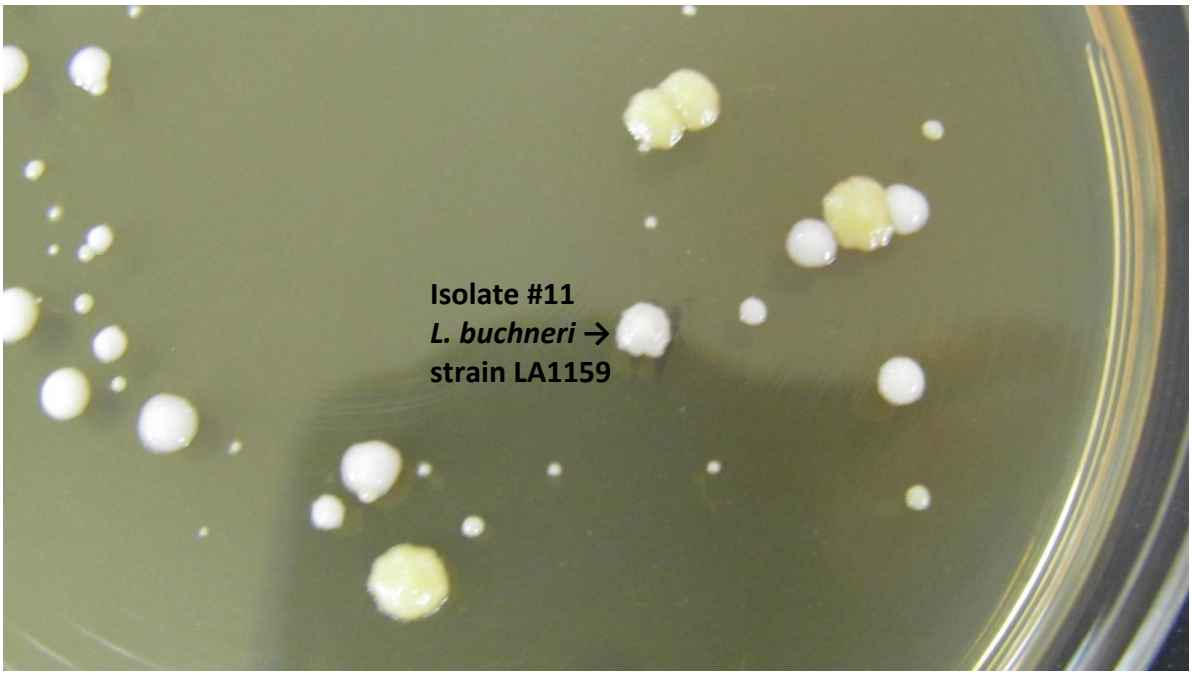
Reduced NaCl spoilage in FCS (2% NaCl, pH 3.8)



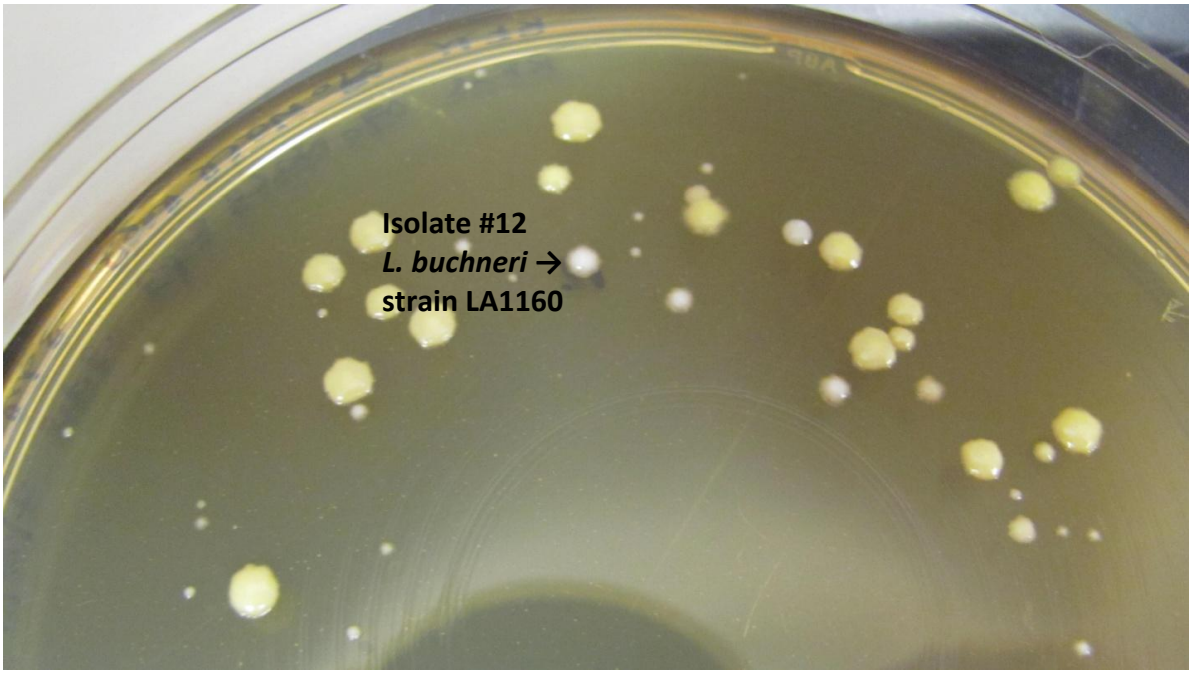
Reduced NaCl spoilage in FCS (4% NaCl, pH 3.8)



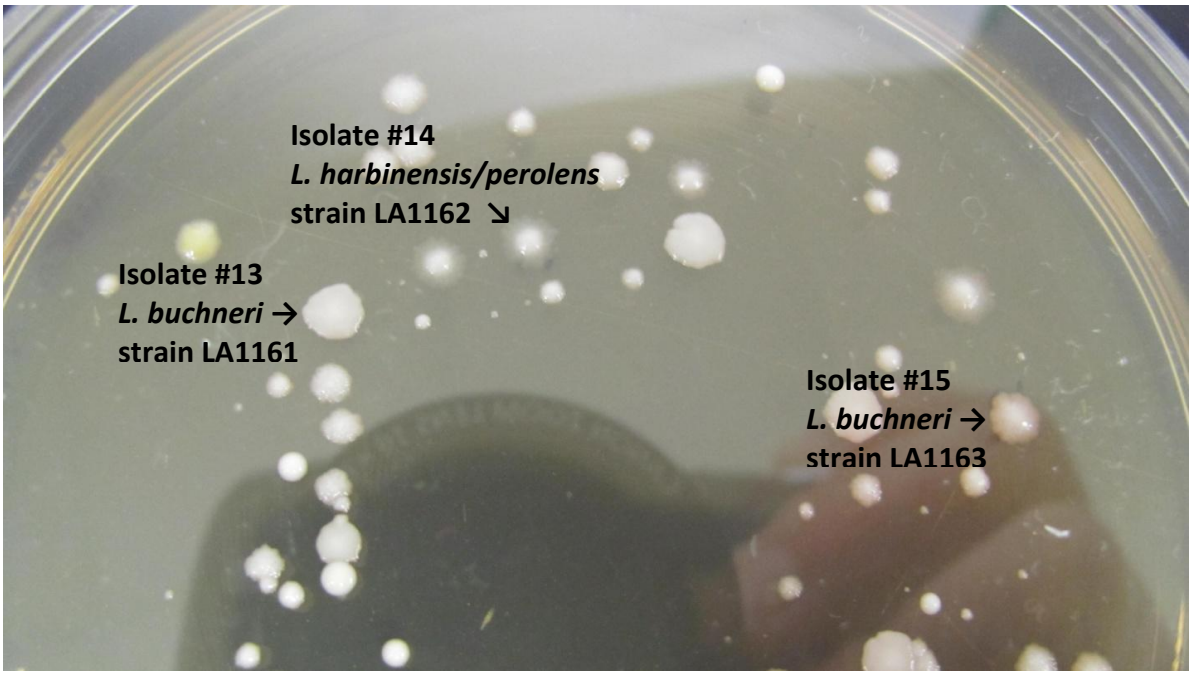
Reduced NaCl spoilage in FCS (6% NaCl, pH 3.8)



