## ABSTRACT

DAUGHTRY, KATHERYNE VIRGINIA. Phenotypic and Genotypic Characterization of *Lactobacillus buchneri* Strains Isolated from Spoiled Fermented Cucumber. (Under the direction of Dr. Rodolphe Barrangou and Dr. Suzanne D. Johanningsmeier).

*Lactobacillus buchneri* is a facultative anaerobe and member of the lactic acid bacteria. *L. buchneri* has been isolated from various environments, but most commonly from decomposing plant material, such as silage and spoiled food products, including wine, beer, Swiss cheese, mayonnaise, and fermented cucumber. Recently, the metabolic pathway for the conversion of lactic acid to acetic acid and 1,2-propanediol was annotated in this species. Although this metabolic pathway is not common in most lactic acid bacteria, *L. buchneri* degrades lactate under various conditions. Lactic acid utilization in fermented cucumbers leads to a rise in pH, ultimately spoiling the product. In previous studies, strains of *L. buchneri* isolated from fermented cucumber spoiled displayed variation in colony morphologies. It was predicted the isolates were phenotypically and genotypically diverse, and that the abilities to degrade lactic acid may be strain specific. To examine this hypothesis, thirty-five *L. buchneri* cultures isolated from spoiled fermented cucumber and the type strain isolated from tomato pulp were characterized and unique strains were subjected to whole genome sequencing. Each isolate was genotypically and phenotypically characterized using 16S rDNA sequencing, DiversiLab® rep-PCR, colony morphology on MRS agar, carbohydrate profiling, growth rates in MRS media, and the ability to degrade lactic acid in a modified MRS medium. Great diversity in colony morphology revealed variations of color (ranging from opaque yellow to white), texture (brittle, viscous, or powdery), shape

(umbonate, flat, circular, or irregular) and size (1 mm- 11mm). API® 50CHL revealed a diverse spectrum of carbohydrate utilization among the isolates. Some strains metabolized as many as 16 or as few as 9 carbohydrates, including xylose, mannitol, and lactose. Glucose and maltose were the only carbohydrates fermented by all of the isolates. DiversiLab® rep-PCR revealed 8 distinct clusters of isolates with 20/36 of the isolates exhibiting more than 97 % similarity. Each isolate was able to degrade lactic acid in both a modified MRS medium and fermented cucumber medium, but differences in the rate of lactic acid utilization were observed. Although isolated from similar environmental niches, significant phenotypic and genotypic diversity was found among the *L. buchneri* strains. Eight unique isolates were selected for genome sequencing, and a preliminary analysis revealed shared synteny across each of the strains. The G+C content of the organisms ranged from 44.0-45.2, and genome sizes were 2.49 Mbp to 2.72 Mbp. Using the RAST (Rapid Annotation using Subsystem Technology) pipeline, subsystems were annotated for each of the 8 sequenced strains of *L. buchneri*. Strain differences were found in numerous genes for carbohydrate metabolism, cell wall and capsule elements, and presence of phage, prophage, plasmids and other transmissible elements. This is the first study to explore the diversity of this species from a single environmental niche.

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Phenotypic and Genotypic Characterization of *Lactobacillus buchneri* Strains Isolated from Spoiled, Fermented Cucumber

by
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_______________________________
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## DEDICATION

I dedicate my thesis to the following:

My wonderful father, **Rudy Daughtry**, for his unyielding love and support.

My sister, **Trish**, for always playing "devil's advocate" and pushing me to be my best every day.

My "adopted" family, **Julia, Sarah**, and **Mary Wall**, for welcoming me into your home and loving me as your own.

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# BIOGRAPHY

Katheryne Daughtry was born to Deborah and Rudy Daughtry in Wrightsville Beach, North Carolina on July 26th, 1991. Katheryne spent her early days building many a sandcastle, attempting to play piano, and toting around her younger brother and accomplice, Parker, in all her endeavors. In 2013, she graduated with a B.S. in Food Science and a minor in Agribusiness Management at North Carolina State University. During this time, she conducted undergraduate research in the USDA-ARS Food Science Research Unit under the direction of Dr. Ilenys Perez-Diaz and Dr. Suzanne Johanningsmeier. In the summer of 2013, Katheryne started an internship at S&D Coffee and Tea, working under the “direction” of a bitter Michigan State fan (David Landis) and curmudgeon (Joe Bremser), where she successfully earned the title “BEST (and only) INTERN EVER.”

Two weeks post-graduation, Katheryne started her Master’s degree with Dr. Suzanne Johanningsmeier and Dr. Rodolphe Barrangou. During her time spent as a proud member of the Wolfpack, Katheryne was an active member of the Food Science Club, serving as the Dairy Bar Fundraising Co-chair for 2 terms, and served on the Outreach, Wine & Cheese, and Public Service Committees. Katheryne enjoys SCUBA diving, yoga, traveling, baking, volunteering, and all things wine, chocolate and coffee-related.

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**TABLE OF CONTENTS**

LIST OF TABLES ..... vii
LIST OF FIGURES ..... viii

1 CHAPTER 1: *LACTOBACILLUS BUCHNERI* METABOLISM: IMPLICATIONS FOR FOOD SPOILAGE AND INDUSTRIAL APPLICATIONS ..... 1

1.1 INTRODUCTION ..... 2

1.2 TAXONOMY OF LACTIC ACID BACTERIA ..... 3

1.3 PHYLOGENY OF THE *LACTOBACILLUS BUCHNERI* GROUP ..... 4

1.4 OVERVIEW OF CARBOHYDRATE METABOLISM IN LACTIC ACID BACTERIA ..... 6

1.5 FUNCTIONAL METABOLIC FEATURES OF *LACTOBACILLUS BUCHNERI* .. 7

*1.5.1 CARBOHYDRATE UTILIZATION ..... 7*

*1.5.2 LACTATE DEGRADATION ..... 8*

*1.5.3 ARGININE DEIMINASE PATHWAY ..... 11*

1.6 THE ROLE OF *LACTOBACILLUS BUCHNERI* IN FOOD SPOILAGE ..... 14

*1.6.1 SWISS CHEESE HISTAMINE POISONING ..... 14*

*1.6.2 LACTATE DEGRADATION IN FERMENTED CUCUMBER ..... 15*

1.7 INDUSTRIAL APPLICATIONS OF *LACTOBACILLUS BUCHNERI* ..... 18

*1.7.1 SILAGE STARTER CULTURE ADJUNCT TO IMPROVE AEROBIC STABILITY 18*

*1.7.2 BIOCONVERSION OF BIOMASS SUBSTRATES FOR ALTERNATIVE FUEL PRODUCTION ..... 20*

1.8 SUMMARY ........ 21

1.9 OVERVIEW OF RESEARCH ........ 22

*1.9.1 HYPOTHESIS ........ 22*

*1.9.2 OBJECTIVES ........ 22*

*1.9.3 SIGNIFICANCE OF RESEARCH ........ 23*

1.10 REFERENCES ........ 24

2 CHAPTER 2: PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *LACTOBACILLUS BUCHNERI* STRAINS ISOLATED FROM SPOILED, FERMENTED CUCUMBER ........ 30

2.1 ABSTRACT ........ 31

2.2 INTRODUCTION ........ 33

2.3 MATERIALS AND METHODS ........ 35

*2.3.1 BACTERIAL STRAIN CULTIVATION ........ 35*

*2.3.2 16S rDNA SEQUENCING ........ 36*

*2.3.3 COLONY AND CELLULAR MORPHOLOGY ........ 37*

*2.3.4 GROWTH CURVES IN DE MAN ROGOSA AND SHARPE (MRS) BROTH ..... 37*

*2.3.5 CARBOHYDRATE FERMENTATION PROFILES ........ 38*

*2.3.6 LACTATE UTILIZATION IN FERMENTED CUCUMBER MEDIA ........ 38*

*2.3.7 LACTATE UTILIZATION IN A MODIFIED MRS MEDIUM (MMRS) ........ 39*

*2.3.8 DIVERSILAB® STRAIN TYPING ........ 40*

2.4 RESULTS ........ 40

*2.4.1 16S rDNA SEQUENCING ........ 40*

*2.4.2 COLONY MORPHOLOGY ........ 41*

*2.4.3 CELLULAR MORPHOLOGY ........ 41*

*2.4.4 GROWTH CURVES IN DE MAN, ROGOSA AND SHARPE (MRS) MEDIUM 42*

*2.4.5 CARBOHYDRATE UTILIZATION ........ 42*

*2.4.6 LACTIC ACID UTILIZATION ........ 44*

*2.4.7 DIVERSILAB® STRAIN TYPING ........ 45*

2.5 DISCUSSION ........ 46

2.6 ACKNOWLEDGEMENTS ........ 53

2.7 REFERENCES ........ 55

3 CHAPTER 3: DRAFT GENOME SEQUENCES OF *LACTOBACILLUS BUCHNERI* 69

3.1 ABSTRACT ........ 70

3.2 INTRODUCTION ........ 72

3.3 METHODS ........ 73

3.4 RESULTS ........ 74

3.5 ACKNOWLEDGEMENTS ........ 77

3.6 REFERENCES ........ 78

CHAPTER 4: CONCLUSIONS ........ 84

A. APPENDICES ........ 87

## LIST OF TABLES

TABLE 2.1: *LACTOBACILLUS BUCHNERI* ISOLATES .................................................... 60

TABLE 2.2: COLONY MORPHOLOGIES OF 8 *LACTOBACILLUS BUCHNERI* ISOLATES FROM SPOILED, FERMENTED CUCUMBER ...................................... 63

TABLE 3.1: OVERVIEW OF GENOMIC DATA FROM 8 SEQUENCED GENOMES OF *LACTOBACILLUS BUCHNERI* .......................................................................... 70

TABLE 3.2: SUBSYSTEM OVERVIEW OF EIGHT *LACTOBACILLUS BUCHNERI* GENOMES ................................................................................................ 81

**APPENDIX**

**TABLE A.1:** CARBOHYDRATE PROFLING OF 36 ISOLATES OF *LACTOBACILLUS BUCHNERI*

**TABLE A.2**: CARBOHYDRATE PROFILING OF 36 ISOLATES OF *LACTOBACILLUS BUCHNERI* (CONTINUED)

**TABLE A.3**: *LACTOBACILLUS BUCHNERI* ISOLATES AFTER 28 DAYS IN FERMENTED CUCUMBER JUICE .................................................................. 92

## LIST OF FIGURES

**FIGURE 2.1**: MORPHOLOGICAL DIVERSITY OF SELECTED ISOLATES OF *LACTOBACILLUS BUCHNERI* ........ 62

**FIGURE 2.2**: CELLULAR MORPHOLOGY OF SELECTED *LACTOBACILLUS BUCHNERI* ISOLATES: PRESENCE OF AUTO-AGGREGATION PHENOTYPE IN STRAINS LA1184 AND LA1167 AFTER 36 H OF GROWTH IN MRS MEDIA USING PHASE CONTRAST MICROSCOPY. ABSENCE OF PHENOTYPE WAS OBSERVED IN STRAINS LA0030 AND LA1181.

64

**FIGURE 2.3**: HIERARCHIAL CLUSTER ANALYSIS OF CARBOHYDRATE UTILIZATION ........ 65

**FIGURE 2.4**: GROWTH CURVES OF SELECTED ISOLATES OF *LACTOBACILLUS BUCHNERI* ........ 66

**FIGURE 2.5**: DIVERSILAB® DENDROGRAM DISPLAYING 36 ISOLATES OF *L. BUCHNERI* ........ 67

**FIGURE 2.6**: LACTIC ACID DEGRADATION OF 8 SELECTED ISOLATES OF *LACTOBACILLUS BUCHNERI* IN MODIFIED MRS MEDIA ........ 68

**FIGURE 3.1** MAUVE ALIGNMENT OF 8 *LACTOBACILLUS BUCHNERI* STRAINS ... 83

# CHAPTER 1: *LACTOBACILLUS BUCHNERI* METABOLISM: IMPLICATIONS FOR FOOD SPOILAGE AND INDUSTRIAL APPLICATIONS

## 1.1 INTRODUCTION

*Lactobacillus buchneri* is a member of the lactobacillaceae, a family of lactic acid bacteria. Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive, non-spore forming, catalase-negative microorganisms producing lactic acid as one of the primary end products of carbohydrate metabolism. LAB emerged around 3 billion years ago and began to co-evolve with milk-producing mammals around 65 million years ago (Champomier-Verges, Maguin et al. 2002, Makarova, Slesarev et al. 2006). Evidence of sourdough consumption in a typical diet and references of cheese and yogurt consumption have been documented from present day Iraq (Mesopotamia) dating back to 3200 BC (Stiles and Holzapfel 1997). A variety of genera are included within the LAB occupying environmental niches relevant to food production and preservation. Consequently, these organisms are classified as GRAS, 'Generally Recognized as Safe'. Since the establishment of the agro-food industry, domesticated LAB have become widely used as starter cultures and adjuncts for fermented food products. These cultures have been cultivated for traits such as bacteriophage resistance, survival in various conditions, and ability to produce organic acids, and have since adapted to these environments.

While ubiquitous in food and feed fermentations, certain LAB species are more often affiliated with spoilage. *Lactobacillus buchneri* is a facultative anaerobe most often isolated from spoiled or aged food and beverage environments. *L. buchneri* are non-motile, rod-shaped cells with rounded ends ranging from 0.8-1.0 $u$m wide and 2.0-3.0 $u$m long, occurring singly, in pairs, and in chains (Hammes and Vogel 1995). This bacterium has been

isolated from various environments, including spoiled fermented cucumbers, cheese, sourdough bread, wine, beer wort, human intestines, aerobically stable silage, and commercial ethanol fermentations (Sumner, Speckhard et al. 1985, Sakamoto and Konings 2003, Liu, Skinner-Nemec et al. 2008, Zeng, Pan et al. 2010, Franco, Perez-Diaz et al. 2012, Johanningsmeier, Franco et al. 2012). *L. buchneri* strains can metabolize a diverse spectrum of carbohydrates, degrade lactic acid under oxic and anoxic conditions, and degrade arginine through the arginine deiminase (ADI) pathway (Liu, Pritchard et al. 1994, Liu, Skinner-Nemec et al. 2008). Therefore, *L. buchneri* has been recognized as an organism of interest in industrial applications such as silage fermentations and alternative biofuel production. This organism has also been identified as a causative agent in spoilage of fermented cucumbers (Franco, Perez-Diaz et al. 2012, Johanningsmeier and McFeeters 2013).

## 1.2 TAXONOMY OF LACTIC ACID BACTERIA

Traditionally, LAB have been separated into three taxonomic groups based on preference for pentose fermentation and the pathways utilized to metabolize carbohydrates. Preference of pathway is family-specific. Members of the families *Enterococcaceae*, *Lactobacillaceae,* and *Streptococcaceae* all use the glycolytic pathway except for Group III lactobacilli. Classification of metabolic groups depends on the presence or absence of the enzymes fructose 1,6-diphosphate (FDP) aldolase (glycolytic pathway) or phosphoketolase (phosphoketolase pathway) (Pot, Felis et al. 2014, Vandamme, De Bruyne et al. 2014). Obligate homolactic LAB possess FDP aldolase (no phosphoketolase), while obligate

heterofermentative LAB produce only phosphoketolase (typically no FDP aldolase). Facultative heterolactic LAB lack FDP aldolase, and ferment hexoses exclusively via the EMP pathway, while pentoses are metabolized with an inducible phosphoketolase (Vandamme, De Bruyne et al. 2014).

The first taxonomic group is defined as obligately homofermentative. Included in this group are Group I lactobacilli, which metabolize glucose via the glycolytic pathway, but cannot metabolize pentose carbohydrates (Kandler 1983). Facultative heterofermentative LAB make up the second group, metabolizing glucose exclusively via glycolysis and pentoses via the phosphoketolase pathway. *Enterococcus*, *Lactococcus*, *Lactovum*, *Paralactobacillus*, *Pediococcus*, *Streptococcus*, *Vagococcus* and Group II *Lactobacillus spp* are present in this group (Endo and Dicks 2014). The third group consists of obligate heterofermentative LAB, including *Leuconostoc sp, Oenococcus sp, Weissella sp* and Group III *Lactobacillus sp*., which includes *L. buchneri*. Obligate heterolactic organisms metabolize pentoses, hexoses and related compounds solely through the phosphoketolase pathway.

