ABSTRACT

UCAR, REDIFE ASLIHAN. Utilization of Alternate Energy Sources in Cucumber Fermentations by Certain Lactic Acid Bacteria, Including Starter Culture Candidates and Spoilage-Associated Species. (Under the direction of Dr. Ilenys Pérez-Díaz).

Carbohydrate utilization by lactic acid bacteria (LAB) defines the extent of cucumber fermentations, their quality and long-term stability. Aside from glucose and fructose, alternate energy sources such as citrulline, trehalose, cellobiose, xylose, lyxose, gentiobiose, and furfural were detected in cucumber fermentations using metabolomics. These compounds remained present in cucumber fermentations after glucose and fructose were utilized. We hypothesize that the removal of alternate energy sources by starter cultures during the most active period of the bioconversion prevents the proliferation of spoilage-associated microbes such as *L. buchneri*. The nature of the alternate energy sources in fresh and fermented cucumbers was investigated using HPLC. The presence of putative pathways for the utilization of alternate energy sources in the LAB listed above was investigated using the publically available genome sequences and the KEGG Orthology and Integrated Microbial Genomes tools. The ability of starter or adjunct culture candidates, including *Lactobacillus plantarum*, *L. pentosus*, *L. brevis*, *L. buchneri* and *Pediococcus pentosaceous* to utilize alternate energy sources in a fermented cucumber juice model system (FCJM) simulating conditions post-fermentation conditions was studied. The influence of oxygen availability and pH on the metabolism of some of the targeted compounds was also studied in FCJM. The metabolic conversion of the targeted substrates was monitored using HPLC analysis as well. While the presence of gentiobiose, cellobiose, and lyxose was unconfirmed in fresh cucumber juice and fermentation cover brines collected on day 3 and 38 of commercial fermentations, trehalose and xylose were sporadically detected in the fresh fruit at 15.5 ± 1.6 and 36 mM, respectively, while citrulline was consistently found in fresh cucumbers at 1.6 ± 0.6 mM and in lower concentrations in fermentations. Even though, the putative

metabolic potential of the selected LAB to utilize alternate energy sources was clearly suggested by the bioinformatics analysis, limited correlation was found with the phenotypes observed. *L. plantarum* and *L. pentosus* were able to utilize cellobiose, gentiobiose and trehalose in FCJM, but not citrulline, furfural or xylose. *L. brevis* was able to only utilize gentiobiose, xylose and furfural in FCJM at pH 4.7. *P. pentosaceous* utilized trehalose in FCJM at pH 4.7, and it is still uncertain if this species can utilize the other compounds. *L. buchneri* was able to utilize all the compounds tested in FCJM and was unique in converting citrulline to ammonia and ornithine in the presence of limiting glucose. The presence of excess glucose in the FCJM prevented the production of the biogenic amine, ornithine by *L. buchneri*. Additionally, *L. plantarum*, *L. pentosus* and *L. brevis* were able to produce citrulline presumably from arginine. Differences were observed in the ability of specific *L. plantarum* and *L. pentosus* strains to convert cellobiose or gentiobiose to lactic acid. Inoculation of *L. brevis* and *L. buchneri* in fresh cucumber juice medium (FrCJ) supplemented with 18.25 ± 0.49 mM trehalose, 51.97 ± 1.30 mM xylose, and 4.23 ± 0.61 mM citrulline resulted in a complete removal of the alternate energy sources prior to reaching a pH inhibitory for the fermentation itself (pH 3.3). Inoculation of unsupplemented FrCJ with a tripartite starter culture of *L. pentosus*, *L. brevis* and *L. buchneri* resulted in the removal of citrulline between days 10 and 30 of the fermentation and a stable pH (3.57 ± 0.01) and lactic acid and acetic acid concentrations for 55 days of long-term storage under anaerobiosis. Further studies are needed to determine if the use of a tripartite starter culture can aid in achieving a complete cucumber fermentation that is microbiologically stable as a function of time.

Evaluating the Utilization of Organic Compounds by Certain Lactic Acid Bacteria Including Starter Culture Candidates in the Cucumber Fermentation

by
Redife Aslihan Ucar

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Food Science

Raleigh, North Carolina
2018

APPROVED BY:

Dr. Ilenys M. Pérez-Díaz Committee Chair	Dr. Lisa L. Dean
Dr. Sophia Kathariou	

DEDICATION

I dedicate this thesis to my husband, Erhan Ucar and our two babies. This thesis is also dedicated to my wonderful family, my father, Ali Yucel Keles, my mother, Gulgun Keles, my sister Nihan Keles, and my brother Hasan Enes Keles currently living in Turkey.

BIOGRAPHY

Redife Aslihan Ucar was born in Aydin, Turkey on March 25, 1988 and has a wonderful sister and smart brother. She received her B.S. degree from the Pamukkale University (PAU) in Food Engineering. While at PAU, she did the internships at Taris Olive Oil Company, Yorukoglu Dairy Product Company, and a food research laboratory. Upon graduation, she worked as a food engineer at Akimpex and Company (dry fruits), while studying towards a master degree at Adnan Menderes University in Food Technology-Veterinary Medicine. She transferred to FASDAT (logistic) Company to work as a Quality Control Manager. While at FASDAT, Ms. Ucar won a scholarship for graduate education in the United State from the Republic of Turkey. She married Mr. Erhan Ucar before relocating to the U.S.A to complete her second Master Degree in Food Science. She successfully completed the Intensive English Program at Dokuz Eylul University (TURKEY) and the University of Georgia (USA) before starting her Master Degree program at Nort Carolina State University. She has a handsome son and is expecting a baby girl in January 2019.

ACKNOWLEDGMENTS

First, I would like to thank my advisor, Dr. Ilenys M. Pérez-Díaz for her constant support and encouragement throughout my graduate education. Second, I would like to thank my committee members Dr. Lisa Dean and Dr. Sophia Kathariou for their comments, suggestions and technical support. I am also thankful to Janet Hayes for providing help with the laboratory techniques. I am very thankful to Robert Price for his technical support and Sandra Parker for her administrative support. I would like to acknowledge all my co-workers and friends including Rasi Fitria, Yawen Zhai, Trisha Bhatia, Morgan Caudil, Rong Reynolds, Lesley Montalvo-Sandoval and Dr. Blanca Escudero-Abarca. I would also like to acknowledge the Ministry of Food with the Turkish government for their financial support and help throughout my education in the U.S.