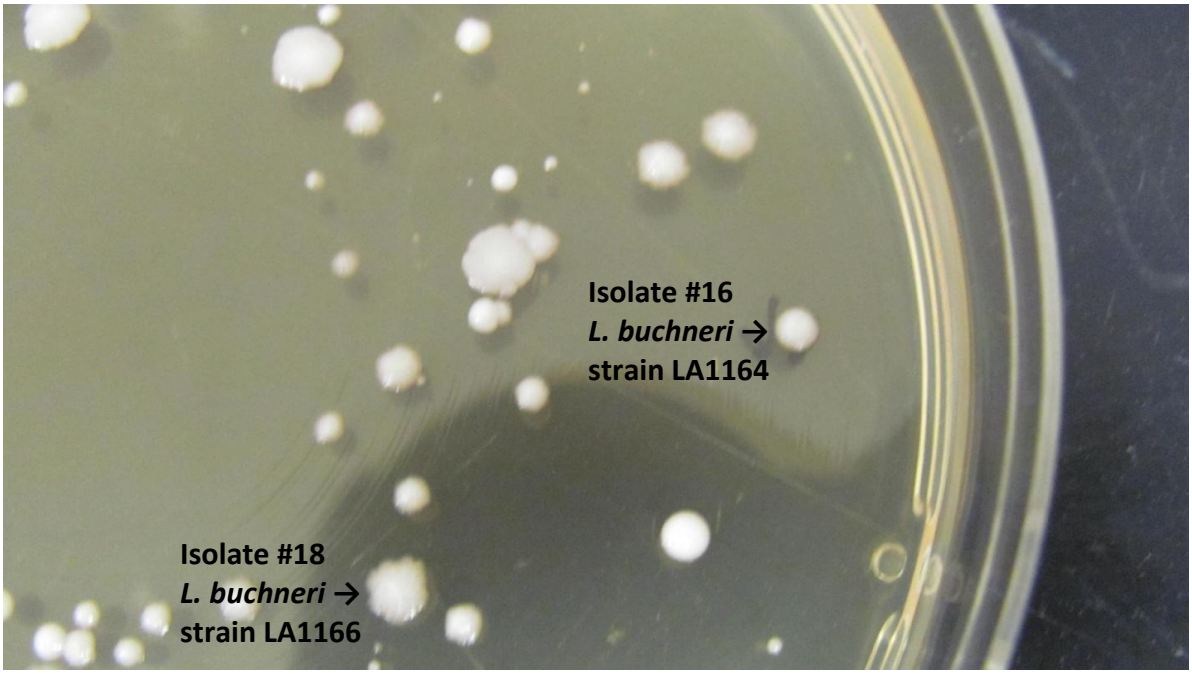
Reduced NaCl spoilage in FCS (0% NaCl, pH 3.8)



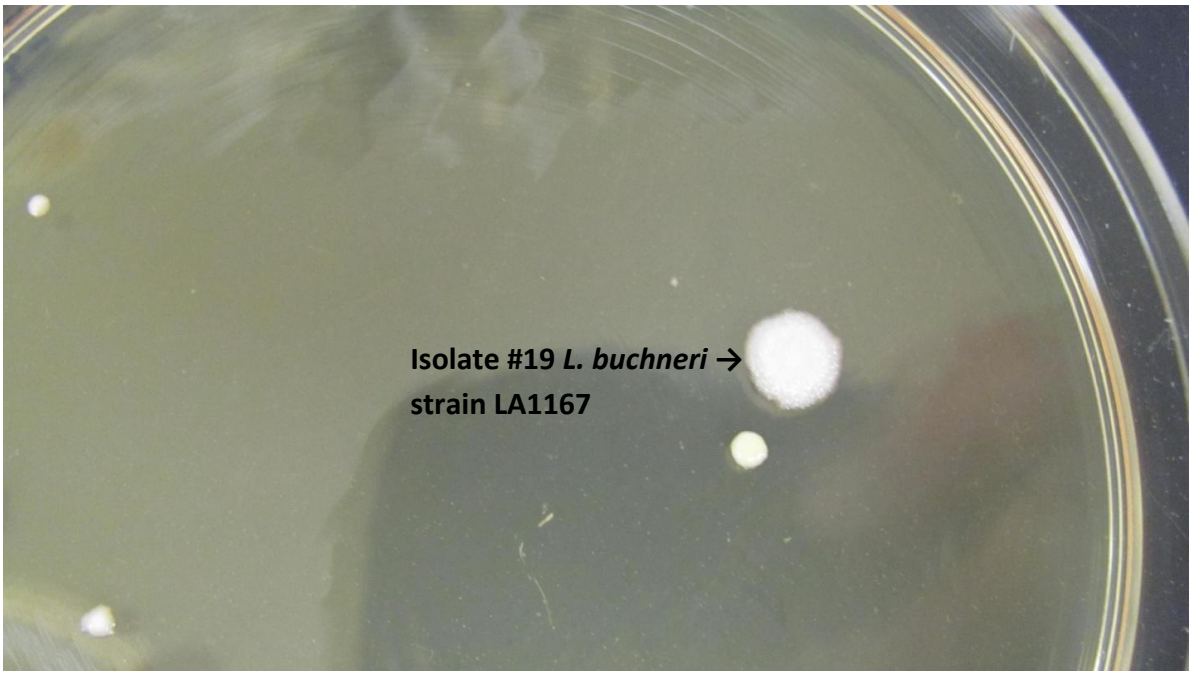
Commercial spoilage 2 in FCS (4% NaCl, pH 3.8)



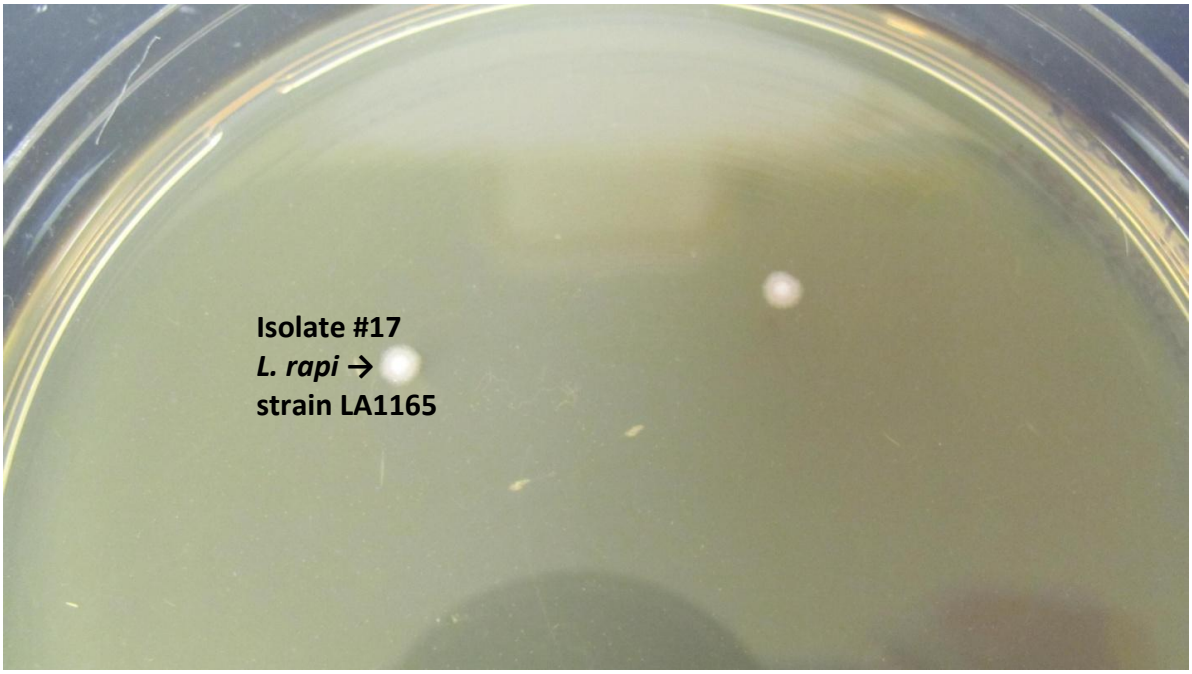
Commercial spoilage 2 in FCS (4% NaCl, pH 3.8)



Commercial spoilage 2 in FCS (2% NaCl, pH 3.8)



Cross-contaminated sample of FCS that spoiled



Appendix 2

Detection of volatile spoilage metabolites in fermented cucumbers using nontargeted, comprehensive 2-dimensional gas chromatography-time-of-flight mass spectrometry (GCxGC-ToFMS) Publication Reprint

Detection of Volatile Spoilage Metabolites in Fermented Cucumbers Using Nontargeted, Comprehensive 2-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry (GC×GC-TOFMS)

Suzanne D. Johanningsmeier and Roger F. McFeeters

Abstract: A nontargeted, comprehensive 2-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOFMS) method was developed for the analysis of fermented cucumber volatiles before and after anaerobic spoilage. Volatile compounds extracted by solid-phase microextraction were separated on a polyethylene glycol 1st-dimension column and 14% cyanopropylphenyl 2nd-dimension column. Among 314 components detected in fermented cucumber brine, 199 had peak areas with coefficients of variation below 30%. Peak identifications established by mass spectral library matching were 92% accurate based on 63 authentic standards. Analysis of variance of analytes' log peak areas revealed 33 metabolites changed in concentration after spoilage ($P < 0.05$), including increases in acetic, propanoic, and butyric acids, n-propyl acetate, several alcohols, and a decrease in furfural. GC×GC-TOFMS with a nontargeted, semi-automated approach to data analysis made possible the separation, identification, and determination of differences in polar volatile components, facilitating the discovery of several metabolites related to fermented cucumber spoilage.