### 1.3 PHYLOGENY OF THE *LACTOBACILLUS BUCHNERI* GROUP

Phylogenetic groups are constructed based on comparative analysis of the 16S rRNA gene to determine the genetic relatedness of the LAB species. The *L. buchneri* group is one of the largest phylogenetic groups within the *Lactobacillus* genus. Species within this group are mostly obligate heterofermenters, with two facultative heterofermentative members, *L. parafarraginis* and *L. farraginis* (Endo and Okada 2007) and two obligate homofermentative

organisms *L. keferi* and *L. senioris* (Takizawa, Kojima et al. 1994). The G+C content of the DNA ranges between 38.8 % and 42.0 % and each of the group members produces both L-(+) and D-(-) isomers of lactic acid, except for *L. parakefiri*, which produces only the L-(+) isomer (Pot, Felis et al. 2014). The organisms within the *Lactobacillus buchneri* group have mostly been isolated from food fermentation environments. The first and oldest species within the group is *Lactobacillus buchneri*, originally named *Bacillus buchneri.* It was isolated from various sources including pressed yeast, milk, cheese, and the oral cavity in 1903 by German scientist E. Buchner. *L. buchneri* has been previously mischaracterized as *L. brevis*. While not a member of the *Lactobacillus buchneri* group, *L. brevis* is phenotypically similar to *L. buchneri,* but it is unable to metabolize melezitose (Pot, Felis et al. 2014). One species from the group includes *Lactobacillus diolivorans,* which has been repeatedly isolated in maize silage in The Netherlands along with *L. buchneri,* and can metabolize 1,2-propanediol under anoxic conditions to produce propanol and propionic acid (Zhang, Brandt et al. 2010, Pot, Felis et al. 2014). Other species from the *L. buchneri* group include *L. parabuchneri,* isolated from saliva, cheese and contaminated brewery yeast (Farrow, Phillips et al. 1988, Hammes and Vogel 1995, Pot, Felis et al. 2014), *L. kefiri* and *L. parakefiri* isolated from kefir grains (Masuda and Kawata 1985), and *L. farraginis* and *L. parafarraginis* isolated from a compost of distilled shochu residue, an alcoholic Japanese beverage made from sweet potatoes, barley, or rice (Endo and Okada 2007, Pot, Felis et al. 2014). Other members of the *L. buchneri* group include *L. kisonensis, L. otakiensis, L. rapi* and *L. sunkii*. Each of these species was isolated from unsalted pickle brine used in

production of sunki, an unsalted fermented vegetable product native to the Kiso Valley region of Japan (Watanabe, Fujimoto et al. 2009). *L. rapi* was also isolated from spoiled fermented cucumber and shown to convert 1,2-propanediol to propanol and propionic acid (Johanningsmeier et al. 2012). Other species within the *L. buchneri* group include *L. senioris* and *L. leiae*, isolated from human fecal matter and putrid tofu brine (Pot, Felis et al. 2014).

## 1.4 OVERVIEW OF CARBOHYDRATE METABOLISM IN LACTIC ACID BACTERIA

Lactic acid bacteria proliferate in environments high in available nutrients, particularly with carbohydrates as the primary carbon source. A diverse continuum of carbohydrates is metabolized via a homofermentative or heterofermentative pathway, with D-(+) or L-(-) lactic acid generated as the primary metabolic end product. Homolactic organisms metabolize hexoses via the Embden-Meyerhof-Parnas (glycolysis) pathway, producing a majority of lactic acid (>80 % vol/vol) as the fermentation product. In this pathway, 2 molecules of lactic acid are generated per molecule of glucose consumed, involving the key enzyme FDP aldolase (Pot, Felis et al. 2014). During substrate-level phosphorylation, homofermentative LAB produce 2 molecules of ATP from one molecule of glucose (Zaunmüller, Eichert et al. 2006). Heterofermentative bacteria metabolize pentoses and hexoses via the 6-P-gluconate/phosphoketolase pathway, resulting in production of lactic acid, ethanol and carbon dioxide. Depending on the substrate, acetate may also be produced. The ratio to which these end products are produced is dependent on the species, as well as the

presence of electron acceptors and pH of the growth medium (Endo and Dicks 2014). For some heterofermentative LAB, hexoses can be used for the production of exopolysaccharides such as dextran, levan, and fructan (Cerning 1990, Zaunmüller, Eichert et al. 2006).

## 1.5 METABOLIC FEATURES OF *LACTOBACILLUS BUCHNERI*

### 1.5.1 *CARBOHYDRATE UTILIZATION*

*L. buchneri* is classified under the Group III lactobacilli as obligately heterolactic, metabolizing hexoses and pentoses through the phosphoketolase pathway into lactic acid, acetic acid and carbon dioxide, and traces of ethanol (Hammes and Vogel 1995). About 90 % of *L. buchneri* strains ferment arabinose, maltose, melezitose, ribose and melibiose, while between 11 % and 89 % of strains utilize esculin, galactose, raffinose, sucrose and xylose (Wood and Holzapfel 1995). It has been reported that less than 10 % of *L. buchneri* strains metabolize cellobiose, mannose and trehalose as a sole carbon source (Wood and Holzapfel 1995, Liu, Skinner-Nemec et al. 2008). A study aiming to identify differences in carbohydrate utilization examined three strains of *L. buchneri*, NRRL B-30929, DSM 5987, and NRRL 1837 using a simplified MRS medium supplemented with the carbon source of interest (Liu, Bischoff et al. 2009). The results from the study showed that each of the three strains utilized L-arabinose, D-galactose, D-gluconic acid, D-ribose, D-fructose, α-D-glucose, maltose, sucrose, adenosine, methyl pyruvate, D-psicose, L-xylose, pyruvic acid, D-glucosamine, oxalomalic acid, dihydroxy acetone, D-melibiose, inosine, uridine, and D-melezitose. However, none of the strains were able to metabolize D-trehalose, maltotriose, mannose and *N*-acetyl-glucosamine as a sole carbon source. NRRL B-20929 could not

ferment sucrose, while NRRL 1837 and DSM 5987 could not utilize cellobiose. DSM 5987 could not metabolize turanose (Liu, Bischoff et al. 2009). A similar study profiled carbohydrate utilization analyzing various isolates of *L. buchneri* from tomato pulp, mayonnaise, and dressings. In a period of 19 days, isolates fermented amygdalin, L-arabinose, dulcitol, potassium gluconate, D-lactose, D- melezitose, methyl-D-glucopyranoside, methyl-D-mannopyranoside, D-raffinose, L-rhamnose, D-sorbitol, D-trehalose, D-turanose, and D-xylose (Sanders, Oomes et al. 2014). Overall, *L. buchneri* strains have shown a diverse range of carbohydrate metabolism.

### 1.5.2 *LACTATE DEGRADATION*

*L. buchneri* is noteworthy for its diverse metabolic activities within food and feed matrices. Of most recent significance is *L. buchneri*'s ability to degrade lactic acid to 1,2-propanediol and acetic acid without the use of an external electron acceptor. (Elferink, Krooneman et al. 2001). *Lactobacillus parafarraginis* isolated from spoiled fermented cucumber shares this metabolism (Johanningsmeier and McFeeters 2013). However, lactate degradation is unusual for most LAB (Endo and Dicks 2014). Only a few species including *Lactobacillus bifermentans, Lactobacillus brevis, Lactobacillus plantarum, L. buchneri* and *Lactobacillus parabuchneri* have been shown to degrade lactate in a microbiological medium. Under certain pH conditions, ($pH > 4.0$) the facultative heterolactic *L. bifermentans* degrades lactate to acetate, ethanol, $CO_2$ and $H_2$ after carbohydrate sources have been depleted (Kandler 1983). *Lactobacillus plantarum* can consume lactate under anoxic

conditions using citrate as an electron acceptor (Bobillo and Marshall 1991, Bobillo and Marshall 1992). Veiga da Cunha and Foster found that *L. buchneri* strain B190 and *L. brevis* strain B22 used glycerol as an electron acceptor to metabolize lactic acid into 1,3-propanediol, acetate and $CO_2$ (Veiga-Da-Cunha and Foster 1992). However, strains of *L. buchneri* and *L. parabuchneri* isolated from silage were found to degrade lactate into acetate and 1,2-propanediol at pH 4.0 without the need for an external electron acceptor. Lactate degradation is pH and temperature dependent. It is proposed that the primary role of this pathway is to preserve cell viability by increasing the extracellular pH (Elferink, Krooneman et al. 2001).

A set of putative genes deemed necessary for the novel lactate utilization pathway proposed by Elferink et al. were annotated in the recently sequenced genome of *L. buchneri* CD034, isolated from stable grass silage (Elferink, Krooneman et al. 2001; Heinl, Wibberg et al. 2012). Under anoxic conditions, *L. buchneri* forms acetate from lactate by generation of an acetyl phosphate from acetyl-CoA by phosphate acetyltransferase (Elferink, Krooneman et al. 2001). One mole of ATP is formed during the hydrolysis of acetyl phosphate by acetate kinase to generate acetic acid (Heinl, Wibberg et al. 2012). Concomitantly, 1,2-propanediol and ethanol are produced to regenerate $NAD^+$ from NADH (Heinl, Wibberg et al. 2012). Lactaldehyde dehydrogenase transforms lactate to lactaldehyde, which is then converted to 1,2-propanediol by a putative lactaldehyde reductase (Elferink, Krooneman et al. 2001, Heinl, Wibberg et al. 2012). It is proposed that ethanol is generated from acetyl-CoA and catalyzed by an acetaldehyde-coA/ alcohol dehydrogenase (Heinl, Wibberg et al. 2012).

Currently, it is unknown if this lactate degradation pathway exists in all strains of *L. buchneri*.

Elferink et al. found that lactic acid utilization by *L. buchneri* occured under anoxic conditions (Elferink, Krooneman et al. 2001) . In a similar study, Johanningsmeier et al. found that this organism can metabolize lactic acid equally well in fermented cucumber under both anaerobic and aerobic conditions (Johanningsmeier, Franco et al. 2012; Johanningsmeier and McFeeters 2013). Once all residual carbon sources had been metabolized, *L. buchneri* was able to degrade lactic acid in a fermented cucumber juice medium at a pH of 3.2 and 3.8. However, lactate degradation was not observed when the pH was raised to 5.0 (Johanningsmeier, Franco et al. 2012, Johanningsmeier and McFeeters 2013). These studies exhibited the significance of sodium chloride (NaCl) on lactic acid metabolism by *L. buchneri* in fermented cucumber media (FCM). Concentrations of 0, 2 4, and 6 (at pH 3.8) % NaCl in FCM did not inhibit this specific metabolic pathway for the organism. Instead, *L. buchneri* degraded the largest amount of lactic acid into its byproducts of acetic and 1, 2-propanediol in 2 % NaCl FCM. This suggests the use of a sodium-dependent electron acceptor/transporter may be involved in spoilage of FCM (Johanningsmeier, Franco et al. 2012, Johanningsmeier and McFeeters 2013).

Lactate degradation by *L. buchneri* was found to be strongly pH dependent, as acidic conditions are required to induce lactic acid degradation in microbiological media and fermented cucumber (Elferink, Krooneman et al. 2001; Johanningsmeier and McFeeters et al. 2013). Undissociated organic acids such as lactate diffuse through the cell membrane,

resulting in a decrease of the internal pH. After accumulating within the cytoplasm, the acid degrades the cell wall components and renders the cell non- viable. In order to prevent this, the bacteria maintain the proton motive force by hydrolyzing ATP to expel protons from the cells and increase the internal pH (Brink and Konings 1980, Kashket 1985). In a similar protective mechanism to counter the acidic internal environment, *L. buchneri* may decrease the concentration of undissociated acids by degrading lactate into a fatty acid with a higher $pK_a$ and an alcohol. Thus, a proton is consumed, increasing the intracellular pH. This response may be a protective mechanism to increase the extracellular pH as triggered by the surrounding acidic conditions (Elferink, Krooneman et al. 2001). While this reaction does produce or require ATP, it does not support cell growth, but instead is a method for maintaining cell viability under acidic conditions (Kashet 1985).

### 1.5.3 ARGININE DEIMINASE PATHWAY

Many species of naturally occurring homo and heterofermentative lactic acid bacteria degrade malic acid into lactic acid in red and white wines after the primary fermentation, leading to a reduction in acidity and a modification of the flavor properties (Mira de Orduna, Liu et al. 2000). These organisms, known as malolactic bacteria (MLB) include homo- and heterofermentative lactobacilli. Aside from metabolizing malic acid, only some species of heterofermentative malolactic bacteria will degrade arginine, a vital amino acid in wines, leading to the formation of ammonia and citrulline. The arginine deiminase pathway is an acid tolerance mechanism involving three enzymes: arginine deiminase, ornithine

transcarbamylase, and carbamate kinase (Zimmerli and Schlatter 1991). Citrulline is an amino acid precursor to the carcinogenic ethyl carbamate (also known as urethane) (Mira de Orduna, Liu et al. 2000). Arginine is metabolized through the arginine deiminase pathway. When arginine is degraded through the ADI (arginine deiminase) pathway, ammonia, ornithine, ATP, and $CO_2$ are formed. Ammonia increases the pH of the medium, thus allowing for outgrowth of spoilage bacteria.

One particular MLB is *L. buchneri*, which has been isolated from several wines undergoing malolactic fermentation (MLF) and has been considered undesirable because of its ability to degrade arginine. In two separate studies conducted by Liu *et al* (Liu, Pritchard et al. 1994) and Orduna *et al* (Mira de Orduna, Liu et al. 2000), a strain of *L. buchneri* isolated from a California winery was shown to metabolize both malate and arginine, but similarly to lactic acid degradation studies with this species, the results were pH dependent (Liu, Pritchard et al. 1994, Mira de Orduna, Liu et al. 2000). At higher initial pH levels (3.6 and 3.9), the *L. buchneri* strain CUC-3 degraded arginine more readily compared to only metabolizing 50 % of available arginine at a lower pH of 3.3. Higher initial concentrations of arginine (1.5 g arginine $L^{-1}$) were degraded in the same amount of time as the lower concentrations (0.5 g arginine $L^{-1}$), leading to a faster degradation of fructose and glucose. It was also found that growth was dependent on arginine. Arginine cannot be used as a primary source of energy for a bacterium in the absence of a fermentable carbohydrate. Seemingly, energy generated from carbohydrate metabolism is necessary to induce the initial uptake of

arginine (Liu and Pilone 1998). Growth concluded as arginine sources were depleted from the media, even in the presence of residual hexoses.

Citrulline, an intermediate in the arginine deiminase pathway, can be metabolized by other MLB in wine fermentations. *Oenococcus oeni*, a common starter culture for MLF, metabolized arginine into citrulline only at a pH of 3.9 (de Orduña, Patchett et al. 2001). Furthermore, only 2.2 – 3.9 % of arginine was converted to citrulline by *L. buchneri* (de Nadra, Chaud et al. 1986). Unlike *O. oeni, L. buchneri* CUC-3 re-catabolized the emitted citrulline once the arginine supply was exhausted, possibly indicating the presence of a citrulline-ornithine antiporter (Liu, Pritchard et al. 1996, Liu and Pilone 1998). Further research is necessary to determine the factors affecting the re-assimilation of excreted citrulline. Unlike other malolactic bacteria, it is suspected that *L. buchneri* possesses the full complement of enzymes in the ADI pathway (de Orduña, Patchett et al. 2001). Specific activity of the three enzymes, arginine deiminase, orthinine transcarbamylase, and carbamate kinase, were higher when *L. buchneri* cells were grown in galactose as opposed to glucose or fructose (Manca, Nadra et al. 1986). This indicates that glucose and fructose may be repressing the enzyme activity. Small amounts of citrulline (18.2 µmole g/ dry wt) and arginine (12.5 µmole g/ dry wt) are naturally present in most cucurbits (Fish and Bruton 2010). In studies with cucumber fermentations, it is proposed that *L. buchneri* does not initiate citrulline degradation until after residual carbohydrates are metabolized (Johanningsmeier and McFeeters 2015). Arginine to citrulline exchange increased significantly with increasing concentrations of glucose in the medium (de Orduña, Patchett et

al. 2001).While other MLB increase acid tolerance by depleting malic acid, *L. buchneri* CUC-3 does so by exhausting arginine in the wine medium, thus making this strain a competitive contaminant in malolactic fermentation (Marquis, Bender et al. 1987).

## 1.6 THE ROLE OF *LACTOBACILLUS BUCHNERI* IN FOOD SPOILAGE

*Lactobacillus buchneri*'s diverse metabolic activity makes it a detrimental spoilage organism in various fermented foods, particularly as it pertains to lactic acid catabolism. This organism has been isolated from several other environments including beer, wine, mayonnaise, and sauerkraut where spoilage occurred due to the typical fermentative metabolism of lactic acid bacteria. *L. buchneri* has also been found as a primary spoilage organism in both cheese and fermented cucumbers for it's unique metabolic capabilities.