Many thanks to my family for being supportive, positive, and loving from afar. Finally, I would like to thank my husband, Erhan Ucar for his endless support, love, patience and understanding. And of course, I am very grateful to be a mom of two babies Uras and a baby girl for making my life fun during this stressful period.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Literature Review

1.1 Introduction

1.1.1 Cucumber Fermentation

Cucumbers are commercially fermented by the indigenous microbiota in cover brines containing at least 6% NaCl (Breidt and others, 2013). The lactic acid bacteria (LAB) naturally present in cucumbers ferment the carbohydrates present in the fruits to lactic acid, while reducing the initial pH (5-6.5) to at least 3.5 (Jones and Etchells, 1943). On average 0.8 to 1.2% lactic acid is produced in a cucumber fermentation (McFeeters, 1993; Lu and others, 2002; Franco and others, 2012; personal communications with processors, 2012). The average colony counts for total aerobes and LAB in fresh cucumbers (10^5 CFU/g and 10^3 CFU/g, respectively) is substantially increased in a fermentation to 10^7 CFU/g and 10^8 CFU/g, respectively (Hutkins, 2006; Pérez-Díaz and others, 2014). Generally, cucumber fermentation occurs in four different stages including initiation, primary, secondary, and post-fermentation (Fleming, 1984). A diversity of gram positive and gram negative bacteria initiate cucumber fermentations including *Pseudomonas, Pantoea*, *Stenotrophomonas*, and *Acinetobacter*, *Comamonas*, *Wautersiella*, *Microbacterium*, *Flavobacterium* and *Enterobacteriaceae* (Fleming, 1984; Pérez-Díaz, submitted). The number of undesirable nitrate-reducing bacteria increase rapidly during the initiation of a natural cucumber fermentation due to the absence of LAB in significant numbers (Yan and others, 2008). LAB such as *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus buchneri, Pediococcus pentosaceus, Pediococcus cerevisiae, Lactobacillus pentosus, Leuconostoc mesenteroides, Lactobacillus casei, Lactobacillus paracasei*, and *Weissella* spp. and yeasts prevail in the primary stage of cucumber fermentations (Etchells and others, 1968; Tamang and Tamang, 2010; Breidt and others, 2013; Pérez-Díaz and others, 2014; Soltan Dallal and others, 2017; Yu and others, 2012). Primary fermentation results in the utilization of fermentable sugars, decreasing pH in the

cover brine and increasing lactic acid concentrations and the population of LAB (Tamang and Tamang, 2010; Franco and Perez-Diaz, 2013). Moreover, the growth of *Enterobacteriaceae* and *Bacillus* species is inhibited by the acid produced by the prevailing LAB, the limiting dissolved oxygen levels and the NaCl added in cover brines (Fleming, 1984; Panagou and Katsaboxakis, 2006; Con and Karasu, 2009; Franco and Pérez-Díaz, 2012; Yu and others, 2012; Soltan Dallal and others, 2017). Mostly yeasts such as *Debaryomyces* sp., *Endomycopsis ohmeri*, *Zygosaccharomyces* spp., *Candida krusei*, *Pichia* spp., *Torulopsis* spp., *Brettanomyces versatilis*, *Hansenula subpelliculosa Torulaspora rosei* and *Kloeckera magna* are present in the secondary stage of cucumber fermentation (Etchells and Bells,1950a and 1950b). The last stage of cucumber fermentation is dominated by film-forming oxidative yeasts and bacteria in outdoor-open-top tanks (Etchells and Bells,1950a). Microbial activity during the post-fermentation period often results in rising pH spoilage producing acetic, propionic and/or butyric acids and decreasing lactic acid concentration (Franco and others, 2012). The main spoilage organisms in cucumber fermentations include *Acetobacter peroxydans, Acetobacter aceti, Acetobacter pasteurianus*, *L. rapi* and *L. buchneri* (Medina and others, 2016). Controlled cucumber fermentations have been achieved by eliminating the initiation, secondary fermentation, and post-fermentation stages (Fleming, 1984).

1.1.2 Sugars in Fresh and Fermenting Cucumbers

Fermentable sugars in pickling cucumbers: While glucose and fructose are the main sugars present in cucumbers, the fruit also contains residual sucrose in the endocarp (Handley and others, 1983). Glucose and fructose concentrations decrease in the seeds as a function of development, concomitantly with an increase in galactinol synthase activity and the accumulation of sucrose, raffinose, and stachyose (Handley and others, 1983). Although, a number of sugars have been

extracted from cucumbers mesocarp, including rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and galacturonic acid, at concentrations between 0.1 and 7 mM, no changes occur as a function of tissue softening induced by heating or fermentation (McFeeters, 1992). While glucose, fructose, and sucrose are the major water-soluble sugars in cucumbers, stachyose has been also detected in the smaller fruits (Pharr and others, 1977). Selected strains of *L. plantarum*, *L. brevis*, *Leuconostoc mesenteroides* and *Pediococcus pentosaceous* are able to utilize stachyose and raffinose (Fleming and Pharr, 1980). Raffinose can also be utilized by *Lactobacillus buchneri* (Sanders and others, 2015).

Fermentable sugars in cucumber fermentations: Monitoring of the progression of cucumber fermentations have been focused on the conversion of the primary sugars naturally present in the fruits to lactic acid, acetic acid, and ethanol by lactic acid bacteria. The disappearance of 100% of the fructose and glucose naturally present in the fruits is considered the desired end-point of a cucumber fermentation. Thus, commercial production has capitalized on the use of reductive sugars strips to routinely monitor the completion of fermentation in batches prior to processing. The development of chromatography methods such as HPLC, enabling the concomitant detection of glucose and fructose to organic acids and ethanol, expanded the sensitivity threshold and ability to determine specific yields of the deriving fermentation end products (McFeeters and Barish, 2003).

With the advent of omics technologies, the possibility to go beyond the detection of the carbon balance resulting from cucumber fermentations, now includes the ability to comprehensively detect the chemical composition of the fresh fruits, the fermentation and processing environments. Initial exploratory research employing an omics approach for volatile compounds used two dimensional gas chromatography-time-of-flight mass spectrometry (GC x

GC-TFMS) for fermented cucumber brines and attributed 314 peaks to the process itself (Johanningsmeier and McFeeters, 2011). Thirty-three metabolites were found to change in laboratory scale fermentations that underwent spoilage, characterized by an increase in pH, reduction in lactic acid, increase in acetic acid and production of propionic acid (Johanningsmeier and McFeeters, 2011). Additional metabolite concentrations found to fluctuate as the result of fermentation spoilage included those corresponding to butyric acid, alcohols, n-propyl acetate and furfural. Furthermore, metabolic profiling of anaerobic fermented cucumbers spoilage by *Lactobacillus buchneri* revealed changes in 92 compounds including citrulline, trehalose, cellobiose, xylose, lyxose, gentiobiose and lactic acid (Johanningsmeier and McFeeters, 2015). Incremental concentrations were observed in alcohols and butanoic and pentanoic acids and were accompanied by decreases in the concentration of monosaccharides, disaccharides, amino acids, nucleosides, long chain fatty acids and ketones (Johanningsmeier and McFeeters, 2015). Especially, citrulline, D-trehalose, and D-cellobiose were utilized by *L. buchneri* prior to lactic acid degradation (Johanningsmeier and McFeeters, 2015).

1.1.3 Lactic Acid Bacteria Requirements for Growth and Metabolism

Lactobacilli growth requirements

Carbohydrate utilization is the main energy generating metabolic activity in LAB (Poolman and others, 2008). The Bergey's manual was compiled to distinguish physiological and biochemical properties of different *Lactobacillus* species according to carbohydrate fermentation reaction. However, since carbohydrates are not the only potential energy source for LAB, studies have been performed to understand the role of nucleotides, amino acids, and vitamins as fuels (Elli and others, 2000; Hammes and Hertel, 2015). Most lactobacilli need four ribonucleosides or deoxyribonucleosides for growth (Elli and others, 2000). Adenine and guanine or thymine and

cytosine can be replaced by inosine or uracil, respectively (Elli and others, 2000). Iron is critical in the *de novo* synthesis of purines and pyrimidines (Elli and others, 2000). Three amino acids are essential for growth of lactobacilli including L-glutamic acid, L-valine and L-leucine (Ledesma and others, 1977). Some lactobacilli also need L-arginine, L-tyrosine and L-tryptophan (Ledesma and others, 1977). Some strains of *Lactobacillus plantarum* also need L-cysteine and L-phenylalanine (Ledesma and others, 1977). When compared to *L. plantarum*, *L brevis* and *L. buchneri* have more amino acid requirements (Ledesma and others, 1977). *L. plantarum, L. buchneri* and *L. brevis* require niacin and pantothenate for growth in synthetic medium (Ledesma and others, 1977). While *L. plantarum* needs riboflavin for growth in synthetic medium, *L. brevis* and *L. buchneri* require thiamin (Ledesma and others, 1977; Rogosa and others, 1961). The minerals manganese and potassium are essential for growth of most lactobacilli while magnesium is stimulatory (McLeod and Snell, 1947). Although, differences among amino acid and vitamin requirements of LAB have been shown among species and strains, fermentable carbohydrates are essential for growth of lactobacilli (Morishita and others, 1981).