Keywords: comprehensive 2D GC-MS, fermented cucumber volatiles, GC×GC-TOFMS, metabolites, nontargeted data analysis, 2-dimensional gas chromatography

Practical Application: An optimized method for the chemical analysis of volatile food components is described and applied to the profiling of volatile compounds in fermented cucumbers, resulting in the identification of 137 components, many of which are being reported for the first time in fermented cucumbers. This nontargeted GC×GC-TOFMS method and inclusive data analysis platform facilitated the discovery of several metabolites that were formed or utilized during anaerobic spoilage of fermented cucumbers. Further study of these metabolites will enhance our ability to understand and potentially control the metabolism of spoilage bacteria that can degrade lactic acid under the restrictive environmental conditions present in fermented cucumbers.

Introduction

Fermentation and storage in bulk tanks is used to preserve cucumbers for extended periods of time. The fermented cucumbers are then converted into a variety of processed pickle products, most notably hamburger dill chips. Fresh cucumbers of various sizes are typically brined in sodium chloride (NaCl) solutions so that the equilibrated concentration of NaCl is between 5% and 8% (wt/wt). This concentration of salt inhibits softening enzymes (Bell and Etchells 1961) and favors the growth of the naturally occurring lactic acid bacteria (Etchells and Jones 1943). Cucumber fruits contain approximately 2% to 3% fermentable sugars (Lu and others 2002), which are metabolized by lactic acid bacteria to predominantly lactic acid, thereby reducing the pH and the readily available energy sources for microbial growth. The combination of salt, acid pH, and lack of sugars results in a naturally preserved product that can typically be held for many months prior to final processing into pickle products.

One disadvantage of this fermentation process is the high concentration of NaCl in the waste stream. Efforts to reduce the NaCl used in fermentation and storage of cucumbers have resulted in the increased incidence of fermented cucumber spoilage. This spoilage has been characterized by a normal lactic acid fermentation followed by a gradual rise in pH and decrease in lactic acid concentration (Fleming and others 1989, 2002; Kim and Breidt 2007). The production of volatile compounds and increased pH compromise

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the quality of the product, often necessitating early processing of the tank or discarding the product if spoilage proceeds. If the pH rises above 4.6, clostridial spoilage may occur (Fleming and others 1989), so the possibility of germination and growth of *Clostridium botulinum* spores cannot be ruled out. The currently unpredictable nature of this spoilage contributes to increased production costs for the pickling industry, mainly in the form of increased monitoring of fermentation tanks. In cases where the pH has risen beyond control, product losses and increased waste disposal costs are also incurred.

Given the potential diversity of chemical components in a food fermentation system, a discovery-based approach may provide new insight into the changes in volatile compounds that occur due to microbiological spoilage after the normal fermentation process has been completed. Advances in gas chromatography-mass spectrometry (GC-MS) systems and data collection capability provide the potential to carry out separations of volatile chemical components using 2 different separation mechanisms by connecting columns with different bonded phases in series. The benefits and challenges associated with this technology have been the subject of recent reviews (Marriott and Shellie 2002; Adahchour and others 2008; Mondello and others 2008; Cortes and others 2009). This comprehensive 2-dimensional (2D) GC (GC×GC) methodology has been applied to the analysis of volatiles in a number of complex food matrices, including roasted coffee beans, butter, essential oils, grapes, roast beef, sugarcane spirits, honey, pepper, roasted barley, hazelnuts, olive oil, potato chips, basil, and Chinese liquor. Compared to chromatography with a single column, 2D chromatography resulted in resolution of more components and in improved mass spectral matches when a time-of-flight (TOF) MS detector was used for the analysis of butter volatiles and grape monoterpenoids (Adahchour and others 2005; Rocha and others 2007). Analysis of lavender essential oil using an orthogonal 2D separation consisting of a nonpolar 1st-dimension column followed by a polar 2nd-dimension column resulted in a 25-fold increase in sensitivity and a 3-fold increase in the number of resolved components as compared to traditional GC analysis (Shellie and others 2001). Orthogonal 2D separation of Cheddar cheese volatiles using a comprehensive, 2D GC-TOFMS (GC×GC-TOFMS) showed that separation in the 2nd dimension was necessary to resolve octane from hexanal and ethyl lactate from 3-octanol (Gogus and others 2006). Several other studies have reported separation of volatile compounds from complex food matrices with a nonpolar 1st-dimension column followed by a polar 2nd-dimension column (Cardeal and others 2006, 2008; Čajka and others 2007; Eyres and others 2007; Rocha and others 2007; Rochat and others 2007; Klimánková and others 2008; Cardeal and Marriott 2009; de Souza and others 2009; Lojzova and others 2009; Torres Vaz-Freire and others 2009). However, the reverse column combination as well as nonorthogonal polar-semipolar column combinations have also been demonstrated as viable alternatives for separation of volatile compounds in foods (Adahchour and others 2004, 2005; Mondello and others 2004; Ryan and others 2004; Bianchi and others 2007; Zhu and others 2007; Cordero and others 2008). Although the orthogonal, nonpolar-polar column combination was suitable for separating coffee bean volatiles, the reversed column combination showed a comparable structured order of the components and yielded a volatile compound profile that utilized more of the available separation space (Ryan and others 2004). Adahchour and others (2004) found that improved peak shapes and retention behavior for acids and alcohols were obtained on a polar-semipolar column combination. In addition, a useful pattern of separation for homologous series of compounds with different functional groups was obtained (Adahchour and others 2004; Cordero and others 2008). Therefore, this "reverse-type" GC×GC separation may have advantages for some analyses of food volatiles.

While GC×GC-TOFMS offers greatly increased capability for separating and detecting volatile components present in complex samples, the datasets generated are large and cumbersome. In metabolite profiling studies, target compounds are unknown and the goal is to identify a set of metabolites associated with a particular treatment or phenotype (also known as biological markers) among the hundreds to thousands of metabolites detected. The size and complexity of these types of datasets requires automation of the data analysis process. This study describes a nontargeted, comprehensive GC×GC-TOFMS method for separating and identifying volatile compounds in fermented cucumbers, and detecting changes in volatile metabolites occurring as a result of fermented cucumber spoilage.

Materials and Methods

Cucumber fermentation

Size 2B cucumbers (32 to 38 mm in diameter) were washed, packed into 3 3.84-L glass jars, and covered with brine (55:45 cucumber:brine ratio) containing calcium chloride ($CaCl_2$) and NaCl so that the equilibrated concentrations were 0.25% and 6% (w/w), respectively. Brined cucumbers were inoculated with 10^6 CFU/g *Lactobacillus plantarum* MOP3 starter culture (Culture Collection ID LA0219, USDA-ARS Food Science Research Unit, Raleigh, N.C., U.S.A.). Jars were closed with lids that were heated in boiling water to soften the plastisol liner, and a rubber septum was inserted into the lid of each jar to allow sampling of the brine with a syringe. The jars were stored at ambient temperature (21 to 25 °C) for 11 mo. Fermentation progressed normally in all 3 jars as indicated by decreases in pH and changes in organic acids and sugars as measured by high-performance liquid chromatography (HPLC) with ultraviolet light (UV) and refractive index (R.I) detection (McFeeters and Barish 2003). Seven replicate samples of fermented cucumber brine from a single fermentation jar were analyzed in random order among 12 other fermented cucumber brine samples over the course of a 3-d run of the instrument to assess the analytical reproducibility for the nontargeted analysis of volatile components. Volatile compounds were also analyzed in triplicate for brine samples from the other 2 replicate fermentation jars.

Media preparation

Fermented cucumbers as described above were cut into pieces and blended into a slurry to prepare sterile, fermented cucumber slurry (FCS) as a medium for inoculation with spoilage microorganisms. The FCS was pressed through cheesecloth and centrifuged in 250-mL bottles at 12000 rpm for 15 min to remove particulate matter. The pH of the clarified slurry was raised from 3.1 to 3.8 by addition of 6 N NaOH to increase the rate at which spoilage occurred (Fleming and others 2002; Kim and Breidt 2007). The pH-adjusted, clarified FCS was sterile-filtered with a Nalgene FAST PES 0.2-μm pore size, 90 mm dia membrane, bottle-top filter apparatus (Daigger, Vernon Hills, Ill., U.S.A.). Twelve mL of sterile-filtered FCS was then aseptically transferred into sterile 15-mL conical tubes. The loosely capped tubes were placed into an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, Mich., U.S.A.) for 3 d prior to inoculation to remove dissolved oxygen from the media.