### 1.6.1 SWISS CHEESE HISTAMINE POISONING

In 1985, *Lactobacillus buchneri* was isolated from Swiss cheese implicated in a small outbreak of histamine poisoning in New Hampshire (Sumner, Speckhard et al. 1985). Thirty-seven strains were isolated for bacteriological analysis of the Swiss cheese from the Department of Pathology at the Brooke Army Medical Center in Fort Sam Houston, TX. Of these, only two isolates were shown to produce large amounts of histamine when tested *in vitro* in a MRS medium supplemented with histidine. Within 24 h, these isolates produced 0.00407 and 0.00373 mM of histamine from 0.1 % histidine, equating to 42 mg/ 100 g of histamine in the medium (Sumner, Speckhard et al. 1985, Sumner, Roche et al. 1990). Since

the U.S. FDA's hazard action levels for histamine are 50 mg/100 g (Bodmer, Imark et al. 1999), this could be a cause for concern. While levels of free histidine in fresh milk are typically low, milk protein can contain almost 9.6 g of histidine per 100 ml, and aged Swiss cheese may contain upwards of 370 mg of histidine per 100 g (via proteolysis) (Sumner, Roche et al. 1990). It is possible that factors such as high pH, low salt (0.5 %), and higher temperatures (18-21°C) may accelerate histamine formation in Swiss cheese (Sumner, Roche et al. 1990).

Histidine decarboxlase activity has been characterized among other species of LAB, including closely related *L. brevis* (Veiga-Da-Cunha and Foster 1992). Although histamine production is relatively rare for both *L. buchneri* and *L. brevis* (Sumner, Speckhard et al. 1985), histidine decarboxylase activity has been reported in other lactobacilli such as *Oenococcus oenos, Lactobacillus. casei, Lactboacillus bavaricus, Lactobacillus alimentarius,* and *Lactobacillus reuteri* (Bodmer, Imark et al. 1999).

### 1.6.2 LACTATE DEGRADATION IN FERMENTED CUCUMBER

Recently, *L. buchneri* was recognized as one of the primary spoilage organisms in industrial cucumber fermentations (Franco, Perez-Diaz et al. 2012, Johanningsmeier, Franco et al. 2012). Cucumbers are typically fermented in 30,000 to 40,000 L open-top, fiberglass tanks (Breidt, McFeeters et al. 2013). A sodium chloride brine is added to the tanks (1.03 M NaCl, equilibrated), with wooden boards to keep the cucumbers submerged below the surface (Breidt, Medina et al. 2013). Because these tanks are typically maintained outdoors, UV light

from the sun assists in preventing mold and yeast buildup on the brine surface (Harris 1997). Potassium sorbate (2.3 mM) or acetic acid (26 mM) is also added to inhibit yeast and molds, respectively (Breidt, McFeeters et al. 2013). Calcium chloride ($CaCl_2$) is added to the cover brine in minute amounts (9 to 36 mM, equilibrated) to prevent texture degradation during fermentation (Breidt, McFeeters et al. 2013). LAB are naturally present on the fruits in low numbers, $10^2 – 10^3$ CFU/g, and are selected for by the high concentrations of salt in the brine. Since these microorganisms are more acid-tolerant than their gram-negative spoilage counterparts, they dominate salty vegetable fermentations. LAB selected by the NaCl concentration ferment carbohydrate sources present in the cucumbers and lactic acid accumulates to lower the pH (3.3-3.5) and preserve the fruit. Completion of fermentation is marked by a terminal pH of 3.2-3.6 and 80-120 mM lactate with minimal residual carbohydrates. In colder climates, additional salt (1.71-2.56 M) is added to the storage tanks to prevent freeze damage during the harsh winters. Cucumbers are subsequently formulated into various pickle products, or sent to bulk storage for upwards of 1-2 years until processing. Prior to processing, cucumbers are desalted with water (Breidt, McFeeters et al. 2013). Fermented cucumber pickles may also be pasteurized at 70-80°C for 5-10 minutes to increase the shelf life of the finished product (Marquis, Bender et al. 1987).

The occurrence of secondary fermentation in fermented cucumbers result in economic losses for pickle processors (Franco, Perez-Diaz et al. 2012). Fermented cucumbers can enter into secondary spoilage at the start of the spring season, as ambient air temperature rises (Fleming, Daeschel et al. 1989). Yeast, molds, and LAB are all primary spoilage agents of

acidified and fermented vegetable products. Fermented cucumber spoilage is typically characterized by an increase in acetic, butyric and propionic acids at the expense of lactic acid (Fleming, Daeschel et al. 1989). Spoilage yeasts, such as *Pichia manshurica* and *Issatchenkia occidentalis* have been isolated concomitantly with lactate degradation in a fermented cucumber medium (Franco, Perez-Diaz et al. 2012, Franco and Pérez-Díaz 2013). Cell populations for these spoilage organisms may reach between $10^4$ – $10^6$ CFU/g in a fermentation system.

*L. buchneri* has been repeatedly isolated from spoiled, fermented cucumber (Franco, Perez-Diaz et al. 2012; Johanningsmeier et al. 2012, Johannginsmeier et al. 2013). Uniquely, *L. buchneri* does not produce propionic acid and propanol in pure culture, unlike other spoilage organisms frequently isolated from this environment (Franco and Perez-Diaz 2013). While not exclusively responsible for spoilage in this environment, *L. buchneri* may create conditions more suitable to other spoilage organisms that produce butyric and propionic acids (Franco and Perez-Diaz 2013**)**. One such organism is *Lactobacillus rapi*, which has been demonstrated to metabolize 1,2-propanediol into propionic acid and propanol. Thus, *L. buchneri* and *L. rapi* may act syntrophically to influence spoilage in fermented cucumbers (Johanningsmeier and McFeeters 2013).

Other organisms isolated from spoilage environments include lactic acid bacteria *Lactobacillus parafarraginis* and *Pediococcus ethanolidurans* (Franco, Perez-Diaz et al. 2012, Johanningsmeier and McFeeters 2013, Franco and Pérez-Díaz 2013). It is also possible for *Clostridium tertium* to metabolize organic acids in pH 5.0 environments (Fleming et al.

1989). However, *C. bifermentans* has been shown to produce butyric acid from lactic acid in conditions less than a pH of 5. (Franco, Perez-Diaz et al. 2012). Environmental factors including temperature, oxygen concentration, terminal pH and salt (NaCl) content significantly influence secondary cucumber fermentation (Fleming, Humphries et al. 2002, Kim and Breidt 2007, Johanningsmeier, Franco et al. 2012, Franco, Perez-Diaz et al. 2012, Johanningsmeier and McFeeters 2013). With the elevated pH, the environment becomes more suitable for additional spoilage organisms such as *Clostridium* species. These microorganisms produce enzymes such as proteases, lipases, amylases, and nucleases responsible for the degradation of the cucumber fruits (Franco, Perez-Diaz et al. 2012).

## 1.7 INDUSTRIAL APPLICATIONS OF *LACTOBACILLUS BUCHNERI*

### 1.7.1 SILAGE STARTER CULTURE ADJUNCT TO IMPROVE AEROBIC STABILITY

Notwithstanding the involvement of *L. buchneri* in food spoilage environments, some of the metabolic activities of this species can be exploited for industrial purposes. The lactate degradation pathway of *L. buchneri* found to spoil some foods is beneficial for silage fermentations. Cattle feed (fodder), is often preserved by fermentation into silage in large closed-top silos to increase stability and nutritional value of forage crops (Muck 1996, Driehuis, Oude Elferink et al. 2001). Traditionally, lactic acid bacteria carry out the fermentation process, metabolizing water-soluble carbohydrates and result in a sharp decrease in pH. While silage fermentations are traditionally an uncontrolled process,

advancements in starter culture technology have allowed for use of homolactic bacteria such as *Propionibacterium freudenreichii*, *Lactobacillus plantarum,* and *Pediococci pentosaceus* in order to improve fermentation yields (Kung and Ranjit 2001, Taylor, Ranjit et al. 2002). These homofermentative LAB generate lactic acid to reduce the pH, with much lower concentrations of acetate, butyrate, and ammonia. Heterolactic fermentation was originally thought to be disadvantageous because of the prospect of large dry matter (DM) losses (Driehuis, Oude Elferink et al. 2001). However, excretion of acetic acid from heterofermentative bacteria assists in preventing the growth of many species of yeasts and molds such as *Pichia, Hansenula, Endomycopsis,* and *Candida* that are responsible for lactate assimilation causing aerobic instability, and thus loss of dry matter after the silos have been exposed to air (Woolford 1990, Whitlock, Wistuba et al. 2000). These organisms degrade lactate under aerobic conditions into carbon dioxide and water, heating the silage and degrading nutrients, allowing for other non-ideal opportunistic microbes to flourish (Kleinschmit, Schmidt et al. 2005). The fodder becomes exposed to air as the silos are periodically emptied to feed livestock, resulting in loss of dry matter and a steep loss in product nutrients (Kung and Ranjit 2001, Kleinschmit, Schmidt et al. 2005).

Alternative methods to improve aerobic stability of the silage have been utilized, such as the addition of yeast-inhibiting preservatives. Chemical additives sodium benzoate and potassium sorbate dissociate into the cellular membranes of yeast and molds, acidifying the intracellular region and inhibiting growth (Kleinschmit, Schmidt et al. 2005). In 1996, scientist R.E. Muck isolated a strain of *L. buchneri,* TY16, from aerobically stable silage and

proposed its use as a silage adjunct (Muck 1996, Holzer, Mayrhuber et al. 2003). Hence, several subsequent studies have been conducted to study the effects of *L. buchneri* on the fermentation of silage (Driehuis, Oude Elferink et al. 2001, Elferink, Krooneman et al. 2001). Two strains of *L. buchneri,* 40788 and 11A44, became commercially available in the United States with different application rates, raising the opportunity for exploiting other strain-dependent metabolic processes of this organism. In 2001, the FDA approved *L. buchneri* for use as a silage inoculant. Its strict heterofermentative metabolism allows for the formation of both lactic and acetic acids (Kung and Ranjit 2001). *L. buchneri*'s ability to convert lactic acid to acetic acid and 1,2-propandiol improves aerobic stability by inhibiting aerobic yeasts with minimal loss in dry matter. Currently, *L. buchneri* has been studied as an inoculant for a variety of silages, including barley, corn, sorghum, grass and alfalfa (Driehuis, Elferink et al. 1999, Kung and Ranjit 2001, Taylor, Ranjit et al. 2002, Kung, Taylor et al. 2003).

### 1.7.2 BIOCONVERSION OF BIOMASS SUBSTRATES FOR ALTERNATIVE FUEL PRODUCTION

Current ethanol production levels are below the demand for renewable fuel resources. Lignocellulosic biomass materials have been explored as an alternative and highly accessible resource for biofuel conversion (Liu, Bischoff et al. 2009). Agricultural residues including wheat straw and corn stover containing cellulose and hemicellulose must be pretreated with dilute acid to release C5 and C6 sugars prior to inoculation with microbial biocatalysts for ethanol production (Liu, Skinner-Nemec et al. 2008, Liu, Bischoff et al. 2009). These harsh

pretreatment steps release acetate, furans, and phenols that inhibit most fermenting microbes. Some studies suggest that Gram-positive lactic acid bacteria are the most commonly found contaminants in commercial bioethanol facilities (Liu, Skinner-Nemec et al. 2008, Liu, Bischoff et al. 2009). One such LAB, *L. buchneri* B-30929 has been investigated as a potential biocatalyst for the conversion of biomass to renewable fuels and chemicals (Liu, Bischoff et al. 2009). *L. buchneri* NRRL B-30929 can simultaneously metabolize high concentrations of xylose and glucose to produce lactate and acetate, and tolerates up to 12 % ethanol (Liu, Skinner-Nemec et al. 2008, Liu, Bischoff et al. 2009, Liu, Leathers et al. 2011). Xylose is the main pentose carbohydrate available in the hemicellulose fraction of lignocellulosic biomass (Liu, Skinner-Nemec et al. 2008, Liu, Bischoff et al. 2009). In bioreactor studies with glucose and xylose, *L. buchneri* simultaneously metabolized both carbohydrates. This suggests that xylose metabolism by *L. buchneri* is not suppressed in the presence of glucose (Liu, Skinner-Nemec et al. 2008). A recent study indicates *L. buchneri* can produce ferulate esterases, which break down the cross-links between lignin and hemicellulose, which may suggest applications for lignocellulosic pretreatment and fermentation for alternative fuel production (Nsereko, Rutherford et al. 2010).

## 1.8 SUMMARY

*Lactobacillus buchneri* is a diverse species, occupying various ecological niches. More research is necessary to fully understand the metabolic capabilities and other lactic acid bacteria closely related to this species. Specifically, understanding the mechanism regulating

lactic acid degradation in environments such as fermented cucumber and silage fermentations may allow for enhanced control of these processes.

## 1.9 OVERVIEW OF RESEARCH

### 1.9.1 HYPOTHESIS

The primary objective of this study was to test the hypothesis that phenotypic and genotypic diversity is present among isolates of *Lactobacillus buchneri* from spoiled, fermented cucumber as compared to the type strain of the species. Prior observation of these isolates suggested that phenotypic diversity may exist within the species, especially with regard to colony morphology and lactic acid degradation. Strains of *L. buchneri* have been isolated from various environmental niches, and have distinct metabolic, genotypic and phenotypic differences (Sumner, Speckhard et al. 1985; Cook 1995; de Orduña, Patchett et al. 2001; Sakamoto and Konings 2003; Zeng, Pan et al. 2010; Zhang, Brandt et al. 2010; Franco, Perez-Diaz et al. 2012; Johanningsmeier, Franco et al. 2012).

### 1.9.2 OBJECTIVES

1. To assess phenotypic diversity among 35 isolates of *L. buchneri* from spoiled, fermented cucumber and the type strain of the species
2. To assess the genotypic and genomic diversity among these isolates

3. To determine whether there is a relationship between the phenotypic, genotypic, and genomic characteristics of *Lactobacillus buchneri*

### 1.9.3 SIGNIFICANCE OF RESEARCH

The characterization of *Lactobacillus buchneri* strains from spoiled, fermented cucumber will assist in developing a sense of the metabolic processes and precursors related to lactic acid catabolism. This research will provide insight into the carbohydrate metabolism, growth rate, lactate utilization, and colony and cellular morphology of *L. buchneri* strains. These data will determine whether the selected *L. buchneri* strains are indeed diverse and whether they are phenotypically and genotypically different from one another. Grasping an understanding for this intraspecies diversity will ultimately aid in the prevention of spoilage-associated secondary fermentation by *L. buchneri* by working to preclude the onset of lactic acid utilization. Establishing methods to monitor specific strains of *L. buchneri* responsible for lactate degradation may provide a more directed approach to prevent spoilage. Identifying robust strains of *L. buchneri* that might utilize lactic acid at a faster rate would allow processors to determine strain-specific methods of inhibition, such as adjusting the variety and concentration of preservatives administered after the primary fermentation. Preventing and controlling the rate of spoilage will serve as an economic advantage for fermented cucumber processors.

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# 2 CHAPTER 2: PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *LACTOBACILLUS BUCHNERI* STRAINS ISOLATED FROM SPOILED, FERMENTED CUCUMBER

## 2.1 ABSTRACT

*Lactobacillus buchneri* is a gram-positive, obligate heterofermentative, facultative-anaerobe commonly affiliated with spoilage of food products. Notably, *L. buchneri* is able to metabolize lactic acid into acetic acid and 1,2-propanediol. Although beneficial to the silage industry, this metabolic capability is detrimental to preservation of cucumbers by fermentation. Lactic acid degradation in fermented cucumbers is associated with an increase in pH, providing an environment suitable for growth of other spoilage organisms. The objective of this study was to characterize isolates of *L. buchneri* purified from both industrial and experimental fermented cucumber. Genotypic and phenotypic characterization included 16S rRNA sequencing, DiversiLab® rep-PCR, colony morphology, API® 50 CH carbohydrate analysis, and ability to degrade lactic acid in modified MRS and fermented cucumber media. Distinct groups of isolates were consistently identified with differing colony morphologies that varied in color (translucent white to opaque yellow), diameter (1 mm – 11 mm), and shape (umbonate, flat, circular or irregular). Growth rates in MRS revealed strain differences and a wide spectrum of carbon utilization was observed. Some strains were able to ferment as many as 16 of the 49 carbon sources including lactose, mannitol, potassium ketogluconate, and xylose, while others only metabolized 9 carbohydrates as the sole source of carbon. All isolates degraded lactic acid in both fermented cucumber media and modified MRS, resulting in a rise in pH. Isolates clustered into 8 distinct groups based on rep-PCR fingerprints with 20/36 of the isolates exhibiting more than 97 % similarity. Although isolated from similar environmental niches, significant

phenotypic and genotypic diversity was found among the *L. buchneri* cultures. This work sets the stage for future analysis of metabolic capabilities of this species to allow control of lactic acid degradation and thus avert the onset of spoilage in a fermented cucumber matrix.