Utilization of different carbohydrates in the early exponential growth phase is critical for sugar metabolism in LAB. Growth of LAB in particular lactobacilli is enhanced in the presence of two sugars compared to either sugar alone (Ortakci and others, 2015). *Lactobacillus wasatchii*, a cheese isolate, uses ribose preferentially for energy and galactose for other functions such as cell wall biosynthesis (Ortakci and others, 2015). It is energetically draining to make a six-carbon sugar from pentose sugars and concomitantly use hexoses for biosynthesis of peptidoglycan, other cell building blocks and phosphoenolpyruvate (PEP) products or for the PEP-dependent uptake of ribose.

L. plantarum is a facultative heterofermentor using hexoses and pentoses via the glycolytic and 6-phosphogluconate/phosphoketolase pathway, respectively (Axelsson, 2004; Khalid, 2011). *L. plantarum* NCIMB 8026 is able to utilize ribose, xylose and L-arabinose in the presence of glucose (Westby and others, 2008). When *L. plantarum* NCIMB8026 utilizes ribose and glucose, it produces 3 moles of lactic acid and 1 mole of acetic acid, limits growth to the presence of the hexose and continues to metabolize the pentose when present alone (Westby and others, 2008).

L. plantarum shows lower sugar consumption in cucumber juice containing glucose and fructose as compared to MRS broth containing mainly glucose. Even though, total hexose consumption by *L. plantarum* was low in cucumber juice; the bacterium growth was higher in MRS, as evidenced by colony forming units (Plumed-Ferrer and others, 2008). Such observation suggested a sugar independent growth efficiency in cucumber juice medium, presumably due to the metabolism of malic acid via the malolactic enzyme. In contrast, proteins involved in nucleotide metabolism, were minimally expressed in liquid feed as compared to MRS broth or cucumber juice, suggesting the presence of intrinsic sources for DNA and RNA building blocks in the feed (Plumed-Ferrer and others, 2008). A homolactic acid fermentation occurred in cucumber juice as evidenced by the production of 2 moles of lactic acid from each mole of glucose.

Even though, a *L. plantarum* strain was found able to ferment pentoses, no growth was detected in culture media supplemented with arabinose, xylose, and ribose (Westby and others, 2008; Hammes and Hertel, 2015). It was speculated that other carbohydrates may have been used under the conditions of the study. Hexoses are known to serve for the biosynthesis of peptidoglycan and other building blocks and fructose is required as an electron acceptor rather than as a substrate

for fermentation in *L. plantarum* (Westby and others, 2008; Kandler, 1983; Hammes and Hertel, 2015).

A sugar utilization profile of 120 lactobacilli strains of six species (*L. plantarum, L. brevis, L. buchneri, L. fermentum, L. casei, and L. fructovirons*) under different preservation conditions of acidic pH, the presence of weak acids, and high salt was developed by Sanders and others (2015). *L. plantarum* strains were chosen for the study given the availability of genome sequences and phenotypic trees. All strains of *L. plantarum*, *L. brevis* and *L. buchneri* were inoculated in MRS medium (De Man and others, 1960) supplemented with 2% glucose and other carbon sources (>99% purity) such as D-xylose, amygdalin, glycerol, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, D-melibiose, dulcitol, D-melezitose, D-raffinose, D-lactose, L-rhamnose, starch, D-sorbitol, D-turanose, L-arabinose, potassium gluconate and D-trehalose and incubated at 30 ^{0}C for over 21 days (Sanders and others, 2015). All selected lactobacilli strains were able to grow on D-melezitose, starch, glucose, and D-melibiose and almost all strain were able to utilize amygdalin (118/120), D-raffinose (119/120), D-sorbitol (114/120), methyl-α-D-glucopyranoside (119/120), methyl-α-D-mannopyranoside (117/120), L-arabinose (113/120) (Sanders and others, 2015). Specifically, most of the *L. plantarum, L. brevis*, and *L. buchneri* strains showed an ability to utilize D-xylose and most strains of *L. casei, L. plantarum*, and *L. brevis* were able to grow on D-trehalose. However, none of bacteria tested grew on glycerol (Sanders and others, 2015).

In an attempt to phenotypically and genotypically characterized *Lactobacillus plantarum* strains isolated from various habitat, Siezen and van Hylckama Vlieg (2011) found substantial variability in genomic islands involved in sugar utilization, plantaricin and exopolysaccharide biosynthesis, prophage components and ability to utilize carbohydrates such as D-arabinose, ribitol, ferric citrate, starch glycerol, glycogen, inulin, lyxose, tagatose, L-fucose, arabitol, xylitol,

levan and β-cyclodextrin.

Sugars transport/uptake by lactic acid bacteria:

Obviously, the first step in carbohydrates utilization is the translocation of the molecule into a cell. LAB mostly depend on the ATP-binding cassette transporters to translocate sugars and solutes and excrete toxic products such as xenobiotics and drugs (Poolman, 2002). Secondary transporters, including symporters, uniporters and antiporters are also present in LAB and operate on the free energy difference stored in the electrochemical gradient of the solutes (Poolman, 2002). Additionally, LAB use substrate transport by group translocation via the phosphoenol-pyruvate phosphotransferase systems (PEP-PTSs) (Poolman, 2002).

PTS consists of generic cytoplasmic components (enzyme I (EI) and histidine-phosphorylate protein (HPr), and sugar specific membrane-associated multi-subunits enzymes, Enzyme II (IIA, IIB, IIC and sometimes IID) (Reizer and Saier, 1997). The PTS phosphorylates sugars during transport. A phosphoryl group is transferred from phosphoenol pyruvate (PEP) to enzyme I, HPr and EIIA to EIIB. The enzyme subunit IIB transfers the phosphoryl group to the sugars via the carbohydrate-specific enzyme subunit EIIC, as they are translocated across the membrane and prior to catabolism (Poolman, 2002; Saier, 2000; Postma and others, 1993). The PTSs are an essential component of hierarchical control and autoregulation of carbohydrate utilization by sensing the extracellular sugar concentration and synchronizing the uptake with the ability of the cells to catabolize it (Poolman, 2002). Given that EII systems can be promiscuous to some degree, it is imperative to study PTS transcript expression profiles to define specificity of each transporter rather than using gene annotation based on homology and/or comparative bioinformatics (Francl and others, 2010; Siezen and van Hylckama Vlieg, 2011). The promiscuity of the PTSs in LAB, in particular, *Lc. lactis*, is evidenced by the ability of the chromosomally

encoded cellobiose specific PTS ability to translocate lactose. Both disaccharides consist of at least one glucose molecule joined to a second monomer via a β-glucosidic bond. The cellobiose-specific-PTS in *Lc. lactis* is regulated by its substrate and/or the general catabolite repressor, CcpA (Aleksandrzak-Piekarczyk and others, 2011). While *Lc. lactis* strains are able to utilize the disaccharide cellobiose; the ability to metabolized β-gentiobiose is rare. Lactose assimilation, which is a requirement of PTS system in *Lc. lactis*, can occur when cellobiose is present (Aleksandrzak-Piekarczyk and others, 2011).