Spoilage inoculum source

Brine from a laboratory cucumber fermentation that had undergone an undesirable secondary fermentation was used as spoilage inoculum. Two 5-gallon plastic pails with tightly fitting lids were packed with 9.5 kg size 2B cucumbers (38 to 44 mm in diameter) and covered with an equal volume of brine. One cover brine contained 4% NaCl, 36 mM $CaCl_2$, and 50 mM acetic acid from 20% vinegar to equilibrate at 2% NaCl, 18 mM $CaCl_2$, and 25 mM acetic acid during the fermentation. The 2nd cover brine contained NaCl, $CaCl_2$, KCl, $MgCl_2 \cdot 6H_2O$, and acetic acid to equilibrate at 1.2% NaCl, 0.8% KCl, 30 mM $CaCl_2$, 20 mM $MgCl_2$, and 25 mM acetic acid (McFeeters and Fleming 1997). The pails were inoculated with *L. plantarum* starter culture and fermented normally as indicated by a decrease in pH to 3.2 and typical utilization of sugars and production of lactic acid determined by HPLC 1 mo after initiating the fermentations. However, when the fermentations were sampled after 11-mo storage at ambient temperature, it was noted that the lactic acid had decreased substantially and the pH had risen to 4.1 (Table 1), which is typical of the anaerobic cucumber spoilage described by Fleming and others (1989, 2002) and Kim and Breidt (2007). Brine from the spoiled fermented cucumbers (2% NaCl) was used as the inoculum to reproduce spoilage in filter-sterilized FCS (6% NaCl, pH 3.8). In addition, solid-phase microextraction (SPME) GC×GC-TOFMS was carried out on frozen aliquots of these 1- and 11-mo brine samples that were thawed, diluted, and randomized for run order prior to analysis. Changes in volatile metabolites that occurred during spoilage were determined by comparison of the volatile profile of brine samples taken from the pails after the primary fermentation (1-mo storage) and after the lactic acid had decreased (11-mo storage).

Reproduction of spoilage

Conical centrifuge tubes containing 12-mL sterile-filtered FCS were inoculated in triplicate with 1 mL of spoilage brine and incubated anaerobically at ambient temperature along with triplicate noninoculated FCS controls. Samples were taken immediately after inoculation and after 3 wk, 2 mo, and 6 mo of incubation and stored at −80 °C until analysis. A significant decrease in lactic acid concentration, as measured by HPLC, was used to indicate the appropriate samples to use for analysis of changes in the volatile components that occurred upon spoilage (Table 1). SPME-GC×GC-TOFMS was carried out on initial and 6-mo samples that were thawed, diluted, and randomized for run order. Components that changed during anaerobic incubation of noninoculated FCS were presumed to have been formed as a result of chemical changes that occurred during the extended incubation period and were excluded from the group of compounds that changed as a result of microbial spoilage.

SPME of volatile components

Fermented cucumber brines or spoilage samples (200 μL) were diluted 1:5 with deionized water (796 μL) and acidified with 3 N H_2SO_4 (4 μL) in 10 mL screw-cap headspace vials (Microliter Analytical Supplies, Inc., Suwanee, Ga., U.S.A.). NaCl (0.40 g) was added to "salt out" volatile components from the samples. Spoilage samples were also analyzed at a 1:250 dilution to account for volatile components present in amounts that resulted in column overloading at the 1:5 dilution. Samples were randomized for analysis order (PROC PLAN, version 9.1.3 SAS® software, SAS Inst., Cary, N.C., U.S.A.) and placed into a refrigerated sample tray (2 °C). Automated sampling was performed using a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies, Carrboro, N.C., U.S.A.). Headspace vials containing the diluted samples were agitated at 500 rpm (5 s on and 2 s off) for 15 min at 40 °C prior to extraction. Volatile compounds were collected by insertion of a 1-cm, 50/30 μm DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco, Bellefonte, Pa., U.S.A.) into the headspace above the sample for 30 min at 40 °C with 100 rpm agitation (5 s on and 2 s off). Extracted volatile compounds were desorbed from the SPME fiber into the GC inlet at 250 °C for 15 min. A blank sample (1.0-mL deionized water containing 6 mM sulfuric acid and 0.4 g NaCl) was run between each fermented cucumber sample to reduce carry-over of components on the SPME fiber.

Comprehensive, GC×GC-TOFMS

A LECO® Pegasus III® GC×GC-TOFMS instrument (Model# 614-100-700, Leco Corp., St. Joseph, Mich., U.S.A.) included an Agilent GC (Model# 6890N, Agilent Technologies, Santa Clara, Calif., U.S.A.) fitted with a secondary oven and cryogenic modulator. The 2D separation was achieved using a SolGel-Wax™, 30 m × 0.25 mm i.d. × 0.25 μm film thickness (SGE, Austin, Tex., U.S.A.), polyethylene glycol 1st-dimension column in the primary oven and an RTX 17-01, 1.0 m × 0.1 mm i.d. × 0.1 μm film thickness (Restek, Bellefonte, Pa., U.S.A.), 14% cyanopropylphenyl-86% dimethyl polysiloxane 2nd-dimension column in the secondary oven. Columns were conditioned according to manufacturer recommendations prior to use. A 0.75 mm i.d. Siltek deactivated SPME liner (Restek, Bellefonte) was used in the inlet. It was set at 250 °C and operated in pulsed splitless mode with a pulse pressure of 37 psi for 1 min. The split vent was opened 2 min following injection, and the GC was operated in constant flow mode with 1.3 mL/min helium carrier gas. The primary oven temperature was maintained at 40 °C for 2 min and then increased at 5 °C/min to 140 °C. The temperature ramp was then increased to 10 °C/min to 250 °C and the temperature was held at 250 °C for 3 min. The secondary oven followed the same temperature program except the temperature was maintained at 10 °C higher than the main oven until

Table 1–Changes in organic acids and pH as an indicator of spoilage.

	Time (mo)	pH	Lactic acid (mM)	Acetic acid (mM)	Propanoic acid (mM)
Fermented cucumber spoilage					
After primary fermentation	1	3.17 ± 0.01	116.8 ± 5.6	27.6 ± 0.9	None detected
After spoilage	11	4.08 ± 0.01	10.4 ± 1.0	80.0 ± 1.9	39.5 ± 1.8
Reproduction of spoilage in fermented cucumber slurry					
Noninoculated control	0	3.79 ± 0.00	125.1 ± 12.1	5.7 ± 1.1	None detected
Noninoculated control	6	3.80 ± 0.00	128.6 ± 0.7	5.9 ± 0.1	None detected
Inoculated with spoilage brine	0	3.82 ± 0.00	106.2 ± 2.5	13.2 ± 1.8	2.9 ± 0.8
Inoculated with spoilage brine	6	4.46 ± 0.01	51.8 ± 0.9	62.5 ± 3.8	16.1 ± 1.3

the temperature reached a maximum of 250 °C in the secondary oven. The transfer line temperature was maintained at 250 °C. The modulator offset was +30 °C with a 1.5 s 2nd-dimension separation time and 0.3-s hot pulse. Compressed air (35 psi) was used for the hot pulses, and liquid nitrogen-cooled nitrogen gas (18 psi) was used for the cold pulses.

The mass spectrometer was operated with −70 eV and an ion source temperature of 200 °C. The detector voltage was set at 1500 V and masses 25 to 500 were collected at 200 spectra per second. No solvent delay was employed.

Data processing and analysis

Data analysis involved a series of steps that made use of the instrument software, ChromaTOF® version 3.25 (Leco Corp.) for data processing, Excel® 2003 (Microsoft Corp., Redmond, Wash., U.S.A.) for data compilation, and SAS® version 9.1.3 (SAS Inst.) for statistical analysis. At the time of data acquisition, user fields were created in the ChromaTOF® acquisition menu to include information that uniquely identified each sample injected as to treatment type, replicate number, time of sampling, and so on. This information was then accessible in the peak tables for every peak associated with that sample. Inclusion of this information at the acquisition step was a key element contributing to efficiency in subsequent review and statistical analysis of the peak table data.