## 2.2 INTRODUCTION

*Lactobacillus buchneri* is a gram-positive, facultative anaerobe that has been isolated from a wide range of food, feed, and bioprocessing environments due to its diverse range of metabolic activities. *L. buchneri* has potential for extensive applications in food and feed technology. Most notably, *L. buchneri*'s ability to metabolize lactic acid into acetic acid and 1,2-propanediol makes this organism both detrimental to the cucumber pickling industry and suitable as an adjunct starter culture for the silage industry. Considering the various ecological niches *L. buchneri* strains have occupied, diversity among the species is likely. In addition to fermented cucumber (Franco, Perez-Diaz et al. 2012, Johanningsmeier, Franco et al. 2012) and silage (Cooke 1995, Muck 1996), *L. buchneri* has also been isolated from human intestines (Zeng, Pan et al. 2010), sourdough bread (Zhang, Brandt et al. 2010) , wine (de Orduña, Patchett et al. 2001) beer wort (Sakamoto and Konings 2003), tomato pulp and spoiled Swiss cheese (Sumner, Speckhard et al. 1985).

Pickling cucumbers are fermented in a sodium chloride solution in open-top 40,000 L vats where naturally occurring lactic acid bacteria, principally homolactic *Lactobacillus plantarum*, converts sugars to organic acid to preserve the fruits (Breidt, McFeeters et al. 2013). Prior to processing, some tanks may be held in bulk storage for upwards of 1-2 years. Occasionally, secondary fermentation may ensue, particularly when the salt concentration is too low (Fleming, Daeschel et al. 1989, Johanningsmeier, Franco et al. 2012, Breidt, McFeeters et al. 2013). Spoilage-associated secondary cucumber fermentation occurs during bulk storage after residual carbohydrates have been depleted (Fleming, Daeschel et al. 1989,

Franco, Perez-Diaz et al. 2012, Breidt, McFeeters et al. 2013). This particular spoilage is characterized by an increase in pH and decrease in lactic acid with a concomitant increase in acetic and propionic acids (Fleming, Daeschel et al. 1989, Johanningsmeier, Franco et al. 2012, Franco, Perez-Diaz et al. 2012). In numerous *in vitro* studies, *Lactobacillus buchneri* has been shown to initiate spoilage by degrading lactate to acetate and 1,2- propanediol without the use of an external electron acceptor (Johanningsmeier, Franco et al. 2012, Johanningsmeier and McFeeters 2013). As lactate is depleted, spoilage occurs in a succession of microbial growth. As the pH rises, gram-negative species including *Enterobacter cloacae* and *Clostridium bifermentans* can degrade lactate to butyric acid, creating an undesirable off-odor. Ultimately, this renders the final product unfit for consumption and leads to economic losses (Franco, Perez-Diaz et al. 2012, Breidt, McFeeters et al. 2013).

Potential industrial applications of *L. buchneri* are diverse. One particular strain isolated from pickle juice was shown to have potential probiotic effects, such as the reduction of serum cholesterol levels (Zeng, Pan et al. 2010). This strain of *L. buchneri* produces a bile salt hydrolase (BSH) to catalyze the hydrolysis of conjugated bile salts into amino acid residues and bile acids (Driehuis, Oude Elferink et al. 2001). Another strain of *L. buchneri* was isolated from sauerkraut, and has been exploited for bacteriocin production, targeting several gram-positive bacteria including species from *Listeria, Bacillus, Micrococcus, Enterococcus, Leuconostoc, Lactobacillus, Streptococcus,* and *Pediococcus* (Yildirim and Yildirim 2001, Yıldırım, Avs et al. 2002). *Lactobacillus buchneri* strain NRRL B-30929, found as a contaminant in a commercial ethanol facility, has since been exploited for

bioethanol production, and this strain of *L. buchneri* was the first genome of this species to be fully sequenced and annotated (Krooneman, Faber et al. 2002, Taylor, Ranjit et al. 2002). Maintaining an acidic environment and allowing for anaerobic lactic acid utilization by *L. buchneri* is beneficial for the inhibition of aerobic spoilage yeasts and fungi responsible for heating and degrading dry matter in the silage silo after being exposed to the outside air (Muck 1996, Ranjit and Kung 2000, Driehuis, Oude Elferink et al. 2001, Kung and Ranjit 2001, Krooneman, Faber et al. 2002, Taylor, Ranjit et al. 2002, Kung, Taylor et al. 2003, Kleinschmit, Schmidt et al. 2005). Strains of *L. buchneri* have been sourced from diverse environments and there is much to be investigated for potential applications of this species.

To date, diversity within the *L. buchneri* species has not been explored in great detail. Understanding the genotypic and phenotypic diversity among *L. buchneri* will facilitate the development of strategies to eliminate the onset of secondary cucumber fermentation by this species. Furthermore, this knowledge may also be exploited to develop improved starter culture adjuncts for the silage industry. The objective of this study was to characterize *L. buchneri* cultures isolated from spoiled, fermented cucumber to determine the phenotypic and genotypic diversity of this species from a single environmental niche.

## 2.3 MATERIALS AND METHODS

### 2.3.1 BACTERIAL STRAIN CULTIVATION

Strains of *L. buchneri* isolated from spoiled, fermented cucumber (Johanningsmeier, Franco et al. 2012, Franco and Pérez-Díaz 2012) were obtained from the USDA-ARS Food

Science Research Unit Culture Collection (Raleigh, NC). The sources of the isolates are described in Table 2.1. Species identification was verified using 16S rRNA sequencing. Frozen stocks were prepared in de Man-Rogosa-Sharpe (MRS) broth and 30 % glycerol, and stored at -80°C. Prior to experiments, isolates were streaked onto MRS agar and incubated at 30°C under anoxic conditions for 4-5 days followed by propagation in MRS liquid broth anaerobically for 2-3 days at 30°C for all experiments unless otherwise noted.

### 2.3.2 16S rDNA SEQUENCING

Genomic DNA was isolated from broth cultures of each of the 36 cultures using a DNA purification kit (Ultra-Clean Microbial DNA Isolation Kit, MoBio laboratories, Carlsbad, CA). Polymerase Chain Reaction (PCR) was implemented to amplify a 500 bp region with 16S rRNA primers. Primers were adapted from Kullen et al. and include the highly variable V1 and relatively divergent V2 regions (Kullen, Sanozky-Dawes et al. 2000). The primers used in this study were plb16 5' AGAGTTTGATCCTGGCTCAG 3' and mlb16 5' GGCTGCTGGCACGTAGTTAG 3'. Amplicons were sequenced using Eton Bioscience Inc. (Durham, NC, USA). The NCBI Basic Local Alignment Search Tool (BLAST) was used to identify the bacterial cultures. Alignment matches with > 99 % identity were utilized in this study.

### 2.3.3 COLONY AND CELLULAR MORPHOLOGY

Each of the isolates were streaked onto MRS agar, and incubated anaerobically for 4 days at 30°C. Colony morphology was evaluated based on diameter, pigmentation, form, elevation, margin, texture and opacity of each colony for all isolates of *L. buchneri* using the method found in the Manual of Microbiological Methods (Kleinschmit, Schmidt et al. 2005). Isolates were further propagated in MRS broth, anaerobically for 36 hours at 30°C prior to cellular morphology analysis. Cellular morphology was visualized using a Nikon Eclipse E600 phase contrast microscope with a Q-Imaging Micropublisher Camera attachment (Nikon, Japan).

### 2.3.4 GROWTH CURVES IN DE MAN ROGOSA AND SHARPE (MRS) BROTH

Growth curves were generated by measuring optical density over time in 96-well plates containing standard MRS media using a microtiter plate reader (Spiral Biotech QCount; Advanced Instruments, Inc.; MA, USA) for 64 hours. A 5 µL aliquot of a $10^8$ CFU/mL culture was inoculated into a single well of 245 µL of sterile MRS broth for each of the 36 isolates and incubated at 30°C under modified anaerobic conditions using mineral oil. This experiment was conducted in duplicate. Lag times, growth rates, and max OD were calculated using an algorithm published by Breidt and others (Breidt, Romick et al. 1994).

### 2.3.5 CARBOHYDRATE FERMENTATION PROFILES

The ability to ferment a variety of carbon sources was determined by the API 50 CHL method per the supplier's guidelines (bioMerieux, Marcy I'Etoile, France). Each of the 36 cultures of *L. buchneri* were cultivated in standard MRS broth under anaerobic conditions at 30°C for 4 days prior to inoculation. Inoculum was standardized using optical density ($OD_{600}$). Strains were grown up to $10^8$ CFU/mL in MRS broth anaerobically at 30°C and $OD_{600}$ was measured.

### 2.3.6 LACTATE UTILIZATION IN FERMENTED CUCUMBER MEDIA

Pickling cucumbers (size 2B) were fermented at 25°C in brines containing $CaCl_2$ (100 mM equilibrated). Fermentation was conducted in a 5 gal (~18.9 L) sealed container, with a pack-out ratio (weight cucumbers: volume of cover brine solution) of 60:40. A mixed starter culture of *Lactobacillus plantarum* (Culture Collection ID LA0445, LA0085 and LA0219 USDA-ARS Food Science Research Unit, Raleigh N.C., U.S.A.) was inoculated at $10^6$ CFU/ml in the fermentation container and allowed to ferment at ambient temperature for 5 days, until a brine pH of 3.4 had been reached. Fermented cucumbers and brine were blended into slurry and stored at -4°C until use. Frozen fermented cucumber slurry (FCS) was thawed and pressed through multi-layered-cheesecloth to remove large particular matter and centrifuged for 15 minutes at 17,568 x g at 4°C (Sorvall; RC-5B centrifuge, Dupont Instruments). Supernatant was filter-sterilized using a 500 ml bottle-top Nalgene apparatus (Nalgene FAST PES, 0.2 um pore size, Daigger, Vernon Hills, Ill., USA). FCS was

aseptically aliquoted into 15 ml sterile conical tubes and transferred to a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, MI., U.S.A.) to allow for removal of oxygen for 5 days prior to inoculation with *Lactobacillus buchneri* isolates at $10^6$ CFU/ml. Inoculated medium was incubated anaerobically at 25°C. pH measurements were recorded as an indication of lactate utilization. This experiment was conducted in duplicate. pH was measured using an Accumet AR25 pH meter equipped with a gel-filled pH electrode (Fisher Scientific, Pittsburgh, PA., USA). The pH electrode was calibrated prior to each use using certified standards of pH 2.0, 4.0, and 7.0 (Fisher Scientific).

### 2.3.7 LACTATE UTILIZATION IN A MODIFIED MRS MEDIUM (MMRS)

A modified MRS (mMRS) medium adapted from Oude Elferink was supplemented with 50 mM lactic acid and pH adjusted to 3.8 with no added glucose (Elferink, Krooneman et al. 2001). After aliquoting into 15 mL sterile conical tubes, mMRS media was allowed to equilibrate in the Coy anaerobic chamber for 5 days to allow for oxygen removal. Each *L. buchneri* isolate was grown individually in standard MRS broth under anaerobic conditions at 25°C for 4 days prior to inoculation at $10^6$ CFU/ml into the mMRS medium. Inoculated media was incubated at 25°C anaerobically; samples were aseptically collected over a time course of 107 days, and pH was measured for each of the isolates as an indication of lactic acid utilization. HPLC analysis of select samples was used to quantify lactic acid in modified MRS (mMRS) nutrient media. Each of the samples were separated on an Aminex Fast-Acid

Analysis column (100 x 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) with 0.03 N $H_2SO_4$ eluent at a flow rate of 0.6 mL/min. Lactic acid was quantified using a refractive index detector.

### 2.3.8 DIVERSILAB® STRAIN TYPING

The DiversiLab® System (bioMerieux, Marcy I'Etoile, France) was used according to the manufacturer's instructions. Genomic DNA from each of the isolates was extracted using an Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA) and used with the DiversiLab® *Lactobacillus* Primer Kit for DNA fingerprinting (bioMerieux) according to the manufacturer's instructions. Data was analyzed using automated DiversiLab web-based statistical software (version 3.4) using the Pearson Correlation to generate a proximity matrix to calculate pair-wise similarities between the isolates (Shutt, Pounder et al. 2005, Tenover, Gay et al. 2009).

## 2.4 RESULTS

### 2.4.1 16S rDNA SEQUENCING

All isolates were subjected to 16S rDNA gene sequencing for species verification prior to characterization studies. Selective primers amplified a ~500bp region of the 16S gene containing the variable v1 and v2 regions. The v1 region is ~50 bp, and lies just downstream of the 5' end of the 16S rRNA gene. The ~50 bp v2 region is followed by a ~80 bp segment

of highly conserved sequence (Kullen, Sanozky-Dawes et al. 2000). All isolates were confirmed to be *L. buchneri* (>99 %).

### 2.4.2 COLONY MORPHOLOGY

Each *L. buchneri* isolate was streaked on MRS agar for morphological examination. Comparatively, the isolates revealed phenotypic diversity. Figure 2.1 displays images of selected *L. buchneri* isolates with 8 distinct morphology types including variances in shape, pigmentation, size, and texture. While sizes ranged from 1-11 mm, Figure 2.1 displays only isolates sized 2.5 – 6 mm. The type strain, LA0030 (ATCC 4005) was isolated from tomato pulp and contrasts entirely with the other 7 isolates. LA0030 has a slight depression in the center of the colony, and it was the only strain with a complete circular form and convex margin. Pigmentation varied among the isolates, ranging from white and opaque to yellow and translucent. One strain, LA1147, arguably has the most distinct morphology, with sharp, irregular edges and a small elevation directly in the center of the colony. Two of the strains, LA0030 and LA1161B had a mucoid appearance and viscous texture, suggesting the potential production of exopolysaccharides. Alternatively, two isolates LA1181 and LA1167 had a dry, powdery texture, while the remaining four presented a butyrous-like texture.

### 2.4.3 CELLULAR MORPHOLOGY

Cellular morphology visualized after 36 hours of anaerobic incubation revealed two distinct morpho-types (Figure 2.3) illustrated by LA1167 and LA1147, which display an

auto-aggregation phenotype, compared to strains LA0030 and LA1181, which are non-aggregative. Auto aggregation phenotypes may be influenced by proteinaceous components on the cell surface (Kos, Suskovic et al. 2003). This possibly reflects the presence of putative surface-layer (S-layer) proteins (Hynönen and Palva 2013, Johnson, Selle et al. 2013), which have been previously identified in *Lactobacillus buchneri* (Heinl, Wibberg et al. 2012).

### 2.4.4 GROWTH CURVES IN DE MAN, ROGOSA AND SHARPE (MRS) MEDIUM

Optical density at 600 nm was measured during growth of each of the 36 isolates. Eight of the isolates are displayed in Figure 4, divided into two separate growth patterns. Figure 2.4A displays the slower growing strains, with a longer exponential phase. Comparably, strains in Figure 2.4B entered into stationary phase just after 24 hours of growth, while the three strains LA1167, LA1161B, and LA1175D entered into stationary phase just after 36 hours. In particular, LA1184 grew to a final OD600 nm of 1.91, the highest out of any of the eight strains pictured.

### 2.4.5 CARBOHYDRATE UTILIZATION

Each of the *L. buchneri* isolates were screened using the API 50 CHL fermentation kit. Substrates including carbohydrates, polyalcohols, uronic acids, and heterosides were divided into cupules on separate strips and elicited a color-change when partially or fully fermented by the inoculated organism. This method has been exploited for carbohydrate profiling of various species of lactobacilli to elucidate physiological capabilities. The

facultative heterofermentative nature of *L. buchneri* allowed for 100 % of the isolates to ferment glucose and maltose, while 72 % of the isolates were able to metabolize arabinose, ribose, xylose and fructose (Figure 2.3). In a previous study, approximately 85 % and 64 % of *L. buchneri* strains fermented potassium gluconate and potassium-5-ketogluconate, respectively (Liu, Leathers et al. 2011). However, after 3 days of anaerobic incubation at 30°C, 81 % of the *L. buchneri* strains in this study only partially fermented potassium gluconate. Complete fermentation of this substrate was observed only in strain LA1161B. In the study conducted by Liu and others, 84 % of the isolates partially fermented potassium-5-ketogluconate. Comparably, complete fermentation was observed in two strains, LA1161B and LA1161C within the present study.