Lactobacilli are lactic acid bacteria known to utilize a variety of sugars via the phosphotransferase system (PTS) (Lorca and others, 2007), which are sugar-uptake systems unique to bacteria (Saier, 2000). The *Lactobacillus plantarum* genome encodes for 25 PTS, which correspond to a broad sugar utilization profile (Kleerebezem and others, 2003). Carbohydrate utilization profiles of lactobacilli are significantly defined by the sugar content in their particular habitat. The *Lactobacillus plantarum* sugar metabolism related genes are encoded in 'lifestyle' adaptation regions located near the genomes origin of replication with a lower GC content (Molenaar and others, 2005; Siezen and van Hylckama Vlieg, 2011). The significant variability of the genomic lifestyle adaptation region confirms the ability of *L. plantarum* to proliferate in diverse environments (Siezen and van Hylckama Vlieg, 2011). While a number of lactobacilli, including *Lactobacillus fermentum, Lactobacillus casei, L. plantarum, Lactobacillus fructivorans, L. brevis* and *L. buchneri*, are capable of using glucose, their growth under stress conditions in the presence of a variety of sugars is strain specific with no unique phenotypes associated at the species level (Sanders and others, 2015). Variability in carbohydrate utilization ability is influenced by the loss of plasmids encoded traits and gene acquisition by horizontal gene transfer (De Vos and Vaughan, 1994; Barrangou and others, 2003). However, most of the

strains belonging to the *L. casei, L. plantarum*, *L. buchneri* and *L. brevis* species are able to grow on D-trehalose and D-xylose (Sanders and others, 2015). In an attempt to classify 185 *Lactobacillus plantarum* strains, Siezen and others (2011) determined that aside from glucose, such strains could utilize D-trehalose but not xylose. Xylose utilization was observed by co-inoculating 2 or more strains in MRS supplemented with the sugar (Siezen and others, 2011). Utilization of key sugars in fermented foods such as glucose, fructose, mannitol, and lactose by *Lactobacillus pentosus LPS26,* a green olives fermentation isolate, frequently dominates the bioconversion of vegetables for the production of exopolysaccahrides (EPS) (Sanchez and others, 2006).

1.1.4 Carbohydrates Metabolism in LAB

Glycolysis is the main metabolic pathway used by lactic acid bacteria (LAB) to convert sugars into lactic acid and generate adenosine triphosphate (ATP) for growth and maintenance of homeostasis. During glycolysis one mole of hexose is converted to 2 moles of lactic acid. For LAB, efficient carbohydrate fermentation also includes substrate-level phosphorylation to produce additional ATP and regenerate the cofactor NAD(P)H/H. Selected LAB preferentially utilize the 6-phosphogluconate pathway to convert one mole of hexose to 1 mole of carbon dioxide, 1 mole of ethanol (or acetic acid), and 1 mole of lactic acid, while generating two NAD(P)H/H^+ molecules and two ATPs (Spector, 2009).

The ability of LAB such as lactobacilli, lactococci, streptococci, and enterococci to produce lactic acid from sugars has relevance in the fermentation of a variety of foods including vegetables, meats, fruits, and dairy products and feed products (Koebmann and others, 2002; Plumed-Ferrer and others, 2008). LAB degrade carbons into pyruvate via glycolysis, which is converted to 2 molecules of lactic acid by lactate dehydrogenase in homolactic fermentation. Such

metabolic conversion generates 2 molecules of ATPs from one molecule of glucose. Heterofermentative LAB convert pentoses or hexoses to a mixture of lactic acid, formic acid, acetic acid and ethanol generating up to 3 molecules of ATP (Kleerebezem and others, 2003). Facultative heterofermentative bacteria, such as *L. plantarum*, are able to convert sugars to lactic acid, formic acid, acetic acid, ethanol, acetoin, and 2,3-butanediol via pyruvate using the Embden-Meyerhoff-Parnas (EMP) for hexoses or the phosphoketolase pathway for pentoses (Kleerebezem and others, 2003). However, *L. plantarum* is known to utilize the phosphoketolase pathway for the conversion of hexoses as well (Pieterse and others, 2005).

The phosphoenol pyruvate-phosphotransferase system (PEP-PTS) is used by homofermentative LAB to utilize glucose through the EMP (Romano and others 1979). As stated above, the phosphoenolpyruvate-dependent phosphate transferase system (PEP-PTS) provides transport of carbohydrates across the majority of bacterial membranes, an energetically efficient translocation that catalyzes the phosphorylation of the substrate (Aleksandrzak-Piekarczyk and others, 2011; Poolman, 2002). A wide range of monosaccharides, disaccharides, glucosides, and polyols are transported by PEP-PTS (Aleksandrzak-Piekarczyk and others, 2011).

LAB utilizing the Emden-Meyerhoff pathway to catabolize glucose preferentially transport carbohydrates via the PTS system, however, metabolism of sugars other than glucose is subject to carbon catabolite repression (Gänzle, 2015). In LAB fermenting glucose via the phosphoketolase pathway instead of the Emden-Meyerhoff pathway, the PTSs are dysfunctional, disaccharides are preferred over glucose, acetyl-phosphate is a central metabolic branching point and fructose is reduced to mannitol (Gänzle, 2015).

1.1.5 Secondary Energy Sources for LAB in Cucumber Pickles

Malic acid has been the most commonly studied alternate energy source for microbes in

cucumber fermentations. Lactic acid bacteria, in particular, *L. plantarum* catabolizes malic acid to lactic acid and CO_2 by the malo-lactic enzyme during the first 7 days of cucumber fermentation (McFeeters and others, 1982). Production of CO_2 in cucumber fermentations is associated with bloater defect (McFeeters and others, 1982; McFeeters and others, 1984). Once 12.5 mM of CO_2 is produced from sources other than malic acid degradation (McFeeters and others, 1984), the internal tissue pressure increases producing enough force to displace the endocarp and/or seed cavity tissue towards the exocarp or skin, forming hollow cavities or gas pockets within the fruit. Malic acid is also a buffer in lactic acid fermentation, enabling increase utilization of the carbohydrates in cucumber juice fermentation (McFeeters and others, 1982). Utilization of malic acid in cucumber fermentations is defined by transport across the bacterial membrane, activity of the malolactic enzyme, the sugar usage, availability of energy, and the concentration of the organic acid.

Citrulline, trehalose, cellobiose were identified as potential energy sources during spoilage associated secondary cucumber fermentation (Johanningsmeier and McFeeters, 2015). Non-targeted metabolomics analysis of the volatile and nonvolatile compounds was applied to end of fermentation and spoiled cucumber fermentation samples identifying more than 600 metabolite peaks that changed as a function of anaerobic spoilage after 146-days (Johanningsmeier and McFeeters, 2015).