ChromaTOF® software data processing methods were used to detect and quantify peaks based on unique masses as determined by the deconvolution algorithm. Data processing parameters are shown in Table 2. A library search of the NIST/EPA/NIH Mass Spectra Library (National Inst. of Standards and Technology [NIST], Gaithersburg, Md., U.S.A., 2005) was utilized for tentative identification of deconvoluted chromatographic peaks. Chemical names were assigned to peaks that had a minimum mass spectral similarity ≥800 (1000 is an exact match). The unique mass (U) for each peak, as assigned by the ChromaTOF® deconvolution algorithm, was used for peak area calculations. All samples were processed in comparison to a single run of a composite brine sample. The composite sample for each experiment was prepared by mixing equal volumes of samples from each treatment of the experiment. Therefore, the composite sample peak table should theoretically contain most components that are present in the experimental samples. In ChromaTOF®, a reference table was created using the composite sample peak table as a standard. Criteria for the reference table were set as detailed in Table 2, and peak tables for each sample were standardized against this reference using the compare function in the ChromaTOF® data processing method. The resulting standardized peak tables containing each peak associated with a quantification name and peak area, based on the respective unique mass, were copied into an Excel® spreadsheet for further analysis. Creation of a reference in ChromaTOF® was necessary to standardize the name assignment for a given peak (including unknowns that were named unknown 1, unknown 2, and so on) and to allow standardized quantification of the peak area with the same specific unique mass for each component in all chromatograms of an experiment. Even in replicate chromatograms of brine from a single sample, the ChromaTOF® algorithm may select different unique masses for quantification of the same analyte, resulting in the inability to make comparisons of peak areas among chromatograms for a given component. This inconsistency is beyond the control of the instrument operator and has been noted by other researchers (O'Hagan and others 2007). In addition to stipulating a single mass per analyte for peak area quantification, employing the reference chromatogram for standardizing peak tables had the advantage of assigning the same unknown number to the matching components in all chromatograms. Therefore, it was possible to do peak area comparisons of unidentified metabolites that without standardization would have been variably numbered depending on the number of unknowns detected in each chromatogram.

Table 2–Data processing parameters used to create standardized peak tables in ChromaTOF®.

Data step	Parameter	Value
Peak detection	Baseline offset	0.8
	Number of points averaged for smoothing	3
	Peak width (second)	0.1
	Signal to noise (S/N)	250
	Number of apexing masses	2
GC×GC parameters	Match required to combine	500
	Override the allowed retention time shift for combine (early and late) (second)	0.1
	First-dimension peak width (second)	15
Library identification	Search mode	Normal, forward
	Number of library hits to return	10
	Molecular weight range	40 to 1000
	Mass threshold	10
	Minimum similarity match before name is assigned	800
	Library	NIST mainlib
Quantification	Mass to use for area/height calculation	U (unique mass)
Reference (compare criteria)	Name, 1st-dimension retention time (s), 2nd-dimension retention time (s), and masses (unique mass in this case)	Fields populated from peak table of the composite sample
	R. T. deviation (s)	4.5
	Quantitate	Area
	Match threshold	500
	S/N threshold	5.0

Peaks not found in a sample chromatogram that were included in the reference table resulted in blank cells for the peak area value of that analyte. These missing values represented the absence of a component within the detection limits of the analytical method, referred to as left-censored data, and needed to be replaced prior to statistical analysis to avoid the loss of fundamental information. Substitution of left-censored data with a random number between zero and the detection limit has been shown to be an adequate statistical alternative in environmental data analysis where observations below the instrumental detection limit constituted less than 70% of the data (Antweiler and Taylor 2008). To obtain an estimate of the experiment-wide detection limit, the minimum reported peak area from all chromatograms within an experiment was located. For example, from the fermented cucumber spoilage experiment, this area was 196. Therefore, blank peak area cells for undetected analytes in the dataset were replaced with a random number between 1 and 195 (<196) to provide substitution data that reflected possible responses below the method's detection limit for undetected components.

Peak areas of volatile components ranged from 196 to $>10^8$ in magnitude and peak area variability within replicate analyses increased as peak area increased. Since the standard deviation of peak areas was generally found to be proportional to the mean peak

areas, log transformation was used to homogenize the variances prior to analysis of variance (ANOVA) (Steel and Torrie 1980). An ANOVA of log peak areas by quantification name was conducted to detect differences in volatile compounds among treatments (version 9.1.3 SAS® software, SAS Inst.). Significance was established at $P < 0.05$ after adjustment of P-values to control the false discovery rate using the method of Benjamini and Hochberg (1995).

Reference compounds

With the exception of the following, all chemicals were obtained from Sigma-Aldrich in their purest available form (Sigma-Aldrich, St. Louis, Mo., U.S.A.). Pentane, methyl propionate, 3-methyl-2-butanone, benzene, methyl isobutyl ketone, 3-penten-2-ol, 1-pentanol, and 3-hydroxy-2-butanone were acquired from Fluka (Sigma-Aldrich, St. Louis). 3-octanol was sourced from Alfa Aesar (Ward Hill, Mass., U.S.A.), 3-pentanol was obtained from Riedel-de-Haen (Seelze, Germany), and 4-methyl-2-heptanol was purchased from ChemSampCo (Trenton, N.J., U.S.A.).

Results and Discussion

Volatile components in fermented cucumbers

Approximately 477 peaks with S/N ≥250 were detected in the brine of cucumbers fermented with 6% NaCl (Figure 1). Of these, 314 peaks were attributed to the fermented cucumber brine based on manual inspection of the chromatograms and peak table data for brine samples compared to water blank chromatograms. The 163 artifact peaks included siloxanes, other system contaminants,

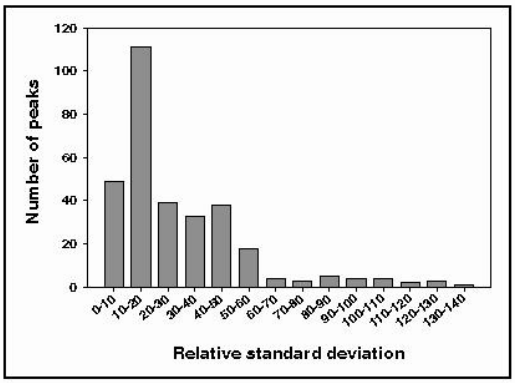

Figure 2–Peak area reproducibility ($n = 7$) for volatile components detected in fermented cucumber brine with SPME GC×GC-TOFMS.

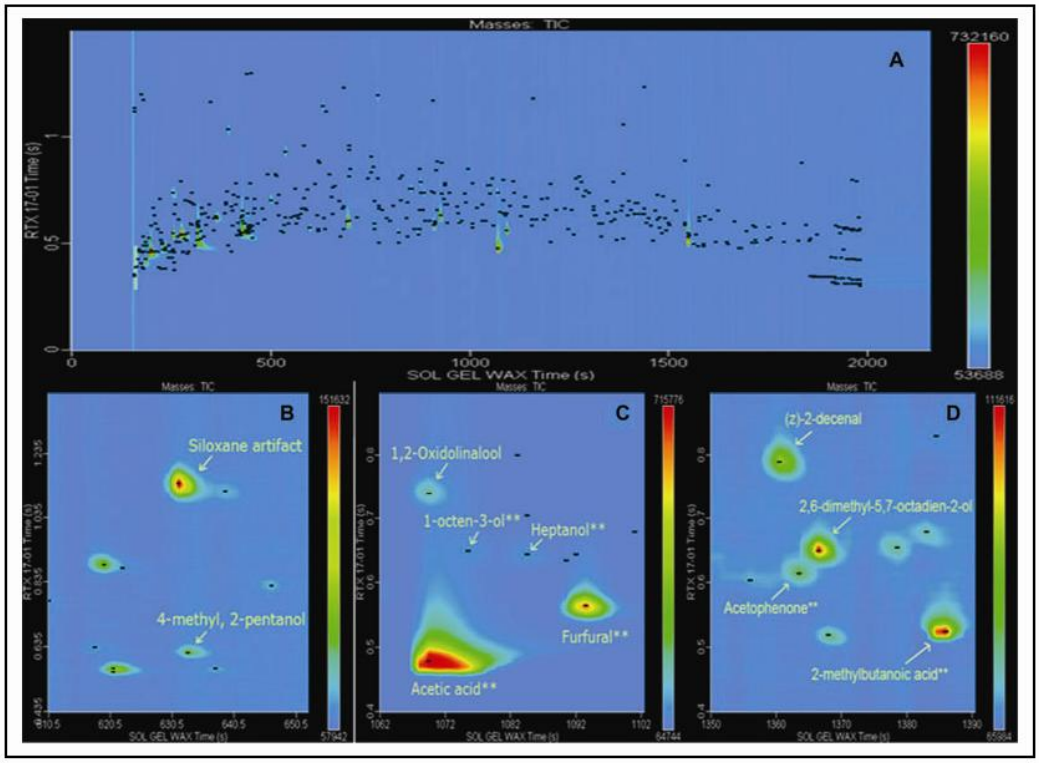

Figure 1–GC×GC-TOFMS total ion current (TIC) contour plot of volatile components in fermented cucumber brine (A). Three detail regions of the 2D separation of volatile components in fermented cucumber brine with a polar-semipolar column combination are shown, illustrating increased separation capacity (B), resolution of siloxane artifacts from metabolites of interest (C), and resolution of low intensity metabolite peaks in the 2nd dimension from an overloaded acetic acid peak (D). Peaks detected with S/N ≥ 250 are indicated by black peak markers.