Figure 3 displays hierarchal clustering of the API 50CHL results for 36 *L. buchneri* cultures and 28 out of the 49 carbohydrates. A total of 27 carbohydrates were successfully fermented (either partial or complete fermentation), while the remaining 22 carbohydrates were not metabolized by the isolates. Fully fermented carbohydrates are shown in red, while blue is displayed in the absence of a reaction. Intermediate (partial metabolism of a carbon source) is indicated with gray. The API 50 CHL data was divided into 8 main groupings of isolates. The top cluster, which included ATCC type strain 4005 (LA0030) and LA1175D, did not metabolize a majority of the carbohydrates as compared to the rest of the 36 cultures. The second cluster contained LA1158A, which failed to metabolize 18 out of the 28 carbohydrates, and only fully metabolized six carbohydrates. Conversely, isolate LA1161B found within the 6$^{th}$ and largest cluster encompassing 18 isolates, assimilated a total of 16

carbohydrates, the largest amount of any isolate screened. Also in the 6th cluster was isolate LA1167, which was the only isolate able to metabolize cellobiose during the 3 day incubation period.

LA1159A and LA1156B made up the third cluster, and were the only isolates able to metabolize trehalose. The sole isolate in the 8th cluster, LA1185B, was perhaps the most unique from this assay. LA1185B was the only isolate to degrade gentiobiose and amidon, and along with LA1136, metabolized fucose. Cellobiose and trehalose, two carbohydrates shown to be depleted prior to the onset of fermented cucumber spoilage (Johanningsmeier and McFeeters 2015) were present in the API 50 CHL strips. However, only one isolate, LA1167, metabolized enough cellobiose during the short term incubation to acidify the medium and produce a color change. In a recent study, *L. buchneri* was observed to metabolize L-arabinose, potassium-gluconate, D-melezitose, and D- melibiose (reaching an OD of 0.7) within 2.4 days. Other carbohydrates such as D-lactose, methyl-D-glucopyranoside, methyl-D-mannopyranoside, raffinose, D-sorbitol, D-turanose, and D-xylose were metabolized after 7 days. Particularly, D-trehalose was metabolized after 12 days (Sanders, Oomes et al. 2014).

### 2.4.6 LACTIC ACID UTILIZATION

In this study, we investigated the variation in lactic acid utilization among each of the *L. buchneri* isolates screened. Conversion of lactic acid by *L. buchneri* strains was indicated by a rise in media pH. Lactic acid utilization was measured in two different types of media, a

modified MRS (mMRS) adjusted to pH 3.8 and a fermented cucumber slurry medium (FCS) with a pH of 3.3. Each medium was incubated under anaerobic conditions at ambient temperature (25°C). Within 3 days post-inoculation, all strains had initiated lactate metabolism in the mMRS media. Conversely, lactate metabolism in the FCS media did not begin until 69 days after inoculation (Appendix A.3). This delay in lactic acid degradation is congruent with similar studies conducted in fermented cucumber media (Franco, Perez-Diaz et al. 2012, Johanningsmeier and McFeeters 2013). Figure 2.6 displays lactate degradation in mMRS medium containing 50 mM lactic acid in a time course from 3, 21, 42, 62, 76, and 107 days. The three strains displayed in Figure 2.6A, LA0030, LA1147, and LA1184 each have a slower rate of lactate degradation as compared to the five strains listed in Figure 2.6B. Each of the five strains LA1161B, LA1161C, LA1167, LA1175D, and LA1181 in Figure 2.6B degraded over 8 mM more lactate than the three isolates in Figure 2.6A over the course of 107 days. In particular, LA1181 utilized the most lactic acid out of all of the strains, degrading over 45 mM lactate.

### 2.4.7 DIVERSILAB® STRAIN TYPING

DiversiLab® is a commercially available repetitive-PCR based method to differentiate bacterial isolates on the subspecies and strain level through noncoding, recurring elements interspersed throughout the genome (Shutt, Pounder et al. 2005). Outward-facing genus-specific primers amplify the sequence between these DNA fragments, as opposed to inward-facing primers that amplify the repetitive element itself, generating amplicons of

varying sizes to create a virtual gel image to demonstrate similarities between each isolate (Healy, Huong et al. 2005). Statistical, web-based software analyzes the data using the Pearson Correlation and generates a dendrogram to elucidate hierarchal clustering.

DiversiLab® results display the separation of 8 diverse groups of isolates based on their respective rep-PCR fingerprint patterns. The first cluster consisted of twenty isolates with > 97 % similarity (Figure 2.5A). Isolates within this cluster have similar colony morphology, with a flat surface, irregular edges, powdery texture and yellow opacity (see Figure 2.1G). However, the remaining 17 isolates tested were divided into eight clusters with > 80 % similarity (Figure 2.5B). Each of these clusters is more diverse in carbohydrate metabolism and colony morphology. Particularly, isolates LA1161A and LA1161C are the outliers of the clusters (Figure 2.6B), with only 80 % similarity to the other groups. Interestingly, the type strain LA0030, LA1167, and LA1161B are all within the same genotypic cluster, yet have very distinct colony morphologies (Figure 2.1) and vary in lactate degradation (Figure 2.6).

## 2.5 DISCUSSION

Characterization of secondary cucumber fermentation reveals a diverse array of microorganisms associated with different stages throughout the spoilage process. While it is difficult to monitor the occurrence of industrial secondary fermentation because of its irregular manifestation, reproducing spoilage under contained laboratory conditions to isolate the responsible organisms also presents limitations as various uncharacterized species may be

contributing to spoilage. In previous studies, 26 cultures of *L. buchneri* were isolated from spoiled and laboratory-scale reproduced fermented cucumber spoilage, where anaerobic lactic acid degradation was observed (Johanningsmeier, Franco et al. 2012). The present study further purified these cultures into 36 pure isolates to evaluate the diversity of the species from this industrially relevant environmental niche.

While there are many microorganisms capable of metabolizing lactic acid, recent studies have shown that *L. buchneri* is the organism primarily responsible for the initiation of anaerobic lactate utilization in fermented cucumber media under varying conditions (Johanningsmeier, Franco et al. 2012, Johanningsmeier and McFeeters 2013). Factors such as sodium chloride (NaCl) concentration and pH influence lactic acid metabolism for *L. buchneri*. In one study, *L. buchneri* was able to degrade lactic acid at NaCl ranges from (0-6 %) from an initial pH of 3.8 (Johanningsmeier, Franco et al. 2012). The most substantial lactic acid degradation was observed in reduced NaCl (2 %) pilot scale fermentation experiments, which may suggest the involvement of sodium dependent transporters (Johanningsmeier, Franco et al. 2012). Increased lactic acid metabolism by *Lactobacillus buchneri* at reduced NaCl concentrations proves to be an emerging problem for pickle processors (Franco, Perez-Diaz et al. 2012, Johanningsmeier, Franco et al. 2012, Johanningsmeier and McFeeters 2013). Efforts by the pickle industry to reduce the amount of sodium chloride in the waste water have led to the development of a fermentation process in brines containing only calcium chloride ($CaCl_2$). Unlike NaCl, $CaCl_2$ is non-toxic to plants,

and therefore would not pose an issue for treated waste disposal. However, complications such as an increased risk of spoilage have proven to be a problem for the industry.

Other organisms in the LAB family have shown similar metabolic capabilities under both aerobic and anaerobic conditions, with various end-products and electron acceptors in several matrices. A strain of *Lactobacillus plantarum* isolated from fermenting green olive brine was shown to degrade L-lactate and citrate simultaneously accompanied by an increase in acetate, formate, succinate and carbon dioxide after prolonged incubation in the absence of fermentable sugars (Bobillo and Marshall 1992). This would suggest the involvement of an oxygen-dependent pathway, converting lactate to acetate via pyruvate and a NADH-independent lactate dehydrogenase (Murphy, O'Connor et al. 1985, Lindgren, Axelsson et al. 1990, Bobillo and Marshall 1991). Under anaerobic conditions, a spoiled wine isolate, *Lactobacillus brevis* used NAD-dependent lactate dehydrogenases to catalyze the conversation of lactate to pyruvate, in the presence of glycerol.

Elferink and others proposed a novel pathway for lactate assimilation by *L. buchneri*, suggesting the presence of NAD-linked 1,2-propandiol-dependent oxidoreductase activity in *L. buchneri* (Elferink, Krooneman et al. 2001). For every two molecules of lactic acid, one molecule of lactic acid is reduced via lactaldehyde to 1,2-propanediol. *L. buchneri* appears to use this pathway for lactate degradation in fermented cucumber in the absence of fermentable sugars, but the regulatory mechanism for this metabolism in a fermented cucumber matrix is not yet fully understood. Typically, lactic acid degradation by *L. buchneri* is induced by acidic conditions and may potentially be a preservation mechanism for the cell. No increase

in cell counts were observed during lactate utilization experiments in fermented cucumber juice or in nutrient growth media supplemented with lactic acid (Elferink, Krooneman et al. 2001, Franco, Perez-Diaz et al. 2012, Johanningsmeier, Franco et al. 2012).

In the present study, the ability to degrade lactic acid was observed for each of the 36 isolates (Appendix A.3) examined. Differences in lactate utilization varied between strains (Figure 2.6). No correlations were observed between growth rate and rate of lactate degradation over time among the eight isolates. Lactate utilization is not initiated until residual glucose has been depleted from the environment (Elferink, Krooneman et al. 2001). Thus, onset of lactate utilization may not occur until >50 days after the end of a primary fermentation (Johanningsmeier 2013).

Specifically, depletion of cellobiose, trehalose, and citrulline may signify imminent lactate degradation by *L. buchneri* strains at the initiation of fermented cucumber spoilage (Johanningsmeier et al 2015). However, LA1167 was the only isolate to metabolize cellobiose as a sole carbon source in carbohydrate profiling assays. In a metabolomics study, one strain of *Lactobacillus buchneri* (LA1147) degraded cellobiose prior to the onset of secondary fermentation (Johanningsmeier and McFeeters 2015). While each strain was isolated from a similar environmental niche (Table 1), the phenotypic differences make each very unique. CD034, a strain of *L. buchneri* isolated from silage fermentation most closely identifies with the colony phenotype of the type strain of the organism, LA0030 (ATCC 4005). However, these are morphologically different than the other isolates. Aggregation abilities of the selected strains of *Lactobacillus buchneri* were analyzed using visual

observation under a microscope. While five out of eight unique strains displayed the auto-aggregative phenotype, the remaining three did not. This may suggest the presence of extracellular proteins, as *Lactobacillus buchneri* is a known producer of S-layers (Heinl, Wibberg et al. 2012, Hynönen and Palva 2013, Johnson, Selle et al. 2013).

*Lactobacillus buchneri* is classified under the Group III obligately heterofermentative lactobacilli, capable of growth by metabolizing hexoses and pentoses to lactic acid, acetic acid and traces of ethanol and carbon dioxide. In this study, the API 50CHL method was utilized to examine specific carbohydrate profiles for each of the 36 isolates. Results of the assay were compared to the documented API sugar profiles for *L. buchneri*. Along with the more readily metabolized carbohydrates, arabinose, ribose, xylose, glucose, and maltose, particular strains of *L. buchneri* were able to metabolize carbon sources such as mannitol, lactose, esculin ferric citrate, and potassium-5-ketogluconate. According to previous studies, over 90 % of *L. buchneri* strains have been shown to readily metabolize arabinose, while a broader range of 11-89 % of strains will ferment galactose, ribose, and sucrose (Hammes and Vogel 1995). Within the same study, fructose, glucose, maltose, melezitose, and D-melibiose were also fully utilized (Wood and Holzapfel 1995). It is hypothesized that *L. buchneri* strains produce several hydrolytic enzymes to break down disaccharides and polysaccharides. Trehalose and cellobiose, both disaccharides, are present in fermented cucumbers (Johanningsmeier and McFeeters 2015) and *L. buchneri* may use β- amylase and β-glucosidase to fully metabolize the carbohydrates (Liu, Skinner-Nemec et al. 2008, Liu, Bischoff et al. 2009). β- glucosidase is also involved with melibiose and salicin metabolism

(Liu, Skinner-Nemec et al. 2008). Furthermore, 25 % of *L. buchneri* isolates utilized lactose, suggesting β- galactosidase activity. Another enzyme, β- fructofuranosidase is responsible for melezitose metabolism (84 % of isolates) and β- glucosidase aids in fermentation of trehalose (0 %) and maltose (100 %) (Liu, Skinner-Nemec et al. 2008, Liu, Bischoff et al. 2009).

*L. buchneri* strain LA1175D is arguably one of the more diverse strains out of the 36 total isolates. Isolated from reduced salt fermented cucumber spoilage, LA1175D was one of four out of the 36 isolates examined that cannot catabolize galactose and one of eight unable to degrade saccharose, as shown in group 1 of Figure 2.3. Furthermore, LA1175D degraded lactate more rapidly between days 21 and 42 compared to six of the other strains examined (Figure 2.6). In appearance, this isolate has slightly translucent edges, with a darker, yellow opaque center. While displaying an auto-aggregative phenotype (results not shown), it has a slower growth rate, similar to LA1167 and LA1161B. Conversely, isolates LA1167 and LA1161B are consistently phenotypically and genotypically similar, with the exception being colony and cellular morphology. LA1161B remains white with a slight raised elevation in the center of the colony, while LA1167 is darker in color, undulate with a granular surface and a slight depression in the center. The auto-aggregation phenotype is present in LA1167, but absent in LA1161B.

In a previous study, 26 cultures of *L. buchneri* isolates from spoiled, fermented cucumber along with two annotated genomes of *L. buchneri* (strains CD034 and NRRL B-2099) were investigated for the occurrence of CRISPR-Cas systems (Briner and Barrangou

2014). CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a form of adaptive immunity that is present in most archaea and in many species of bacteria. Comparative genomic analysis of the strains revealed the universal occurrence of a type II-A system in each of the *L. buchneri* genomes and fermented cucumber spoilage isolates. The type II-A locus was defined by a highly conserved 36-nt sequence, along with the *cas1* and *cas2* genes and the type II specialized *cas9* nuclease (Briner and Barrangou 2014). Comparative genomic analysis of CRISPR loci from each of the 26 isolates revealed 10 unique CRISPR genotypes containing 9-29 spacers. In active CRISPR loci, spacers are acquired in a polarized manner at the leader end. Shared spacers at the ancestral end of the locus provide a snapshot into the phylogenetic origins of a strain (Briner and Barrangou 2014). CRISPR-Cas systems were determined for eight strains of *Lactobacillus buchneri*. It was noted that three strains with the same CRISPR genotype (LA1167, LA1161B, and LA1161C) exhibited various colony morphotypes. This is consistent with DiversiLab® results, in which these strains had differing genotype patterns. A possible explanation for this dichotomy is that DiversiLab® sequencing uses genus-specific primers to amplify regions throughout the genome while CRISPR sequencing is localized.

In this study, 36 cultures of *L. buchneri* originally isolated from spoiled, fermented cucumber were characterized using phenotypic and genotypic analyses to explore the diversity within the species. Variations in colony morphology revealed stark differences in color, size, and shape for the strains. These data present unique information for characterization of strain diversity in *Lactobacillus buchneri*. Exploiting these data may

allow for insight into the metabolic capabilities of the organism as a gateway for further *in vitro* testing of carbohydrate metabolism. This may prove to be relevant for various industries such as silage and alternative fuel production interested in harnessing the diverse metabolic potential of *L. buchneri*. A difference in lactate degradation was noted among all of the isolates, suggesting some strains may be more robust spoilage organisms in fermented cucumber. This information may yield a more effective ability to potentially control and prevent spoilage, as one method may not be effective to inhibit lactate utilization capabilities of each strain. Furthermore, this level of intra-species diversity has not been explored in *L. buchneri*. Eight of the 36 isolates were selected for genome sequencing to further understand the metabolic potential of this organism. Insights into both the phenotypic and genomic data are necessary to fully harness the potential of *Lactobacillus buchneri*, such as in bioethanol or silage applications, or for development of techniques to prevent spoilage in fermented cucumbers.