1.1.5.1 Metabolism and Chemical Characteristics of Cellobiose, Gentiobiose, and Trehalose

Cellobiose, gentiobiose and trehalose are disaccharides composed of two glucose units joined by a β-(1-4) glycosidic linkage, a β-(1-6) linkage or an α-(1-3) linkage, respectively. Gentiobiose is composed of two D-glucose units. Cellobiose is found in some food products such as vegetables, fruits, corn syrups and others (Buckenhüskes, 1997). It yields two molecules of

glucose when β-glucosidase is present in the environment or provided by LAB (Singhvi and others, 2010; Abdel-Rahman and others, 2011). Cellobiose is the product of the degradation of cellulose or related plant β-glucan. Trehalose, a 1-α-disaccharide, is produced by organism to avoid dehydration and respond to osmotic stress (Crowe and others, 2001; Gänzle and Follador, 2012; Goddijn and van Dun, 1999).

In general, disaccharides are preferably metabolized by LAB using glycolysis (Gänzle, 2015). Disaccharides metabolism is regulated via the PTS systems and/or intracellular phospho-glycosyl hydrolases (Andersen and others, 2012; Barrangou and others, 2006; Francl and others, 2010). Various β-glucosides are translocated by the PTS systems, and subsequently cleaved by P-β-glucosidase to produce glucose-6-P and the respective glycon (Bardowski and others, 1994; Bardowski and others, 1995; Schnetz and others, 1987; Tobisch and others, 1997; Aleksandrzak-Piekarczyk and others, 2011). Specific PTS permeases and the 6-phospho-β-glucosidase are regulated by cellobiose and gentiobiose in *Lactobacillus acidophilus* at the transcriptional level (Andersen and others, 2012). The cellobiose PTS permease coding genes in *L. acidophilus* is homologous to the functionally characterized system in *L. gasseri* (Andersen and others, 2012). The cellobiose and gentiobiose induced PTSs form a unique phylogenetic cluster among the *L. acidophilus* PTSs (Andersen and others, 2012). A gentiobiose specific PTS has not been functionally characterized in *L. gasseri* and/or other LAB (Andersen and others, 2012; Francl and others, 2010). A putative cellobiose PTS was found in *L. paracasei* by comparative genomics (Smokvina and others, 2013). Such gene casettes varied among *L. paracasei* strains (Smokvina and others, 2013). In *Lactococcus lactis* the cellobiose-specific PTS system is comprised of CelB, PtcB and PtcA which is CcpA-dependent and able to transport lactose (Aleksandrzak-Piekarczyk and others, 2011).

While *L. plantarum*, *L. johnsonii*, *L. casei* and *L. acidophilus* have a diversity of enzymes such as disaccharide hydrolases or disaccharide-phosphate hydrolases to breakdown disaccharides, some lactobacilli species, such as *L. brevis*, *L. reuteri* and *L. delbrueckii* have a weak enzymatic system yielding a restricted disaccharides utilization spectrum (Gänzle and Follador, 2012; Andersson and others, 2005; Barrangou and others, 2006; Francl and others, 2010). 90% of a group of 72 *L. plantarum* isolates from vegetables and fruits were able to use D-cellobiose, while less than 50% were capable of fermenting gentiobiose. Variability in the ability to utilize cellobiose and gentiobiose by 10 *L. plantarum* isolated from Thai fermented vegetables and fruits was also observed by Tanganurat and others (2009). A number of lactobacilli including *L. paracasei* subsp. *paracasei*, *L. plantarum*, *L. paraplantarum*, *L. curvatus, L. herbarum*, and *L. rhamnosus* as well as *Pediococcus pentosaceous* and *Pediococcus acidilactici* are able to ferment cellobiose producing L- and D-lactic acid as the result of homofermentation (Carr and others, 2002; Fitzsimons and others, 1999; Tamang and others, 2005; Mao and others, 2015; Soltan Dallal and others, 2017; Yu and others, 2012). However, *L. brevis*, *L. buchneri* and *L. spicheri* are unable to use such sugar (Fitzsimons and others, 1999; Yu and others, 2012; Rogosa and others, 1961). Although the machinery for the metabolism of disaccharides often enables the catabolism of tri- and tetrasaccharides, such conversions occur intracellularly and is limited by transport, which differentiates most LAB from members of the gastrointestinal tract microbiome, particularly *Bifidobacterium* spp (Gänzle and Follador, 2012).

Trehalose and gentiobiose are utilized by a number of lactocbacilli and pediococci, but not by *L. brevis*, *L. buchneri*, *L. spicheri*, some leuconostocs and *Enterococcus thailandicus* (Carr and others, 2002; Sterr and others, 2009; Mao and others, 2015; Tamang and others, 2005; Hammes and Hertel, 2015). Trehalose is produced in response to osmotic stress by organisms

across kingdoms (Crowe and others, 2001) and is metabolized by a number of lactic acid bacteria by PTSs and the intracellular phospho-glycosyl hydrolase (Gänzle and Follador, 2012; Andersson and others, 2005; Barrangou and others, 2006; Francl and others, 2010). Bacteria that are freeze-dried in the presence of trehalose recover significantly better that those treated with sucrose and retained viability even after extended exposure to high humidity (Crowe and others, 2001; Leslie and others, 1995). Given that trehalose lacks reducing ends, it is resistant to heat, extreme pH and the Maillard's reaction, it stabilizes biological structures under stress (Goddijn and van Dun, 1999). Trehalose forms a glass-like structure around biological membranes and enzymes after dehydration (Colaço, 1994). Although, some microbes such as *Escherichia coli* and *Saccharomyces cerevisiae* can synthesize trehalose, other studies recognize the ability of a wider spectrum of microbes capable of transporting and utilizing it (Romero and others, 1997). Studies of trehalose biosynthesis by transgenic plants engineered with the microbial genes revealed that limited amounts of trehalose accumulate in plants and the existence of trehalase in the same system (Goddijn and others, 1997). Trehalose is known to accumulate up to 12 % of the plant dry weight, in cryptobiotic species, and impart stress tolerance, particularly drought (Goddijn and van Dun, 1999). Although, plant systems do not seem to accumulate trehalose in significant amounts, a substantial number of plant associated lactic acid bacteria are capable of assimilating the disaccharide.

1.1.5.2 Metabolism and Chemical Characteristics of Xylose and Lyxose

Most LAB are able to breakdown pentoses to lactic acid and acetic acid (Abdel-Rahman and others, 2011; Oshiro and others, 2009). For instance, xylose and lyxose, defined as the opened chain xylose, are converted to lactic and acetic acids via the phosphoketolase (PK) pathway on a mol/mol basis (Antal and others, 1991; Tanaka and others, 2002). LAB may also utilize xylose to

lactic acid via the pentose phosphate (PP)/glycolytic pathway via pyruvate (3-carbon) (Abdel-Rahman and others, 2011). Some LAB species, such as *Lactoccocus lactis*, are able to use the PK and PP pathways to fully metabolize xylose (Okana and others, 2009). However, early studies suggested that pentose sugars are converted to lactate or acetate via 6-P-gluconate pathway by heterofermentative LAB.

Five-carbon sugars such as D-xylose breaks down to D-xylulose-5P, the intermediate of pentose fermentation, prior to the production of lactic or acetic acids (Kandler, 1983). Xylose is converted to xylulose by xylose isomerase (XylA). Xylulose is metabolized by xylulokinase (XylB) to xylulose-5-phosphate (X5P) which is then converted to glyceraldehyde-3-phosphate (GAP) and acetylphosphate (acetyl-P) by a phosphoketolase. GAP is subsequently converted to pyruvate and then lactic and acetic acids using the phosphoketolase (PK) pathway (Tanaka and others, 2002; Okana and others, 2009). A detailed examination of a metabolic flux analysis showed that xylose is fermented via the PK pathway by 6 different strains of *L. brevis* and *L. plantarum* (Zhang and others, 2016). While the typical homofermentative LAB use the EMP to metabolize sugars, most heterofermentative LAB use the PK pathway to ferment carbohydrates into lactic acid, acetic acid, ethanol and/or CO_2 (Zhang and others, 2016; Gänzle, 2015).