Table 3–Volatile compounds in fermented cucumber brines detected using SPME GC×GC-TOFMS.

Compound[1]	CAS[2] registry #	Method of identification[3]	Similarity	RI^{4}_{calc}	RI^{5}_{lit}	Unique mass[6]	Average area	RSD[7]
Hydrocarbons								
Pentane	109-66-0	MS, RI, ST	934	500	500	41	45602	17.6
Hexane	110-54-3	MS, RI, ST	925	600	600	41	655816	27.2
1,4-pentadiene	591-93-5	MS, ST	943	646	nf	67	37400	19.7
Ethylcyclobutane	4806-61-5	MS	896	692	nf	56	472102	17.0
Cyclohexane	110-82-7	MS, RI, ST	904	742	737	56	88364	15.1
cis-5,5-dimethyl-2-hexene	39761-61-0	MS	901	757	nf	41	29255	16.7
Benzene	71-43-2	MS, RI, ST	970	938	936	78	743703	12.6
Toluene	108-88-3	MS, RI, ST	911	1042	1040	91	909212	9.1
m-xylene	108-38-3	MS, RI	909	1144	1132	91	17265	16.5
Cardene	694-87-1	MS, RI	926	1272	1269	104	64928	18.1
Alcohols								
2-methyl-2-pentanol	590-36-3	MS, RI	885	1110	1101	59	200446	11.0
3-pentanol	584-02-1	MS, RI, ST	938	1116	1112	59	81102	17.0
2-pentanol*	6032-29-7	MS, RI, ST	922	1129	1142	45	223939	14.4
2,4-dimethyl-2-pentanol	625-06-9	MS, ST	875	1147	nf	59	40578	18.0
Butanol*	71-36-3	MS, RI, ST	876	1153	1152	56	794956	13.7
2-methyl-3-pentanol	565-67-3	MS, RI	907	1167	1121	59	21068	21.2
1-penten-3-ol	616-25-1	MS, RI, ST	891	1169	1176	57	318459	4.6
3-penten-2-ol	1569-50-2	MS, RI, ST	841	1181	1182	71	11164	27.6
2,4-dimethyl-4-penten-2-ol	19781-53-4	MS	866	1195	nf	59	38954	15.7
Eucalyptol*	470-82-6	MS, RI	852	1223	1216	81	29202	10.5
2-hexanol	52019-78-0	MS, RI, ST	908	1238	1238	45	49865	17.0
3-methyl-3-buten-1-ol	763-32-6	MS, RI	883	1264	1263	68	37794	13.7
Pentanol*	71-41-0	MS, RI, ST	920	1265	1256	42	477300	12.8
2-methyl-2-heptanol	625-25-2	MS	812	1265	nf	59	47995	14.4
trans-2-penten-1-ol	1576-96-1	MS, RI, ST	929	1325	1335	57	22334	4.9
2-methyl-2-buten-1-ol	4675-87-0	MS, RI	862	1333	1315	71	55966	29.2
2-heptanol*	543-49-7	MS, RI, ST	942	1334	1334	45	299671	12.8
2-methyl-2-propen-1-ol	513-42-8	MS	808	1337	nf	72	11391	12.4
Hexanol*	111-27-3	MS, RI	891	1362	1354	43	2564937	9.5
4-methyl-2-heptanol	56298-90-9	MS, ST	928	1369	nf	45	120208	21.1
trans-3-hexen-1-ol	544-12-7	MS, RI	926	1371	1371	67	20351	12.4
2,3-dimethyl-1-pentanol	10143-23-4	MS	834	1388	nf	85	3623	10.8
cis-3-hexen-1-ol	928-96-1	MS, RI, ST	951	1389	1388	67	335213	11.4
2-methyl-2-octanol	628-44-4	MS, ST	893	1397	nf	59	71673	10.8
3-octanol	589-98-0	MS, RI, ST	917	1399	1395	55	19815	16.1
trans-2-hexen-1-ol	928-95-0	MS, RI	862	1408	1410	57	31238	24.4
2-octanol	5978-70-1	MS, RI	904	1421	1430	45	29309	13.6
1-octen-3-ol	3391-86-4	MS, RI, ST	932	1451	1456	57	248344	13.4
Heptanol	53535-33-4	MS, RI, ST	900	1457	1460	56	106479	10.2
2-ethyl-1-hexanol*	104-76-7	MS, RI	932	1495	1492	57	291814	12.3
cis-3-hepten-1-ol	1708-81-2	MS, RI	877	1509	1491	81	18911	15.5
2-nonanol	628-99-9	MS, RI, ST	840	1528	1528	45	34665	17.4
Octanol*	111-87-5	MS, RI	900	1568	1561	56	63772	8.0
4-terpineol	562-74-3	MS, RI	827	1614	1617	93	4391	9.1
Myrcenol	543-39-5	MS, RI	860	1622	1604	59	28202	17.4
cis-2-octen-1-ol	26001-58-1	MS, RI	896	1626	1616	57	15126	12.0
cis-ocimenol	5986-38-9	MS, RI	847	1662	1662	93	55339	12.7
trans-ocimenol	5986-38-9	MS, RI	837	1685	1688	93	72282	11.9
α-terpineol*	98-55-5	MS, RI, ST	914	1703	1718	59	345764	13.3
cis-6-nonen-1-ol	35854-86-5	MS, RI	941	1720	1711	67	19277	11.8
Benzyl alcohol	100-51-6	MS, RI, ST	900	1900	1874	79	48419	9.6
Phenylethyl alcohol*	60-12-8	MS, RI, ST	942	1939	1939	91	54906	10.5
Aldehydes								
Acetaldehyde	75-07-0	MS, RI, ST	928	727	727	44	7442303	12.2
Pivaldehyde	630-19-3	MS, RI	872	807	809	41	146801	11.0
2-methylbutanal	96-17-3	MS, RI, ST	878	914	914	57	90940	17.4
3-methylbutanal	590-86-3	MS, RI, ST	864	918	917	41	431295	5.8
Hexanal*	66-25-1	MS, RI, ST	923	1084	1080	57	664650	7.9
trans-2-methyl-2-butenal	497-03-0	MS, RI	912	1098	1094	84	29451	26.2
2-pentenal	1576-87-0	MS, RI, ST	884	1137	1135	55	175677	6.8
2,4,4-trimethyl-2-pentenal	53907-61-2	MS	800	1254	nf	55	17749	23.2
cis-2-heptenal*	57266-86-1	MS, RI	932	1340	1331	41	540286	14.7
Nonanal*	124-19-6	MS, RI, ST	902	1402	1396	41	283024	16.4
trans-2-octenal	2548-87-0	MS, RI, ST	873	1432	1432	55	317319	19.4
Furfural	98-01-1	MS, RI, ST	892	1464	1474	96	2442243	4.5
2,4-heptadienal	5910-85-0	MS, RI	873	1469	1468	81	30513	10.8