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**Keywords**: lactic acid bacteria, pickled vegetables, spoilage organisms, spoilage metabolites, *Lactobacillus buchneri* strain diversity

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**Table 2.1: Source of *Lactobacillus buchneri* isolates characterized in this study**

| Isolate ID | Source[1] | Isolation conditions[2] | GenBank Accession # | # gaps/total |
|---|---|---|---|---|
| LA0030 | Type Strain ATCC #4005 | N/A | KF624610 | 1/696 |
| LA1138 | Reproduced from commercial spoilage; anaerobic conditions in 6 % NaCl | N/A | N/A | 0/690 |
| LA1149 | Commercial $CaCl_2$ Fermentation Tank | N/A 100 mM CaCl2 | JQ086334 | 0/643 |
| LA1173 | Reduced NaCl Spoilage | 2 % NaCl | JQ249034 | 1/647 |
| LA1175A | Reduced NaCl Spoilage | 2 % NaCl | N/A | 0/700 |
| LA1175D | Reduced NaCl Spoilage | 2 % NaCl | N/A | 0/697 |
| LA1178 | Reduced NaCl Spoilage | 2 % NaCl | JQ249040 | 1/720 |
| LA1181 | Reduced NaCl Spoilage | 2 % NaCl | JQ249043 | 0/663 |
| LA1184 | Reduced NaCl Spoilage | 2 % NaCl | JQ24904 | 1/658 |
| LA1147 | Reduced NaCl Spoilage | 2 % NaCl | JQ249035 | 0/662 |
| LA1161B | Reproduced from commercial spoilage; anaerobic conditions in FCS | 4 % NaCl | N/A | 1/693 |
| LA1161C | Reproduced from commercial spoilage; anaerobic conditions in FCS | 4 % NaCl | N/A | 0/690 |
| LA1163 | Reproduced from commercial spoilage; anaerobic conditions in FCS | 4 % NaCl | JQ249062 | 0/754 |
| LA1164 | Reproduced from commercial spoilage; anaerobic conditions in FCS | 4 % NaCl | JQ249063 | 0/652 |
| LA1166 | Reproduced from commercial spoilage; anaerobic conditions in FCS | 4 % NaCl | JQ249064 | 0/689 |
| LA1167 | Reproduced from commercial spoilage; anaerobic conditions in FCS | 4 % NaCl | JQ249065 | 1/659 |
| LA1161A | Reproduced from commercial spoilage; anaerobic conditions in FCS | 4 % NaCl | N/A | 0/700 |
| LA1160A | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 0 % NaCl | N/A | 0/685 |
| LA1160B | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 0 % NaCl | N/A | 0/710 |
| LA1155A | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 2 % NaCl | N/A | 0/600 |

**Table 2.2 Continued**

| | | | | |
|---|---|---|---|---|
| LA1155B | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 2 % NaCl | N/A | 1/698 |
| LA1155C | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 2 % NaCl | N/A | 0/710 |
| LA1156A | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 2 % NaCl | N/A | 0/695 |
| LA1156B | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 2 % NaCl | N/A | 0/700 |
| LA1157A | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 2 % NaCl | N/A | 0/620 |
| LA1157B | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 2 % NaCl | N/A | 1/684 |
| LA1151 | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 4 % NaCl | JQ249047 | 1/675 |
| LA1152 | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 4 % NaCl | JQ249048 | 0/640 |
| LA1154 | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 4 % NaCl | JQ249052 | 1/660 |
| LA1158A | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 4 % NaCl | N/A | 1/569 |
| LA1158B | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 4 % NaCl | N/A | 0/628 |
| LA1159A | Reproduced spoilage from reduced NaCl (0-6%) ; anaerobic in FCS | 6 % NaCl | N/A | 0/664 |
| LA1185A | Reproduced from commercial spoilage; anaerobic conditions in FCS | 2 % NaCl | N/A | 2/672 |
| LA1185B | Reproduced from commercial spoilage; anaerobic conditions in FCS | 2 % NaCl | N/A | 1/683 |
| LA1187B | Reproduced from commercial spoilage; anaerobic conditions in FCS | 2 % NaCl | N/A | 0/700 |
| LA1188 | Reproduced from commercial spoilage; anaerobic conditions in FCS | 2 % NaCl | N/A | 0/672 |

[1]**Source:** Isolates from Johanningsmeier, Franco et al. 2012 studies involving fermented cucumber spoilage organisms
[2]**Isolate Conditions**: Spoilage LAB were reproduced under various conditions (NaCl, calcium chloride)
[3]**Gaps/Total**: identity to *L. buchneri* based on 16S gene sequencing (NCBI BLAST)

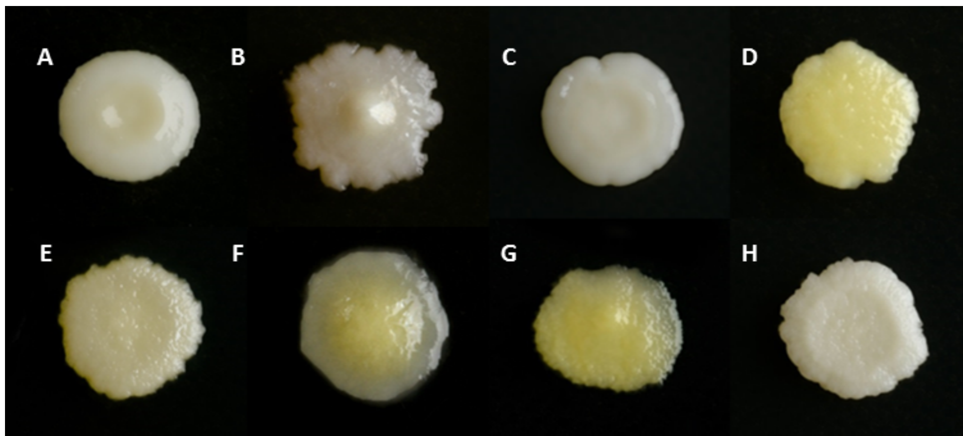


**Figure 2.1: Morphological diversity of selected isolates of *Lactobacillus buchneri***

Eight colony morphologies from selected isolates: (A) LA0030, (B) LA1147 (C) LA1161B (D) LA1184 (E) LA1167 (F) LA1175D (G) LA1181 (H) LA1161C reveal distinct phenotypic differences among the strains.

**Table 2.3: Colony morphologies of 8 *Lactobacillus buchneri* isolates from spoiled, fermented cucumber**

| **Strain** | **LA0030** | **LA1147** | **LA1161B** | **LA1161C** | **LA1167** | **LA1175D** | **LA1181** | **LA1184** |
|---|---|---|---|---|---|---|---|---|
| **Size (mm)** | 3.5 | 3 | 4.5 | 5 | 3 | 2.5 | 6 | 2.5 |
| **Color** | White | White | White | White | White | Yellow | Yellow | Yellow |
| **Form** | Circular | Irregular | Circular | Irregular | Irregular | Irregular | Irregular | Irregular |
| **Elevation** | Convex | Umbonate | Raised | Umbonate | Umbonate | Umbonate | Flat | Flat |
| **Margin** | Entire | Undulate | Entire | Undulate | Undulate | Entire | Lobate | Undulate |
| **Surface** | Mucoid | Bumpy | Dull | Granular | Granular | Smooth | Matte | Matte |
| **Texture** | Butyrous | Butyrous | Powdery | Powdery | Powdery | Butyrous | Powdery | Butyrous |
| **Opacity** | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Translucent | Opaque |

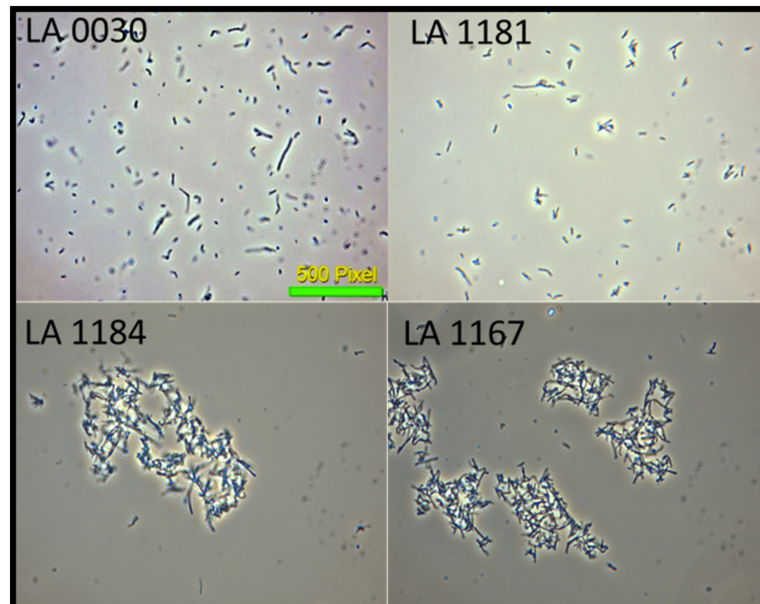


**Figure2.2: Cellular morphology of selected *Lactobacillus buchneri* isolates**

Presence of auto-aggregation phenotype in strains LA1184 and LA1167 after 36 h of growth in MRS media using phase contrast microscopy. Absence of phenotype was observed in strains LA0030 and LA1181.

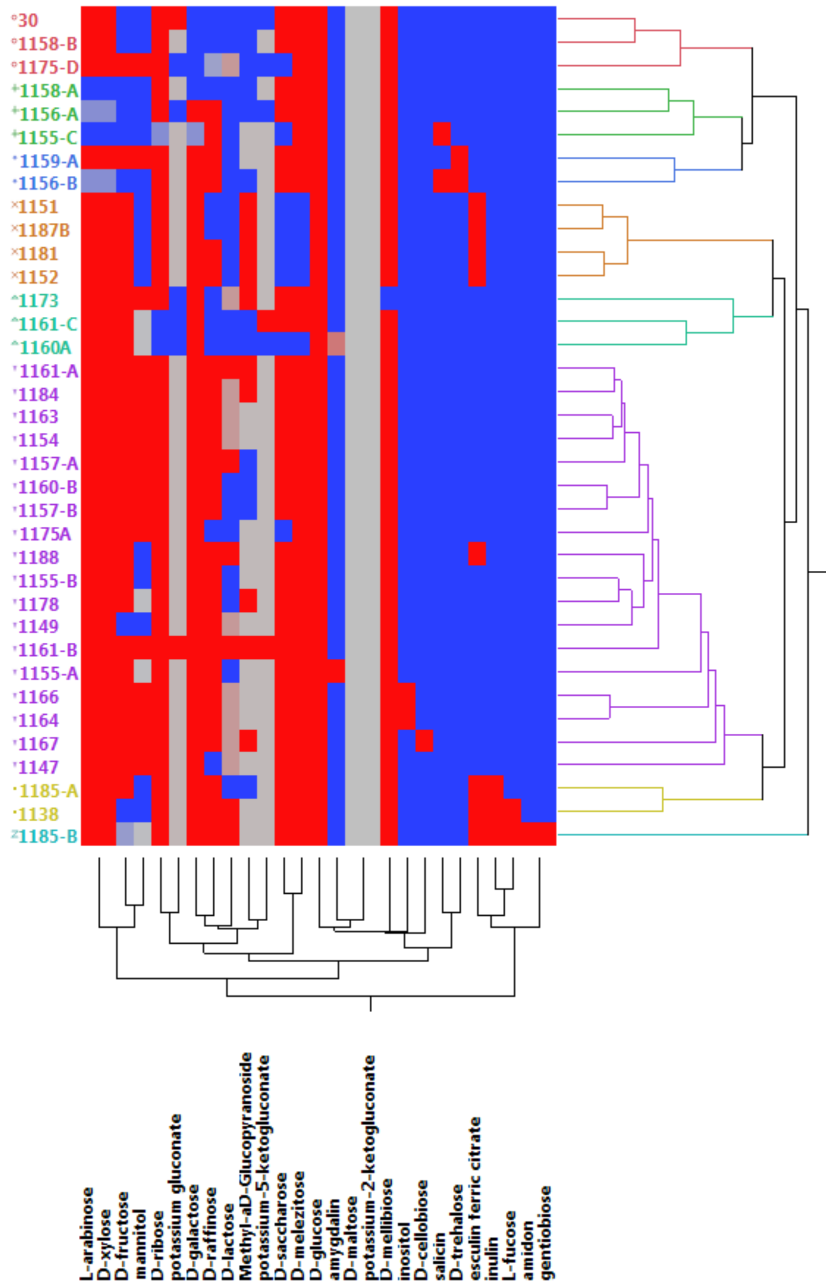


**Figure 2.3: Hierarchical cluster analysis of carbohydrate utilization by *L. buchneri***

Hierarchical clustering analysis was used to group isolates according to differences in carbohydrate utilization

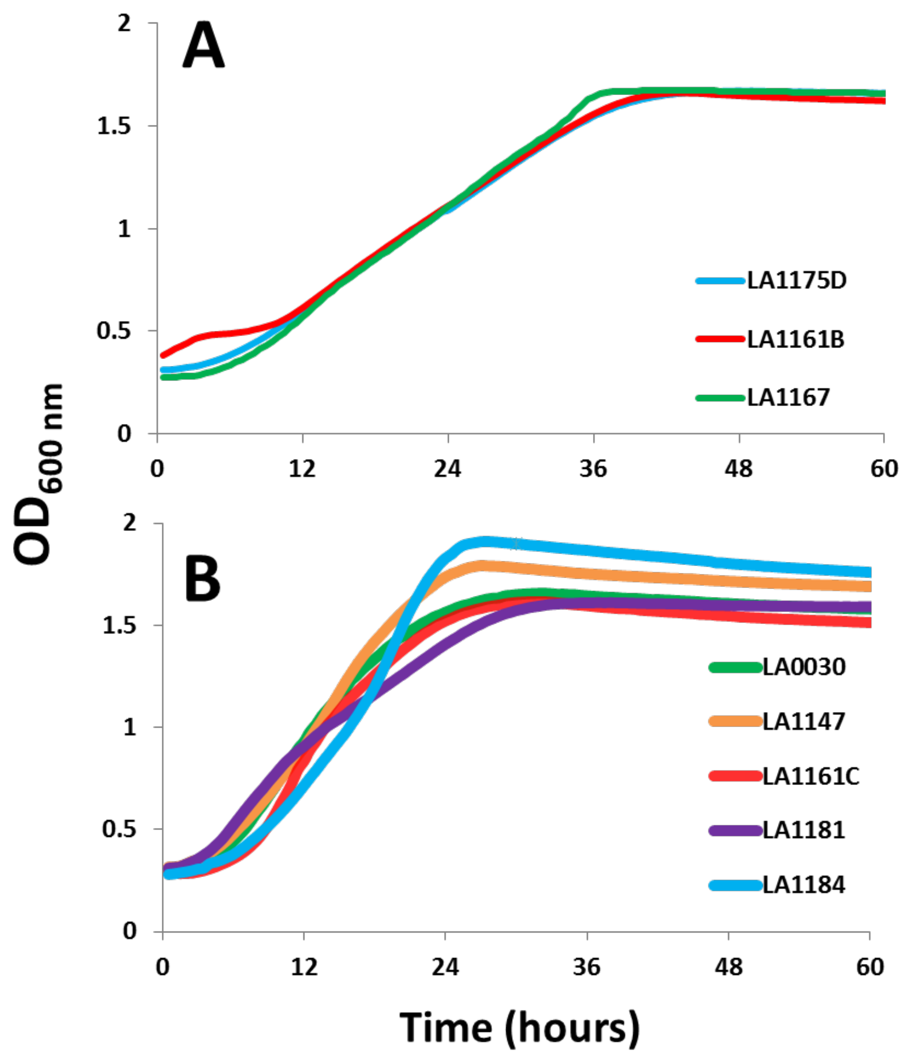


**Figure 2.4: Growth curves of selected isolates of *Lactobacillus buchneri***

The growth rate of eight select *L. buchneri* isolates was monitored over 60 h in MRS media.