While the typical *L. brevis* can utilize glucose, xylose and/or both as carbon sources to produce lactic acid via the PK pathway and some cases through the EMP pathway, selected *L. plantarum* strains cannot ferment xylose alone (Zhang and others, 2016; Yu and others, 2011). Xylose can be also metabolized by *P. pentosaceus* and *L. pentosus*, but not by *L. buchneri* (Carr and others, 2002; Bustos and others, 2005; Bringel and others, 1996; Lokman and others, 1994; Chaillou and others, 1999). In 1991, Lokman and others described three genes involved in D-xylose catabolism in *L. pentosus* strains: D-xylose isomerase (XylA), D-xylose kinase (XylB),

and a regulatory protein (XylR). The translocation of D-xylose is facilitated by XylP and XylQ through the EII^{Man} transporter system in *L. pentosus* (Lokman and others, 1994; Chaillou and others, 1999). According to a proteomic analysis, *L. brevis* can metabolize xylose alone and/or combine with glucose through the heterofermentative pathway (Kim and others, 2009). Proteomic and transcriptomic analyses have also shown that cells supplemented with xylose alone or a combination of glucose and xylose express the *xyl* operon (Kim and others, 2009). Thus, it was concluded that *L. brevis* co-ferments xylose and glucose (Kim and others, 2009; Yu and others, 2012).

While, some *L. plantarum* strains are able to ferment pentoses, no growth was detected in culture media supplemented with arabinose, xylose, and ribose (Westby and others, 1993; Hammes and Hertel, 2015). It was speculated that carbohydrates may be co-metabolized with different purposes. Hexoses are known to participate of the biosynthesis of peptidoglycan and other building blocks and fructose is required as an electron acceptor rather than as a substrate for fermentation in *L. plantarum* (Westby and others, 1993; Kandler, 1983; Hammes and Hertel, 2015).

1.1.5.3 Metabolism and Chemical Characteristics of Furfural

Furfural is a furalaldehyde, formed from the dehydration of pentoses such as xylose in the presence of sulfuric acid at high temperatures (100 °C) (Tuteja and others, 2012; Takagaki and others, 2010; Agirrezabal-Telleria and others, 2014; Antal and others, 1991). The presence of saturating concentrations of sodium chloride in an acidic solution potentiates the production of furfural from xylose (Danon and others, 2014; Le Guenic and others, 2015). Furfural can also be converted to higher molecular weight compounds or polymers by condensation reactions (Agirrezabal-Telleria and others, 2014).

Production of furfural from pentoses in lignocellulose based-fermentations is known to impair some biotransformations (Ran and others, 2014; Pornkamol and Franzen, 2015; Unrean, 2017; Horváth and others, 2003). *Saccharomyces cerevisiae*, an industrially robust fermentative yeast, increases the flux via the pentose phosphate pathway, the tricarboxylic acid cycle and the proline and serine biosynthesis in response to furfural to satisfy the increase need for the co-factor, NAD(P)H (Pornkamol and Franzen, 2015). The fungus *Amorphotheca resinae*, was found able to degrade furfural and 5-hydroxymethylfurfural derived from lignocellulose pre-treatments, preferentially over glucose in the presence of oxygen (Ran and others, 2014). Such metabolic capability enables the utilization of *Amorphotheca resinae* as a biodetoxifier in aerobic fermentation processes. A *Saccharomyces cerevisiae* engineered strain overexpressing isocitrate dehydrogenase, showed enhanced growth and furfural degradation when exposed to furfural with enhanced ethanol production (Unrean, 2017). However, the yeast *Meyerozyma guilliermondii*, a pentose-fermenting yeast, seemed resistance to furfural in concentrations similar to that found in hemicellulosic hydrolysates (Perna and others, 2018). Furfural metabolism by bacteria is not well documented. Significant reduction in furfural concentrations were observed during long term storage of fermented cucumbers prior to spoilage characterized by lactic acid degradation which was attributed to the metabolic activity of *Lactobacillus buchneri* (Johanningsmeier and McFeeters, 2015).

1.1.5.4 Metabolism and Chemical Characteristics of Citrulline

Some LAB, particularly those isolated from wine, convert L-arginine to ornithine and ammonia via the arginine deiminase pathway (Alberto and others, 2012; Bauer and Dicks, 2004; Liu and others, 1996). Citrulline is also a by-product of arginine degradation (Arena and others, 2002). Both products of arginine degradation, ornithine and citrulline are consumed by some

lactobacilli (Arena and others, 2002). In the conversion of L-arginine and water to L-citrulline and ammonia via the arginine deiminase (*arcA*), L-citrulline is phosphorylated to form L-ornithine and carbamyl phosphate via the ornithine transcarbamylase (*arcB*). Carbamyl phosphate and ADP react to form ATP, carbon dioxide and ammonia via the carbamate kinase (*arcC*). Thus, the catabolism of arginine can be used by LAB to derive energy in the form of ATP, particularly in the absence of sugars in acidic environments (Spano and others, 2002; Liu and others, 1996; Gänzle and others, 2007; Gänzle, 2015). However, conversion of ornithine into putrescine, a biogenic amine, and citrulline and/or carbamayl-phospate to ethyl-carbamate, a carcinogen, in the presence of ethanol are two of the undesired consequences of arginine catabolism in fermented foods. The production of ammonia induces an increase in the extracellular pH (Bauer and Dicks, 2004; Gänzle, 2015; Liu and others, 1996).

Several studies focusing on wine fermentations showed that a strain of *L. buchneri* (CUC-3) can metabolize either arginine or citrulline using the ADI pathway (Liu and others, 1995, Liu and others, 1996). Some strains of *L. buchneri, L. brevis, L. hilgardii,* and *P. pentosaceus* can synthesize citrulline from the degradation of arginine in wine fermentations (Araque and others, 2011). Two strains of *L. plantarum* (N8 and N4) have been found capable to utilize both citrulline and arginine in a stressful environment such as orange juice (Arena and others, 1999). On the other hand, citrulline accumulation occurs in soy sauce fermentation during the lactic acid production stage and the alcoholic fermentation (Fang and others, 2018). Strains of *Bacillus amyloliquefaciens* are able to metabolize citrulline and ethyl carbamate in soy sauce fermentation (Zang and others, 2016; Fang and others, 2018).