Continued

Table 3–Continued

Compound[1]	CAS[2] registry #	Method of identification[3]	Similarity	RI^{a}_{ak}	RI^{b}_{lit}	Unique mass[6]	Average area	RSD[7]
(E,E)-2,4-heptadienal	3/5/4313	MS, RI	873	1501	1497	81	156003	12.0
Benzaldehyde*	100-52-7	MS, RI	872	1530	1528	77	77720	3.1
2-decenal	2497-25-8	MS, RI	926	1658	1652	41	77800	23.2
3,5-dimethyl-benzaldehyde	5779-95-3	MS	912	1837	nf	133	100878	13.6
Ketones								
Acetone*	67-64-1	MS, RI, ST	922	814	814	58	1363820	8.2
3-methyl-2-butanone	563-80-4	MS, RI, ST	847	929	929	39	25302	27.9
3,3-dimethyl-2-butanone	75-97-8	MS, RI, ST	870	949	978	57	32611	10.4
2-methyl-3-pentanone	565-69-5	MS, RI	863	997	1003	57	37274	7.6
2,4-dimethyl-3-pentanone	565-80-0	MS, RI, ST	880	1000	995	71	10099	11.9
Methyl isobutyl ketone	108-10-1	MS, RI, ST	926	1008	1008	43	524065	14.1
3-methyl-2-pentanone	565-61-7	MS, RI, ST	902	1019	1016	43	70287	6.4
1-penten-3-one	1629-58-9	MS, RI, ST	837	1024	1024	55	698188	9.8
4,4-dimethyl-2-pentanone	590-50-1	MS	886	1025	nf	43	144329	10.2
3-hexanone	589-38-8	MS, RI, ST	914	1055	1052	57	65095	12.7
2-methyl-1-penten-3-one	25044-01-3	MS, RI	907	1069	1069	69	40626	10.4
trans-3-penten-2-one	3102-33-8	MS, RI, ST	844	1134	1123	69	29502	17.1
4-methyl-3-penten-2-one	141-79-7	MS, RI	886	1140	1131	98	8040	14.0
4-methyl-2-heptanone	6137-06-0	MS, RI	902	1224	1206t	58	124174	13.9
3-hydroxy-2-butanone	513-86-0	MS, RI, ST	863	1301	1289	45	983577	11.4
1-octen-3-one	4312-99-6	MS, RI	905	1319	1299	55	210558	16.0
6-methyl-5-hepten-2-one	110-93-0	MS, RI	838	1351	1340	43	112007	22.2
2-hydroxy-2,4-dimethyl-3-pentanone	3212-67-7	MS	865	1376	nf	59	7580	9.9
Acetophenone	98-86-2	MS, RI, ST	935	1660	1660	77	42629	9.8
p-methylacetophenone	122-00-9	MS, RI	890	1789	1794	119	19819	10.1
Acids								
Acetic acid*	64-19-7	MS, RI, ST	927	1446	1450	60	9802352	17.5
Propanoic acid	79-09-4	MS, RI, ST	938	1543	1534	45	230741	7.3
Pivalic acid	75-98-9	MS, RI	863	1586	1579	57	106234	11.5
Butanoic acid*	107-92-6	MS, RI, ST	846	1636	1620	60	55323	9.8
2-methyl-butanoic acid	116-53-0	MS, RI, ST	861	1677	1682	74	136728	9.5
Pentanoic acid	109-52-4	MS, RI, ST	913	1734	1734	60	81854	8.9
Hexanoic acid*	142-62-1	MS, RI, ST	864	1865	1841	60	721279	6.4
Octanoic Acid	124-07-2	MS, RI, ST	891	2071	2053	60	375641	9.2
Nonanoic acid	112-05-0	MS, RI, ST	892	2157	2157	60	501018	17.3
Decanoic acid	334-48-5	MS, RI	869	2219	2263	60	52296	14.4
Esters								
Methyl acetate	79-20-9	MS, RI, ST	882	825	828	74	1012974	13.7
Methyl propionate	554-12-1	MS, RI, ST	815	905	911	57	57501	14.0
Ethyl propionate	105-37-3	MS, RI	880	956	957	57	81551	11.7
Ethyl nitrate	625-58-1	MS	934	969	nf	76	4708	14.7
Isoamyl acetate	123-92-2	MS, RI, ST	851	1127	1127	43	33883	28.2
Methyl lactate	2155-30-8	MS, RI, ST	948	1331	nf	45	1719881	9.6
Ethyl lactate	97-64-3	MS, RI, ST	949	1354	1353	45	7808306	7.4
Isoamyl lactate	19329-89-6	MS, RI	852	1580	1583	45	27084	22.9
trans-3-hexenyl butanoate	53398-84-8	MS, RI	826	1621	1602	71	42111	20.8
2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl propanoate	74367-34-3	MS	894	1902	nf	71	281380	6.0
2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl propanoate	74367-33-2	MS	854	1921	nf	71	185822	5.4
Ethers								
Oxetane	503-30-0	MS, ST	897	790	nf	58	305941	5.2
tert-amyl methyl ether	994-05-8	MS	871	790	nf	73	32913	14.2
1,2-oxidolinalool	76985-29-0	MS	896	1446	nf	59	379361	8.0
Diphenyl ether	101-84-8	MS, RI	863	2038	2017	51	37843	8.2
Furans								
2-methylfuran	534-22-5	MS, RI, ST	895	864	876	82	54359	9.0
2-ethylfuran	3208-16-0	MS, RI, ST	900	950	945	81	60954	8.8
trans-linalool oxide*	34995-77-2	MS, RI	886	1477	1484	59	112235	7.9
2-acetylfuran	1192-62-7	MS, RI	911	1509	1511	95	19327	12.7
Butyrolactone	96-48-0	MS, RI	962	1637	1635	42	276487	11.3
5-pentyl-*γ*-lactone	104-61-0	MS, RI	882	2056	2055	85	100505	6.8
Pyrans								
Linalool 3,7-oxide	7392-19-0	MS, RI	868	1111	1109	71	255202	10.4
trans-rose oxide	876-18-6	MS, RI	822	1365	1341	139	7646	13.9
Nerol oxide	1786-08-9	MS, RI	831	1476	1466	83	10567	13.3

Continued

Table 3–Continued

Compound[1]	CAS[2] registry #	Method of identification[3]	Similarity	RI^{4}_{ok}	RI^{5}_{lit}	Unique mass[6]	Average area	RSD[7]
Phenols								
Butylated hydroxytoluene	128-37-0	MS, RI	858	1946	1902	205	49914	19.8
p-propylguaiacol	2785-87-7	MS, RI	919	2117	2103	137	11239	11.8
3,5-di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	MS	834	>2219	nf	219	5239	15.9
Nitrogenous compounds								
Methyl isocyanide	593-75-9	MS	980	1002	nf	41	302897	9.9
3-methyl-butanenitrile	625-28-5	MS, RI	797	1132	1120	41	23517	8.5
3,3-dimethyl-butanamide	926-04-5	MS	813	1205	nf	59	134717	14.0
5-methyl-isoxazole	5765-44-6	MS	878	1215	nf	43	25887	14.4
Acetaldoxime	107-29-9	MS	941	1301	nf	59	14668	21.9
Hexanenitrile	628-73-9	MS, RI, ST	872	1315	1303	54	58876	21.4
4-O-acetyl-2,5-di-O-methyl-3,6-dideoxy-d-gluconitrile	N/A	MS	848	1335	nf	129	4639	13.5
2-methoxy-3-isopropyl-pyrazine*	25773-40-4	MS, RI	863	1432	1443	137	51619	8.8
Sulfur compounds								
Dimethyl disulfide*	624-92-0	MS, RI, ST	981	1072	1075	94	128995	27.9
3-methylthiophene	616-44-4	MS, RI, ST	920	1120	1120	97	14082	17.8
Dimethyl sulfoxide	67-68-5	MS, RI, ST	935	1576	1582	63	249017	16.3

[1]Compounds reported previously in fermented cucumber brine are designated with an *.
[2]Chemical Abstracts Service registry number.
[3]MS = identification based on mass spectral match to the NIST 05 library with >800 similarity, RI = comparison with published retention indices on polyethylene glycol column phase, ST = mass spectral and retention index match to authentic standard.
[4]Retention indices based on 1st-dimension retention of components on a SOL-GEL-WAX (polyethylene glycol) column using SPME GC×GC-TOFMS.
[5]Retention indices reported in the literature (nf = not found); References available at the NIST Chemistry WebBook database, http://webbook.nist.gov.
[6]Mass selected by ChromaTOF software during automated data processing to represent an interference free mass for each analyte; The unique mass for each component was used for calculation of peak area.
[7]Relative standard deviation ($n = 7$).

and column bleed at the higher end of the temperature program. Fortunately, with the polar-semipolar column combination, these artifacts were well resolved from sample volatile components (Figure 1), making it possible to detect low-level volatile metabolites in the midst of system contaminants. The presence of contaminant compounds is not unusual and often creates a mass spectral background that can interfere with identification and quantification of sample analytes in one-dimensional (1D) GC chromatograms.

Of the 314 sample peaks detected in fermented cucumber brine, 214 (68%) were tentatively identified by ChromaTOF® data processing based on the best spectral match to the NIST05 library with similarity ≥800. To evaluate the quality of these tentative identifications, authentic standards of 63 compounds were individually chromatographed. The 63 test compounds were chosen from throughout the chromatographic run subject to commercial availability. Based upon retention time and mass spectral matches with components detected in the fermented cucumber brine samples, the best library match was a correct identification in 58 of the 63 cases (92%). The incorrect identification of acetic acid was most likely due to column overload, which has been demonstrated to create problems with the ChromaTOF® deconvolution algorithm (Lisec and others 2006). Although it was incorrectly identified, the overloaded acetic acid peak would have interfered with detection of at least 3 other components in the 1st dimension. These components were clearly resolved in the 2nd dimension, enabling their detection and identification (Figure 1).