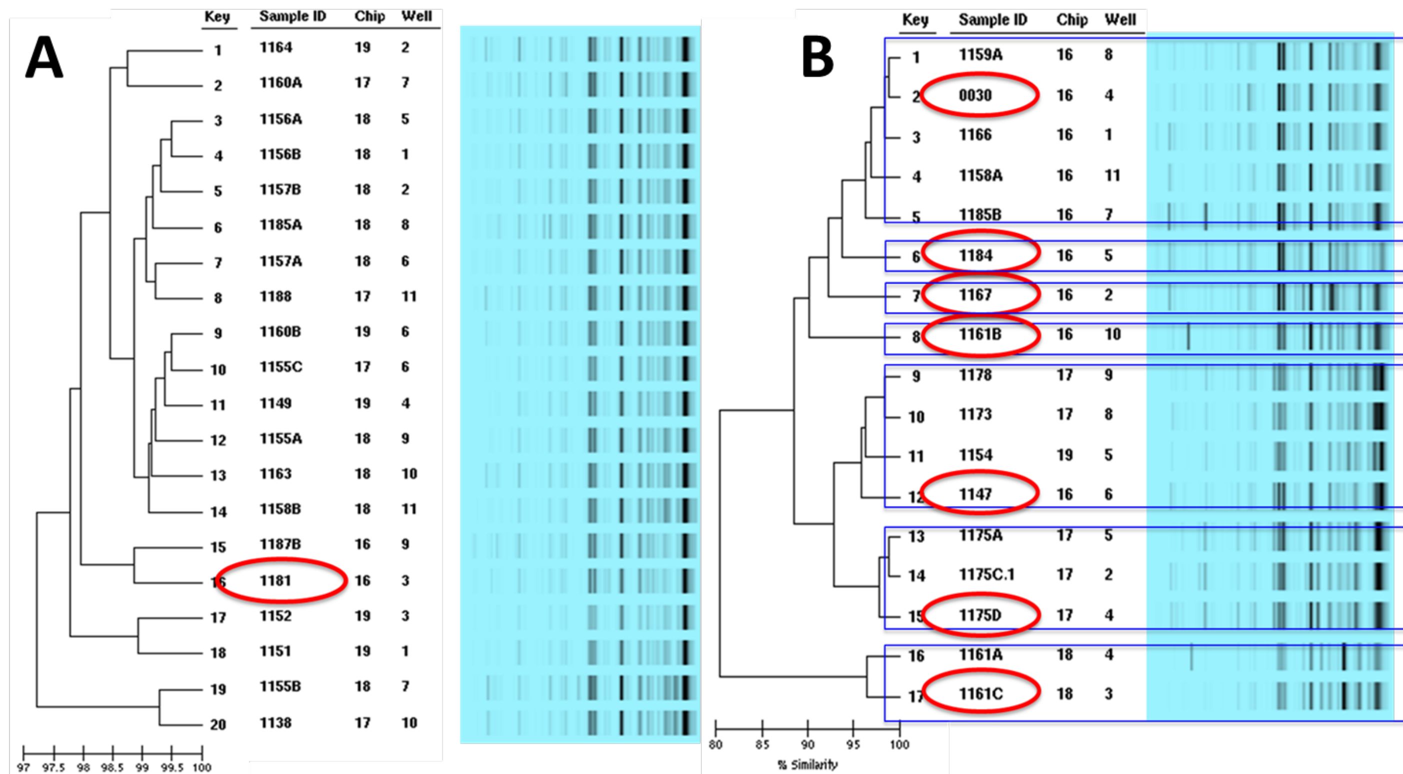


**Figure 2.5: DiversiLab® dendrogram displaying 36 isolates of *L. buchneri***

Dendrogram showing isolates clustered with A showing > 97 % similarity and B showing > 80 % similarity

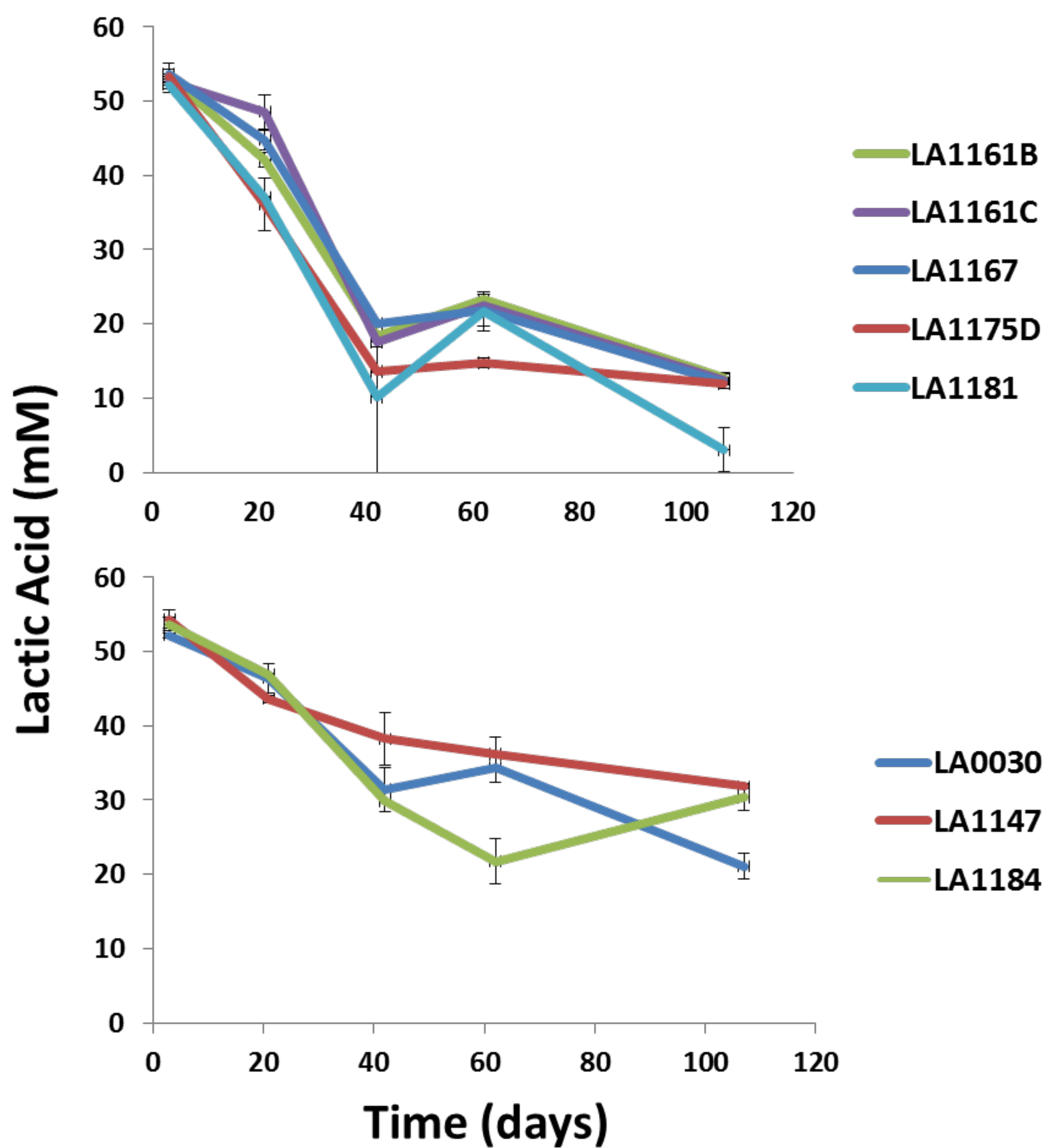


**Figure 2.6: Lactic acid degradation in modified MRS media (supplemented with lactic acid) by 8 strains of *Lactobacillus buchneri***

# 3 CHAPTER 3: DRAFT GENOME SEQUENCES OF EIGHT STRAINS OF *LACTOBACILLUS BUCHNERI*

## 3.1 ABSTRACT

*Lactobacillus buchneri* is a member of the family of lactic acid bacteria (LAB), a ubiquitous group that excretes lactic acid as the primary fermentation product into a medium supplied with a suitable source of carbohydrate. *L. buchneri* is notable for it's unique ability to further metabolize lactic acid into acetic acid and 1,2-propanediol and increase the extracellular pH after the primary fermentation is completed. Although *L. buchneri* has been affiliated with spoilage of fermented cucumbers, it is also used as a starter culture adjunct for silage. This organism has only recently been investigated for its intra-species phenotypic and genotypic diversity. Here, we present the draft genome sequences of seven *L. buchneri* strains isolated from spoiled, fermented cucumber and the ATCC type strain 4005 isolated from tomato pulp. Using the genomic tools RAST (Rapid Annotation Using Subsystem Technology) pipeline, Geneious and Mauve, a comparative analysis was conducted using these eight newly sequenced strains. While a Mauve alignment showed high conservation among the genomes, two variable regions were found between the strains. Although the genomes maintain a high degree of gene synteny overall, the RAST pipeline-generated subsystems displayed variation in the number of carbohydrate metabolism and cell wall and capsule genes among the sequenced genomes. Exploring the genomic differences such as carbohydrate catabolism, cell wall and surface structure as well as further investigating the variable regions within the genomes of the seven strains of *L. buchneri* isolated from spoiled, fermented cucumber and the ATTC type strain will further elucidate the metabolic potential of the species. Identifying genomic diversity among *L. buchneri* strains isolated from various

environments will help to harness potential applications within the species. Furthermore, understanding the lactic acid degradation pathway in this organism will aid the development of tools to prevent the onset of fermented cucumber spoilage, and conversely increase the rate of lactate conversion in silage fermentations.

## 3.2 INTRODUCTION

*L. buchneri* is a gram-positive, facultative anaerobe. Specifically, this organism is classified under the Group C lactobacilli, as an obligate heterofermenter, capable of growth by metabolizing hexoses and pentoses to lactic acid, acetic acid, ethanol and carbon dioxide (Hammes and Vogel 1995). *L. buchneri* has been isolated from a wide range of food, feed, and bioprocessing environments likely due to its diverse range of metabolic activities (Sumner, Speckhard et al. 1985, Sakamoto and Konings 2003, Liu, Skinner-Nemec et al. 2008, Zeng, Pan et al. 2010, Franco, Perez-Diaz et al. 2012, Johanningsmeier, Franco et al. 2012). Most notably, the ability of *L. buchneri* to metabolize lactic acid into acetic acid and 1,2-propanediol makes this organism a candidate as an adjunct starter culture for the silage industry. *L buchneri* has also been isolated spoiled, fermented cucumber (Johanningsmeier, Franco et al. 2012), human intestines (Zeng, Pan et al. 2010), sourdough bread, (Zhang, Brandt et al. 2010), wine (de Nadra, Chaud et al. 1986), beer wort (Sakamoto and Konings 2003), and Swiss cheese implicated in food poisoning (Sumner, Speckhard et al. 1985). Alternatively, a strain of *L. buchneri* isolated from pickled juice was shown to have potential probiotic effects, such as the reduction of serum cholesterol levels (Zeng, Pan et al. 2010). This strain of *L. buchneri* produces a bile salt hydrolase (BSH) to catalyze the hydrolysis of conjugated bile salts into amino acid residues and bile acids (Zeng, Pan et al. 2010). Another strain of *L. buchneri* was isolated from sauerkraut, and has been exploited for bacteriocin production targeting several gram positive bacteria (Yildirim and Yildirim 2001, Yıldırım, Avs et al. 2002). Currently, two *L. buchneri* genomes are available to the public. *L. buchneri*

strain NRRL-30929 found as a contaminant in a commercial ethanol facility was the first genome of its species to be fully sequenced and annotated (Liu, Skinner-Nemec et al. 2008). A second fully annotated genome of a *L. buchneri* isolate from a stable grass silage, strain CD034, became available in 2012 (Heinl, Wibberg et al. 2012). In order to investigate the genomic diversity among the strains, we sequenced seven strains of *L. buchneri* that were selected from a pool of 35 isolates from spoiled, fermented cucumber along with the type strain, ATCC 4005. The eight strains were selected from phenotypic and genotypic characterization experiments, as described in Chapter 2, to represent a diverse selection of *L. buchneri* strains. The objective of this study was to collect genomic information and conduct a preliminary comparative analysis on these seven strains of *L. buchneri* isolated from spoiled, fermented cucumber and the type strain isolated from tomato pulp.

## 3.3 METHODS

Strains of *L. buchneri* were obtained from the USDA-ARS culture collection for comparative analyses. These eight strains were selected from a pool of 36 isolates from previous phenotypic and genotypic characterization experiments described in Chapter 2. These isolates are a representation of the diversity within a species isolated from a single environmental niche. Each strain was propagated under anaerobic conditions at 30°C for 48 h in de Man, Rogosa and Sharpe (MRS) broth. Genomic DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, CA, USA) following the manufacturer's instructions for Gram positive organisms. Genomic DNA purity was quantified using a

NanoDrop 1000 spectrophotometer (Thermo Scientific, CA, USA). Approximately 1.4 to 3.5 μg of DNA per strain was submitted to the Biotechnology Center at the University of Illinois at Urbana-Champaign (UIUC) for genome sequencing**.** Shotgun genomic libraries were prepared with the TruSeq DNA Sample Prep kit (Illumina, CA), and the genomes were sequenced using the Illumina HiSeq platform (UIUC).

Each strain was genome sequenced using Illumina HiSeq technologies. The eight genomes were assembled *de novo* using Geneious software with custom assembly settings as follows: no trimming, do not allow gaps, no minimum overlap identity, use paired reads to improve assembly, and only use paired hits during assembly. *L. buchneri* CD034 was used as a reference genome. The eight strains were uploaded into the Rapid Annotations using Subsystems Technology (RAST) pipeline for fully automated annotation (Aziz, Bartels et al. 2008). The annotations were further explored using Geneious software (BioMatters; Auckland, New Zealand) (Kearse, Moir et al. 2012). A comparative analysis was conducted among the 8 strains using the RAST annotations, and a Mauve alignment (Darling, Mau et al. 2004) was performed to compare these strains to a reference genome of *L. buchneri* strain CD034. Mauve allowed for the recognition of conserved regions of the *L. buchneri* genomes, as well as areas of variability.

## 3.4 RESULTS

Once the genomes were imported into RAST, subsystems were automatically generated from the annotation pipeline. Table 3.2 displays the various categories of

subsystems and the number of corresponding genes for each of the 8 strains. The G+C content of the eight genomes ranged from 44.0 % to 45.2 % and genome size ranged from 2.49 Mbp and 2.71 Mbp. The smallest genome size belongs to LA0030 (ATCC 4005), and the largest is LA1184. A brief overview of the RAST subsystem categories suggests differences in cell wall/capsule and carbohydrate metabolism, which is supported by related information from the literature (Liu, Skinner-Nemec et al. 2008, Heinl, Wibberg et al. 2012, Johanningsmeier, Franco et al. 2012). Overall, a high degree of synteny was observed among the eight draft genomes.

ATCC strain LA0030 has only 198 genes for carbohydrate metabolism, which is correlated with a slightly smaller genome size compared to the other sequenced *L. buchneri* strains. This is consistent with the carbohydrate profiling (API® 30 CHL) results found in Chapter 2. LA0030 utilized the least number of carbohydrates out of all of the isolates examined. Strain LA1175D contains 258 genes for carbohydrate catabolism, the largest amount of the eight strains, and contains the second largest genome at 2.70 Mbp. According to the carbohydrate profiling analysis in Chapter 2, LA1175D did not ferment the largest number of carbohydrates. Variation was found in the cell wall and capsule subsystem of the strains. LA1147 had the smallest amount of genes (99) associated with this subsystem, while LA1161B has the largest amount at 133 genes. The variation among the genes among all of the strains in this specific subsystem may correlate to the observed differences in colony morphology as described in Chapter 2. A Mauve alignment was generated to look for regions

of variability among the spoilage strains compared to the reference genome CD034 (Figure 3.1).

Using the Mauve results in conjunction with Geneious software, putative S-layer proteins and CRISPR loci were found among the 8 strains. S- layer proteins are the outermost cell surface structures composed of a proteinaceous, lattice-like layer covering the cell occurring in most taxonomic groups of bacteria and *Archaea* (Hynönen and Palva 2013). While the functional aspects of *Lactobacillus* S-layers are relatively uncharacterized, S-layer proteins may prove to be advantageous, as they may influence bacterial adherence to host cells or provide a protective function. *L. buchneri* is a known S-layer producer, with eight putative S-layer proteins found in the recently annotated genome of *L. buchneri* strain CD034 (Heinl, Wibberg et al. 2012). CRISPR (Clustered Regularly Interspaced Palindromic Repeats) with CRISPR associated sequences (Cas) constitute an adaptive immune system found in prokaryotes that confers resistance to foreign, invading elements such as phage DNA and plasmids (Barrangou, Fremaux et al. 2007). Genotypic methods using CRISPR have been recently explored in *L. buchneri* strains, revealing ten unique genotypes among isolates from one environmental niche (Briner and Barrangou 2014). In the present genome analyses, most of the *L. buchneri* genome was conserved with two slightly variable regions between 142 kb and 175kb within the genome. One of these regions was the CRISPR array (Briner and Barrangou 2014). While the CRISPR spacer region displayed a common ancestor among the strains, divergent evolution was also observed. Interestingly enough, the subsystem overview displays 0 genes for phage exposure, plasmids, and other transmissible

elements for strain LA1175D. Overall, each of the eight draft genomes of *L. buchneri* are relatively similar, as expected. However, the variability among the strains was observed between carbohydrate utilization and cell wall and capsule genes. These findings may explain the phenotypic diversity observed in Chapter 2.