While the physiological level of citrulline in peach, apple, broccoli, onion, pepper, potato, tomato and squash are minimal, cantaloupe, cucumbers and pumpkin have an average of 22.23 ±

3.69 mM. The physiological levels of citrulline in watermelons, including the Botswana and Domestic varieties (237.3 ± 59.5 mM), are on average 10-fold higher than those found in cucumbers (18.9 mM) and cantaloupe (21.6 mM) (Fish, 2012). Such high levels of L-citrulline in watermelon led to the investigation of the use of the fruit as a natural source for the amino acid, which is involved in the detoxification of catabolic ammonia, in the production of the vasodilator, nitric oxide and the precursor of arginine in the kidney of mammalians (Fish and Bruton, 2014). Thus, although citrulline is a non-proteinaceous and non-essential amino acid in mammalian systems, it has a significant physiological role. In plants, citrulline has been associated with protection against oxidative stress, particularly during periods of drought (Yokota and others, 2002). The L-citrulline content in watermelons can vary 2-fold as a function of growing conditions and can be altered by abiotic stresses (Fish and Bruton, 2014; Rimando and Perkins-Veazie, 2005). Citrulline accumulation in watermelon has also been associated with ripeness (Akashi and others, 2017). A study of fermented cucumber investigated citrulline as a preferred substrate as a residual energy source for anaerobic bacteria such as *L. buchneri* during the spoilage-associated secondary cucumber fermentation (Johanningsmeier and McFeeters, 2015).

1.1.6 Starter Cultures for Cucumber Fermentations

LAB are naturally present on the surface of cucumbers or in the industrial environment in which fermentations occur (Fleming and McFeeters, 1985). Thus, processors mostly rely on the indigenous microbiome to initiate a cucumber fermentation at the commercial scale. Only, a limited number of producers use starter cultures to improve the quality and stability of the finished products (Pérez-Díaz and others, 2014). However, studies have suggested that the physical and chemical characteristics in finished products may be positively impacted by the use of a starter culture (Daeschel and Fleming 1987). The positive effect on quality, safety, and acceptability

improvement for small-scale fermentation have been observed when starter cultures are used (Holzapfel, 2002). In their review of LAB as functional starter cultures for the food fermentation industry, Leroy and De Vuyst (2004) identified four crucial advantages in using starter cultures or co-cultures including effective food preservation and the enhancement of the organoleptic, technological, and nutritional qualities of the products (Leroy and De Vuyst, 2004). LAB are not only effective at converting sugars to lactic acid but also in the fermentation of sugar polymers and other raw plant- and animal-derived materials (De Vos and Hugenholtz 2004).

Several studies have highlighted the essential desirable and features in LAB to be used as starter cultures, which includes rapid and dominant growth, type and extent of acid production, salt tolerance, an extended growth permissible temperature range, CO_2 production, cell sedimentation, bacteriophage resistance, nutritional value, and ability to survive as concentrated cultures (Daeschel and Fleming, 1984; Daeschel and others, 1987; Ammor and Mayo 2007; Pérez-Díaz and others, 2014). *L. plantarum* is the most commonly recommended starter culture for vegetable fermentations, given its ability to meet most of the requirements listed above. Pederson and Albury (1956) determined that the facultative heterofermentative *L. plantarum* is the only LAB able to survive in cucumber fermentations until a complete sugar conversion is achieved, in a pure culture system. Observations made from controlled sliced cucumber fermentations conducted with the *L. plantarum* WSO starter culture inoculated to 10^8 CFU/g suggested an improvement in tissue firmness retention (reduction of softening) regardless of the size of the original fruit (Fleming and others, 1978). In 1973, Etchells and co-workers demonstrated that using a *P. cerevisiae* and *L. plantarum* starter culture in commercial controlled cucumber fermentations led to the reduction of CO_2 accumulation (Etchells and others, 1973). A reduction in the fermentation period was observed in the ethnic fermented vegetable products *Gundruk* (a

fermented leafy vegetable) and *Khalpi* (a fermented cucumber) prepared using mixed starter cultures, including *L. plantarum* and *P. pentosaceus* for *Gundruk,* and *L. plantarum, L. brevis,* and *Leuc. fallax* for *Khalpi* in the Himalayas (Tamang and Tamang, 2010). The antagonistic activity of *L. plantarum* and *L. pentosus* starter cultures isolated from 70 cucumber pickle and 16 olive fermentation samples, collected from the west part of Turkey, over the pathogenic bacteria, *L. monocytogenes* and *E. coli,* was associated with the ability to rapidly produce acid at the start of the fermentation (Çon and Karasu, 2009). Thus, *L. plantarum* is considered one of the most promising genera as a starter culture for vegetables fermentations.

Other LAB, such as *Lc. mesenteroides*, *P. pentosaceous* and *L. brevis* have been considered as potential starter cultures for vegetable fermentations. Singh and Ramesh (2008) proposed the use of *Leuconostoc* and *Lactobacillus* spp. as potential starter cultures given their ability to accelerate cucumber fermentations, after observing the relatively high incidence of these genera among a group of 328 LAB isolates obtained from 30 different salt fermented cucumber samples using a PCR detection method. Research has also shown that using *Lc. mesenteroides* as a starter culture in reduced salt cabbage fermentation may positively impact finished product uniformity, firmness, crunchiness, texture retention and in obtaining the desirable flavor and quality (Johanningsmeier and others, 2007). Yan and others (2008) studied the effects of the LAB starters (*L. pentosus* and *Lc. mesenteroides*) on nitrite formation in the paocai fermentation identifying a decline in the formation of nitrite. However, it has been also reported that *Lc. mesenteroides*, are not capable of producing desirable flavor and aroma properties in a fermented food (Hutkins, 2006). *L. pentosus, L. plantarum,* and *L. brevis* are known to ferment plant material (Tamminen and others, 2004).

1.1.7 Spoilage of Cucumber Fermentation

Analysis of commercial spoilage samples from NaCl fermentations and laboratory reproduced secondary fermentations with NaCl, identified two yeasts, *Pichia manshurica* and *Issatchenkia occidentalis* and three gram-positive bacteria, *Lactobacillus buchneri*, a *Clostridium* sp., *Pediococcus ethanolidurans* and *Acetobacter* spp. as potentially relevant to different stages of secondary fermentation (Franco and others, 2012; Medina and others, 2016). Parallel studies determined that spoilage associated *Lactobacillus buchneri* and *Pediococcus ethanolidurans* are able to produce lactic acid from sugars, but only *L. buchneri* produces acetic acid at the expense of lactic acid under both aerobic and anaerobic conditions regardless of the initial acidic pH of 3.2 in the medium (Johanningsmeier and McFeeter, 2013; and Franco and Perez-Diaz, 2013). The characterization of NaCl cucumber fermentation spoilage bacteria using culture-dependent and independent techniques and an enrichment step revealed that *Propionibacterium*, *Pectinatus*, *Veillonella* and *Dialister propionifacien* play a role in converting lactic acid to propionic acid at pH above 4.2 (Breidt and others, 2013; Medina and others, 2016). Gram-negative anaerobic bacteria supersede Gram-positive Firmicutes species in secondary cucumber fermentation after the pH rises from around 3.2 to pH 5, and propionic and butyric acids are produced. Although the ability of other spoilage associated bacteria, such as *Clostridium bifermentas, Enterobacter cloacae, Propionibacterium* spp. and *Pectinatus sottacetonis,* to utilize lactic acid has been observed under conditions similar to those present in commercially fermented cucumber tanks containing NaCl, such activity proceeds at pH above 4.5. (Breidt and others, 2013; Franco and Perez-Diaz, 2013). Therefore, these microbes are considered as secondary microbiota in secondary cucumber fermentations. Prevention of secondary cucumber fermentations may be achieved by

maintaining the NaCl content at or above 6% at a pH of 3.3 (Johanningsmeier and McFeeters, 2013).