Among the 314 volatile components in fermented cucumber brine, 199 had <30% relative standard deviation (RSD) in their peak areas for 7 replicate analyses randomized among 12 other fermented cucumber brine samples over the course of a 3-d run of the instrument (Figure 2). This volatile compound profile was representative of fermented cucumbers in all 3 of the 6% NaCl fermentations analyzed. The 199 reproducibly detected volatile components in fermented cucumber brines included 40 unknowns and 159 tentatively identified compounds. Further manual inspection of the data and comparison with retention indices reported in the literature resulted in the identification of 137 volatile compounds in fermented cucumber brine (Table 3). Comparison of these metabolites with previously reported volatile compounds in fermented cucumber brines (Zhou and McFeeters 1998; Marsili and Miller 2000) indicated that this method may be more sensitive in the detection of plant terpenoids, esters, alcohols, highly volatile aldehydes, and light hydrocarbons as greater numbers of these compounds were found in the present study. Several volatile compounds previously identified in fermented cucumber brines using 1D GC-MS on nonpolar columns were also found in this study, including butanol, pentanol, hexanol, octanol, 2-pentanol, 2-heptanol, 2-ethyl-1-hexanol, eucalyptol, α-terpineol, phenylethyl alcohol, hexanal, nonanal, 2-heptenal, benzaldehyde, acetone, acetic acid, butanoic acid, hexanoic acid, linalool oxide, 2-methoxy-3-(1-methylethyl) pyrazine, and dimethyl disulfide (Zhou and McFeeters 1998; Marsili and Miller 2000).

Nontargeted detection of volatile metabolites associated with spoilage fermentations

Fermented cucumber slurries before and after spoilage with a mixed culture inoculum obtained from spoiled fermented cucumbers were subjected to the GC×GC-TOFMS analysis described. ANOVA of analyte log peak areas revealed 33 metabolites that changed significantly ($P < 0.05$) in concentration after spoilage (Table 4). The nontargeted data analysis approach narrowed the field of approximately 500 peaks per sample to 33 metabolites of interest without extensive manual inspection of the 2D peak table data and chromatograms. The only manual inspection required was review of representative chromatograms to be certain that an appropriate sample dilution was chosen for analysis. Since several compounds of interest were overloaded at the low dilution and many compounds were undetected at higher dilutions, it was necessary to run the FCS samples at 2 dilutions and compile the results to avoid floor and ceiling effects. Noninoculated, sterile-filtered

FCS controls were used to exclude volatile compounds that may have changed due to chemical reactions during the extended incubation time. Changes in compounds tentatively identified as 1-(2,4-dimethyl-furan-3-yl)-ethanone, 2-methyl-2-pentanol, and amylene hydrate were similar in magnitude and direction in both control and spoilage samples. Therefore, they were excluded from the list of potential spoilage metabolites.

Volatile metabolites that changed during spoilage (Table 4) included increases in acetic acid, propanoic acid, butanoic acid, n-propyl acetate, several alcohols, and a decrease in furfural. The observed increases in acetic, propanoic, and butanoic acids were in accordance with previously published studies that showed increases in these components in fermented cucumbers that had undergone secondary spoilage fermentation, as measured by HPLC and tentatively identified based on retention time match (Fleming and others 1989, 2002; Kim and Breidt 2007). In the present study, the increase in butanoic acid detected by GC×GC-TOFMS was below the detection limit of the HPLC. However, quantitative analysis of acetic and propanoic acids by HPLC confirmed the fold increases detected with this nontargeted volatile analysis method in the anaerobic reproduction of the spoilage in FCS (Table 1). In addition to confirming these 2 primary spoilage metabolites, several other target spoilage metabolites were discovered. Comparison of these metabolite changes to the original brine samples from spoiled fermented cucumbers showed that 11 of the 33 metabolites that changed in cucumber slurries upon controlled reproduction of spoilage coincided with metabolites that were formed or utilized in the brines of spontaneously spoiled fermented cucumbers. In addition to acetic, propanoic, and butanoic acids, there were increases in n-propyl acetate, isoamyl acetate, and 1-butanol. Decreases were observed in components identified as ethyl lactate, acetonitrile, methyl lactate, tetrahydrofuran, and 1-penten-3-ol. The other 22 metabolites observed to change when the spoilage was transferred to sterilize FCS in an anaerobic chamber showed that the transferred bacteria were able to utilize or produce a number of metabolites differently than in the original spoilage. The significance of these metabolites in the overall spoilage process remains to be determined.

Table 4–Changes in volatile metabolites associated with anaerobic spoilage of fermented cucumber slurry (pH 3.8, 6% NaCl).

Metabolite[1]	CAS[2] registry #	RI[3]	Fold change[4]	Direction of change
4-methyl-benzenemethanol	589-18-4	1960	579.4	Increase
Unknown A	N/A	1371	342.2	Increase
4-methyl-2-heptanol	56298-90-9	1375	188.2	Increase
4-methyl-2-pentanol	108-11-2	1173	183.5	Increase
2-hexanol	626-93-7	1231	33.4	Increase
4-methyl-3-hepten-2-one[MS]	22319-25-1	1015	29.4	Increase
Unknown B	N/A	1902	11.7	Increase
2-pentanol	71-41-0	1126	11.4	Increase
3-methylene-2-pentanone[MS]	4359-77-7	1137	6.3	Increase
Isoamyl acetate*	123-92-2	1127	6.0	Increase
n-propyl acetate*	109-60-4	961	6.0	Increase
Propanoic acid*	79-09-4	1543	4.8	Increase
2-heptanol	543-49-7	1334	4.7	Increase
Tert-butyl ethyl ether	637-92-3	710	4.6	Increase
Acetic acid*	64-19-7	1449	4.5	Increase
Unknown C	N/A	920	3.7	Increase
Unknown D	N/A	962	3.3	Increase
Butanoic acid	107-92-6	1636	3.0	Increase
3-pentanol	584-02-1	1110	2.8	Increase
1-butanol*	71-36-3	1153	2.5	Increase
2-methyl-2-propanol	75-65-0	897	2.5	Increase
Methyl acetate	79-20-9	825	2.3	Increase
unknown E		882	1.6	Increase
Ethyl lactate*	97-64-3	1353	1.7	Decrease
Methyl propionate	554-12-1	905	1.9	Decrease
Acetonitrile*	75-05-8	988	2.3	Decrease
Methyl lactate*	2155-30-8	1328	3.2	Decrease
Tetrahydrofuran*	109-99-9	854	3.5	Decrease
1-penten-3-ol*	616-25-1	1166	4.5	Decrease
Unknown F	N/A	1535	6.2	Decrease
Furfural	98-01-1	1464	30.1	Decrease
Unknown G	N/A	967	85.0	Decrease
2,3-butanedione	431-03-8	965	100.1	Decrease

[1]Identification based on mass spectral and retention index match to authentic standards except where noted. [MS]indicates a tentative identification based on mass spectral match to the NIST library. Metabolites marked with an * symbol indicate those compounds that were also found to increase or decrease in the brines from the original spoilage of fermented cucumbers.
[2]Chemical Abstracts Service registry number.
[3]Retention indices based on 1st-dimension retention of components on a SOL-GEL-WAX (polyethylene glycol) column using SPME GC×GC-TOFMS.
[4]Fold change based on ratio of unique mass peak area of a given metabolite in spoiled fermented cucumber slurry as compared to initial fermented cucumber slurry.

Conclusions

Comprehensive GC×GC-TOFMS provides the analytical capability to resolve and identify many more volatile components from food samples than traditional GC-MS methods. However, complex datasets are generated for each sample such that standard approaches to data analysis are impractical when the target analytes are unknown. Optimization of the 2D separation combined with a semi-automated approach to data reduction using the instrument software and basic statistical analysis made it feasible to detect and identify many volatile components in fermented cucumbers. Fermented cucumber brines were found to contain 137 reproducibly detected and identified volatile compounds from a variety of chemical classes including hydrocarbons, aldehydes, ketones, alcohols, acids, esters, furans, and terpenoids. The nontargeted GCGC-TOFMS method and data analysis made possible the separation, identification, and determination of differences in polar volatile components, facilitating the discovery of several metabolites that were formed or utilized during anaerobic spoilage of fermented cucumbers. Further study of these metabolites will enhance our ability to understand and potentially control the metabolism of spoilage bacteria that can degrade lactic acid under the restrictive environmental conditions present in fermented cucumbers.

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