## 3.5 ACKNOWLEDGEMENTS

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**Table 3.1: Overview of genomic data from 8 sequenced genomes of *Lactobacillus buchneri***

| Strain ID | Genome size (bp) | % GC | Contigs (>1000 bp) | N50 length (bp) | tRNA | rRNA |
|---|---|---|---|---|---|---|
| LA1161B | 2614519 | 44 | 77 | 63881 | 57 | 17 |
| LA1181 | 2628753 | 44 | 59 | 95113 | 60 | 15 |
| LA1184 | 2716499 | 44 | 82 | 81855 | 59 | 13 |
| LA1147 | 2608988 | 44.1 | 128 | 36444 | 57 | 15 |
| LA1167 | 2613434 | 44.1 | 73 | 61779 | 59 | 15 |
| LA1175D | 2701261 | 44.1 | 508 | 9728 | 61 | 16 |
| LA0030 | 2493071 | 44.3 | 67 | 64594 | 60 | 16 |
| LA1161C | 2561573 | 45.2 | 60 | 78131 | 59 | 17 |

**Table 3.2: Subsystem overview of eight *Lactobacillus buchneri* genomes**

| Strains | LA1161B | LA1181 | LA1184 | LA1147 | LA1167 | LA1175D | LA0030 | LA1161C | Range |
|---|---|---|---|---|---|---|---|---|---|
| **Subsystem** | | | | | | | | | |
| **Cofactors, Vitamins, Prosthetic Groups, Pigments** | 118 | 121 | 116 | 117 | 118 | 117 | 115 | 118 | 6 |
| **Cell Wall and Capsule** | 133 | 112 | 112 | 99 | 104 | 112 | 110 | 104 | 34 |
| **Virulence, Disease and Defense** | 51 | 48 | 47 | 52 | 50 | 53 | 43 | 51 | 10 |
| **Potassium metabolism** | 5 | 5 | 3 | 4 | 5 | 4 | 3 | 5 | 2 |
| **Miscellaneous** | 17 | 16 | 16 | 16 | 17 | 16 | 16 | 17 | 1 |
| **Phages, Prophages, Transposable elements, Plasmids** | 26 | 21 | 27 | 19 | 25 | 0 | 27 | 25 | 27 |
| **Membrane Transport** | 56 | 56 | 59 | 60 | 62 | 59 | 54 | 56 | 8 |
| **RNA Metabolism** | 74 | 75 | 76 | 76 | 74 | 76 | 74 | 74 | 2 |
| **Nucleosides and Nucleotides** | 96 | 101 | 101 | 97 | 96 | 101 | 95 | 96 | 6 |
| **Protein Metabolism** | 176 | 181 | 180 | 177 | 176 | 179 | 178 | 209 | 33 |
| **Cell Division and Cell Cycle** | 44 | 47 | 49 | 49 | 43 | 47 | 43 | 43 | 6 |
| **Regulation and Cell signaling** | 34 | 36 | 36 | 33 | 36 | 36 | 32 | 33 | 4 |
| **Secondary Metabolism** | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 |
| **DNA Metabolism** | 108 | 119 | 120 | 111 | 113 | 125 | 105 | 108 | 20 |
| **Fatty Acids, Lipids, and Isoprenoids** | 81 | 82 | 85 | 82 | 82 | 87 | 83 | 81 | 6 |
| **Dormancy and Sporulation** | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 0 |
| **Respiration** | 18 | 19 | 18 | 18 | 19 | 17 | 12 | 18 | 7 |
| **Stress Response** | 56 | 55 | 65 | 56 | 56 | 63 | 50 | 56 | 15 |
| **Metabolism of Aromatic Compounds** | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0 |

**Table 3.2 Continued**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Amino Acids and Derivatives** | 227 | 237 | 228 | 230 | 227 | 237 | 224 | 227 | 13 |
| **Sulfur Metabolism** | 7 | 7 | 7 | 8 | 7 | 7 | 7 | 7 | 1 |
| **Phosphorus Metabolism** | 47 | 50 | 48 | 51 | 48 | 49 | 47 | 48 | 4 |
| **Carbohydrates** | 237 | 231 | 237 | 250 | 245 | 258 | 198 | 236 | 60 |

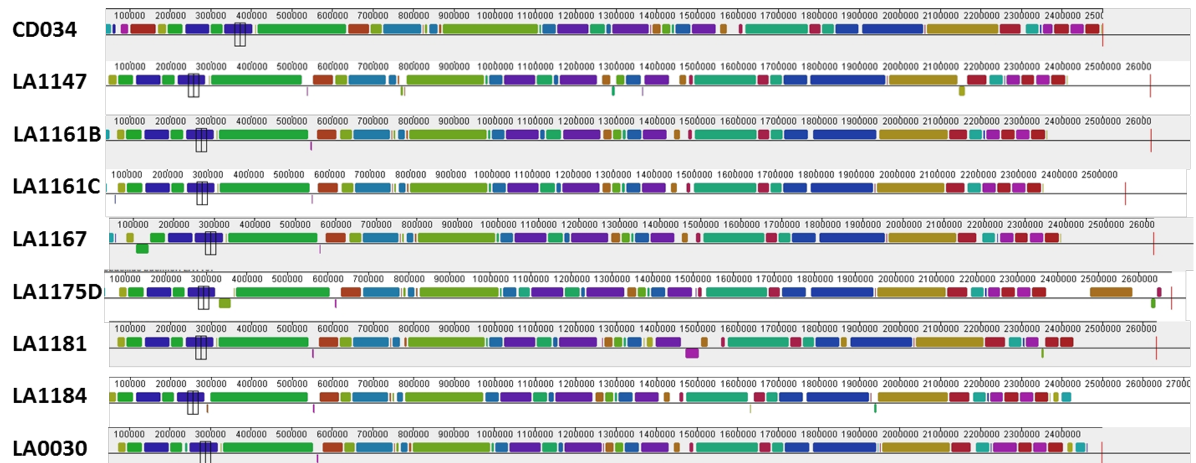


**Figure 3.1 Mauve alignment of 8 *Lactobacillus buchneri* strains**

# 4 CHAPTER 4: CONCLUSIONS

Until recently, the intraspecies diversity of *Lactobacillus buchneri* had not been characterized in great detail. Among 35 isolates from spoiled, fermented cucumber, eight morphotypes were found, ranging in size (1-11 mm in diameter), shape (umbonate vs. entire), and color (yellow vs. white). A wide range of carbohydrate profiles were observed of the 36 isolates, including some strains able to metabolize mannitol, potassium-5-ketogluconate, xylose, and raffinose as a sole carbon source. Lactate degradation revealed differences among the strains in the rate and ability to metabolize lactic acid over a time period of 107 days. Given the extensive diversity within the species, it may prove difficult to predict the onset of spoilage in commercial cucumber fermentation, as some tanks may begin to spoil faster than others due to the observed differences in lactate degradation and growth rates of *Lactobacillus buchneri* strains.

Specifically, further investigating the carbohydrate utilization of *L. buchneri* in both a silage and cucumber fermentation environment may prove to be an integral part of understanding this species' distinct metabolic capabilities. Lactate degradation by *L. buchneri* may take longer than 30 days to initiate. As one study illustrated, components present in fermented cucumber, such as trehalose and citrulline, were a metabolized prior to lactate catabolism. Similar compounds may be present in silage fermentations. Long-term studies in both environments could provide insight to potential regulators of lactate degradation. Long-term (1-2 year), small-scale, bench-top cucumber fermentations inoculated with specific strains of *L. buchneri* may identify other carbohydrates of interest

prior to the onset of lactic acid depletion. This information could be utilized to prevent or delay the onset of spoilage.

Conversely, lactate degradation could be increased in a silage environment. Harnessing the metabolic ability of a rapid growing, heterofermentative starter culture that metabolizes a diverse spectrum of carbohydrates could be inoculated in silage fermentation prior to inoculating the *L. buchneri* adjunct. This process may expedite the lactate degradation process by *L. buchneri.* Also, screening various strains for lactate catabolism ability may be advantageous for the silage industry, as more rapidly lactate-degrading strains could be discovered. Additionally, more extensive strain-monitoring programs should be instated to control spoilage of fermented cucumbers. Identifying and controlling the robust lactic acid-degrading strains of *L. buchneri* will help to delay the onset of spoilage altogether.

# A. APPENDICES

# Appendix A

**Table A.1. Carbohydrate profling of 36 isolates of *Lactobacillus buchneri***

| Fermentable Carbohydrates | L-arabinose | D-ribose | D-xylose | D-galactose | D-fructose | inositol | mannitol | Methyl-αD-Glucopyranoside | amygdalin | esculin ferric citrate | salicin | D-cellobiose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Expected % +** | **92** | **100** | **57** | **85** | **92** | **0** | **0** | **42** | **0** | **7** | **0** | **0** |
| **0030** | + | + | + | - | - | - | - | - | - | - | - | - |
| **1151** | + | + | + | + | + | - | - | + | - | +/- | - | - |
| **1187** | + | + | + | + | + | - | - | + | - | +/- | - | - |
| **1181** | + | + | + | + | + | - | - | + | - | +/- | - | - |
| **1152** | + | + | + | + | + | - | - | + | - | +/- | - | - |
| **1173** | + | + | + | + | + | - | + | + | - | - | - | - |
| **1175D** | + | + | + | - | + | - | + | - | - | - | - | - |
| **1161A** | + | + | + | + | + | - | + | + | - | - | - | - |
| **1161B** | + | + | + | + | + | - | + | + | - | - | - | - |
| **1161C** | + | - | + | + | + | - | +/- | - | - | - | - | - |
| **1160A** | + | - | + | + | + | - | +/- | - | +/- | - | - | - |
| **1167** | + | + | + | + | + | - | + | + | - | - | - | + |
| **1185A** | + | + | + | + | + | - | - | - | - | +/- | - | - |
| **1185B** | + | + | + | + | +/- | - | +/- | +/- | - | +/- | - | - |
| **1138** | + | + | + | + | - | - | - | +/- | - | +/- | - | - |
| **1188** | + | + | + | + | + | - | - | +/- | - | +/- | - | - |
| **1160B** | + | + | + | + | + | - | + | - | - | - | - | - |
| **1158A** | - | + | - | - | - | - | - | - | - | - | - | - |
| **1158B** | + | + | + | - | - | - | - | - | - | - | - | - |
| **1157A** | + | + | + | + | + | - | + | - | - | - | - | - |
| **1157B** | + | + | + | + | + | - | + | - | - | - | - | - |
| **1155A** | + | + | + | + | + | - | +/- | +/- | + | - | - | - |
| **1155B** | + | + | + | + | + | - | - | +/- | - | - | - | - |
| **1155C** | - | +/- | - | +/- | - | - | - | +/- | - | - | + | - |
| **1159A** | + | + | + | + | + | - | + | +/- | - | - | - | - |

**Table A.1 Continued**

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1175A** | + | + | + | + | + | - | + | +/- | - | - | - | - |
| **1163** | + | + | + | + | + | - | + | +/- | - | - | - | - |
| **1147** | + | + | + | + | + | - | + | +/- | - | - | - | - |
| **1149** | + | + | + | + | - | - | - | +/- | - | - | - | - |
| **1166** | + | + | + | + | + | + | + | +/- | - | - | - | - |
| **1164** | + | + | + | + | + | + | + | +/- | - | - | - | - |
| **1156A** | +/- | + | +/- | + | - | - | - | - | - | - | - | - |
| **1156B** | +/- | + | +/- | + | - | - | - | - | - | - | + | - |
| **1184** | + | + | + | + | + | - | + | + | - | - | - | - |
| **1154** | + | + | + | + | + | - | + | +/- | - | - | - | - |
| **1178** | + | + | + | + | + | - | +/- | + | - | - | - | - |

**Table A.2: Carbohydrate profiling of 36 isolates of *Lactobacillus buchneri* (continued)**

| Fermentable Carbohydrates | D-lactose | D-mellibiose | D-saccharose | D-trehalose | inulin | D-melezitose | D-raffinose | amidon | gentiobiose | L-fucose | potassium gluconate | potassium-5-ketogluconate |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Expected % +** | **25** | **85** | **90** | **0** | **0** | **81** | **64** | **7** | **0** | **0** | **85** | **64** |
| **30** | - | + | + | - | - | + | - | - | - | - | + | - |
| **1151** | - | + | - | - | - | - | - | - | - | - | +/- | +/- |
| **1187** | - | + | - | - | - | - | - | - | - | - | +/- | +/- |
| **1181** | - | + | - | - | - | - | + | - | - | - | +/- | +/- |
| **1152** | - | + | - | - | - | - | + | - | - | - | +/- | +/- |
| **1173** | +/- | - | + | - | - | + | - | - | - | - | - | +/- |
| **1175D** | +/- | + | - | - | - | + | +/- | - | - | - | - | - |
| **1161A** | + | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1161B** | + | + | + | - | - | + | + | - | - | - | + | + |
| **1161C** | - | + | + | - | - | + | - | - | - | - | - | + |
| **1160A** | - | + | - | - | - | - | - | - | - | - | - | - |
| **1167** | +/- | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1185A** | - | + | + | - | + | + | + | - | - | - | +/- | +/- |
| **1185B** | + | + | + | - | + | + | + | + | + | + | +/- | +/- |
| **1138** | + | + | + | - | + | + | + | - | - | + | +/- | +/- |
| **1188** | + | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1160B** | - | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1158A** | - | + | + | - | - | + | - | - | - | - | +/- | +/- |
| **1158B** | - | + | + | - | - | + | - | - | - | - | +/- | +/- |
| **1157A** | + | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1157B** | - | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1155A** | - | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1155B** | - | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1155C** | - | + | - | - | - | + | + | - | - | - | +/- | +/- |
| **1159A** | - | + | + | + | - | + | + | - | - | - | +/- | +/- |
| **1175A** | - | + | - | - | - | + | - | - | - | - | +/- | +/- |
| **1163** | +/- | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1147** | +/- | + | + | - | - | + | - | - | - | - | +/- | +/- |

**Table A.2 Continued**

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1149** | +/- | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1166** | +/- | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1164** | +/- | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1156A** | - | + | + | - | - | + | + | - | - | - | - | - |
| **1156B** | - | + | + | + | - | + | + | - | - | - | +/- | +/- |
| **1184** | +/- | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1154** | +/- | + | + | - | - | + | + | - | - | - | +/- | +/- |
| **1178** | - | + | + | - | - | + | + | - | - | - | +/- | +/- |

**Table A.3:** ***Lactobacillus buchneri*** **isolates after 28 days in fermented cucumber juice**

| Isolate ID | Average pH |
|---|---|
| CONTROL | 3.74 ± 0.01 |
| 0030 | 4.29 ± 0.40 |
| 1138 | 4.04 ± 0.14 |
| 1147 | 4.48 ± 0.11 |
| 1149 | 3.96 ± 0.02 |
| 1151 | 4.21 ± 0.05 |
| 1152 | 4.38 ± 0.04 |
| 1154 | 3.76 ± 0.01 |
| 1163 | 4.10 ± 0.16 |
| 1164 | 4.63 ± 0.17 |
| 1166 | 4.70 ± 0.00 |
| 1167 | 3.90 ± 0.00 |
| 1173 | 4.32 ± 0.22 |
| 1178 | 3.91 ± 0.07 |
| 1181 | 4.15 ± 0.05 |
| 1184 | 3.94 ± 0.00 |
| 1187 | 4.30 ± 0.04 |
| 1188 | 3.91 ± 0.02 |
| 1155A | 4.01 ± 0.16 |
| 1155B | 3.97 ± 0.06 |
| 1155C | 4.06 ± 0.07 |
| 1156A | 3.91 ± 0.02 |
| 1156B | 3.97 ± 0.05 |
| 1157A | 4.01 ± 0.00 |
| 1157B | 4.02 ± 0.00 |
| 1158A | 3.93 ± 0.01 |
| 1158B | 3.93 ± 0.11 |
| 1159A | 3.95 ± 0.09 |
| 1160A | 3.94 ± 0.00 |
| 1160B | 3.97 ± 0.01 |
| 1161A | 4.01 ± 0.01 |
| 1161B | 3.91 ± 0.01 |
| 1161C | 3.96 ± 0.02 |
| 1175A | 3.95 ± 0.09 |
| 1175D | 4.07 ± 0.06 |
| 1185A | 3.96 ± 0.02 |

**Table A.3 Continued**

| | |
|---|---|
| 1185B | 4.06 ± 0.01 |