Lactic acid bacteria (LAB) are naturally present in cucumbers and the pickle processing facilities. Carbohydrate utilization is the main energy generating metabolic activity for growth and maintaining homeostasis in LAB. The study evaluates the ability of a selected group of LAB, including, *Lactobacillus plantarum, L. pentosus, L. brevis, L. buchneri* and *Pediococcus pentosaceous*, to utilize the alternate energy sources in the cucumber fermentation: xylose, trehalose, cellobiose, furfural, gentiobiose, lyxose, and citrulline. We hypothesized that fermented cucumber spoilage could be prevented by removing such alternate energy sources by starter cultures of LAB prior to the end of the fermentation. This study aimed to assess the ability of candidates for starter or adjunct cultures for cucumber fermentations to use alternate energy sources.

1.2 References

Abdel-Rahman, M. A., Tashiro, Y., Zendo, T., Shibata, K., and Sonomoto, K. (2011). Isolation and characterisation of lactic acid bacterium for effective fermentation of cellobiose into optically pure homo L-(+)-lactic acid. *Applied Microbiology and Biotechnology*, *89*(4), 1039-1049.

Agirrezabal-Telleria, I., Gandarias, I., and Arias, P. L. (2014). Heterogeneous acid-catalysts for the production of furan-derived compounds (furfural and hydroxymethylfurfural) from renewable carbohydrates: A review. *Catalysis Today*, *234*, 42-58.

Akashi, K., Mifune, Y., Morita, K., Ishitsuka, S., Tsujimoto, H., and Ishihara, T. (2017). Spatial accumulation pattern of citrulline and other nutrients in immature and mature watermelon fruits. *Journal of the Science of Food and Agriculture*, *97*(2), 479-487.

Alberto, M. R., Manca de Nadra, M. C., and Arena, M. E. (2012). Influence of phenolic compounds on the growth and arginine deiminase system in a wine lactic acid bacterium. *Brazilian Journal of Microbiology*, *43*(1), 167-176.

Aleksandrzak-Piekarczyk, T., Polak, J., Jezierska, B., Renault, P., and Bardowski, J. (2011). Genetic characterization of the CcpA-dependent, cellobiose-specific PTS system comprising CelB, PtcB and PtcA that transports lactose in *Lactococcus lactis* IL1403. *International Journal of Food Microbiology*, *145*(1), 186-194.

Ammor, M. S., and Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat science*, *76*(1), 138-146.

Andersen, J. M., Barrangou, R., Hachem, M. A., Lahtinen, S. J., Goh, Y. J., Svensson, B., and Klaenhammer, T. R. (2012). Transcriptional analysis of prebiotic uptake and catabolism by *Lactobacillus acidophilus* NCFM. *PLoS One*, *7*(9), e44409.

Andersson, U., Molenaar, D., Rådström, P., and de Vos, W. M. (2005). Unity in organisation and regulation of catabolic operons in *Lactobacillus plantarum*, *Lactococcus lactis* and *Listeria monocytogenes*. *Systematic and Applied Microbiology*, *28*(3), 187-195.

Antal Jr, M. J., Leesomboon, T., Mok, W. S., and Richards, G. N. (1991). Mechanism of formation of 2-furaldehyde from D-xylose. *Carbohydrate Research*, *217*, 71-85.

Araque, I., Reguant, C., Rozès, N., and Bordons, A. (2011). Influence of wine-like conditions on arginine utilization by lactic acid bacteria. *International Microbioogyl*, *14*, 225-233.

Arena, M. E., and Manca de Nadra, M. C. (2002). Comparative survey in *Lactobacillus plantarum* of the growth and metabolism of arginine and citrulline in different media. *Journal of Agricultural and Food Chemistry*, *50*(22), 6497-6500.

Arena, M. E., Saguir, F. M., and de Nadra, M. M. (1999). Arginine dihydrolase pathway in *Lactobacillus plantarum* from orange. *International Journal of Food Microbiology*, *47*(3), 203-209.

Axelsson, L. (2004) Lactic Acid Bacteria: Classification and physiology. In: Salminen, S., Wright, A.V. and Ouwehand, A., Eds., Lactic Acid Bacteria: *Microbiological and Functional Aspects*, 3rd edition, Marcel Dekker, New York, 1-67. https://doi.org/10.1201/9780824752033.ch1

Bardowski, J., Ehrlich, S. D., and Chopin, A. (1994). BglR protein, which belongs to the BglG family of transcriptional antiterminators, is involved in beta-glucoside utilization in *Lactococcus lactis*. *Journal of Bacteriology*, *176*(18), 5681-5685.

Bardowski, J., Ehrlich, S. D., and Chopin, A. (1995). A protein, belonging to a family of RNA-binding transcriptional anti-terminators, controls beta-glucoside assimilation in *Lactococcus lactis*. *Developments in Biological Standardization*, *85*, 555.

Barrangou, R., Altermann, E., Hutkins, R., Cano, R., and Klaenhammer, T. R. (2003). Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. *Proceedings of the National Academy of Sciences*, *100*(15), 8957-8962.

Barrangou, R., Azcarate-Peril, M. A., Duong, T., Conners, S. B., Kelly, R. M., and Klaenhammer, T. R. (2006). Global analysis of carbohydrate utilization by *Lactobacillus acidophilus* using cDNA microarrays. *Proceedings of the National Academy of Sciences*, *103*(10), 3816-3821.

Bauer, R., and Dicks, L. M. (2017). Control of malolactic fermentation in wine. A review. *South African Journal of Enology and Viticulture*, *25*(2), 74-88.

Breidt, F., McFeeters, R.F., Pérez-Díaz, I.M. and Lee, C. (2013) Fermented vegetables. *In Food Microbiology: Fundamentals and Frontiers*, 4th edition Doyle, M.P. and Buchanan, R.L., Washington, D.C.: ASM Press, 841-855.

Bringel, F., Curk, M. C., and Hubert, J. C. (1996). Characterization of lactobacilli by Southern-type hybridization with a *Lactobacillus plantarum* pyrDFE probe. *International Journal of Systematic and Evolutionary Microbiology*, *46*(2), 588-594.

Buckenhüskes, H.J. (1997) Fermented vegetables. *In Food Microbiology: Fundamentals and Frontiers*, 2nd edition Doyle, P.D. Beuchat, L.R. and Montville, T.J., Washington, D.C.: ASM Press, 595–609.

Bustos, G., Moldes, A. B., Cruz, J. M., and Domínguez, J. M. (2005). Influence of the

metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots hydrolyzates using *Lactobacillus pentosus*. *Biotechnology Progress*, *21*(3), 793-798.

Carr, F. J., Chill, D., and Maida, N. (2002). The lactic acid bacteria: a literature survey. *Critical Reviews in Microbiology*, *28*(4), 281-370.

Chaillou, S., Pouwels, P. H., and Postma, P. W. (1999). Transport of d-Xylose in *Lactobacillus pentosus*, *Lactobacillus casei*, and *Lactobacillus plantarum*: Evidence for a mechanism of facilitated diffusion via the phosphoenolpyruvate: Mannose phosphotransferase System. *Journal of Bacteriology*, *181*(16), 4768-4773.

Colaço, C. A. L. S., Smith, C. J. S., Sen, S., Roser, D. H., Newman, Y., Ring, S., and Roser, B. J. (1994). Chemistry of protein stabilization by trehalose. In *ACS symposium series*, 222-240. DOI: 10.1021/bk-1994-0567.ch014.

Çon, A. H., and Karasu, N. (2009). Determination of antagonistic starter cultures for pickle and olive fermentation processes. *Czech Journal of Food Science, 27*(3), 185-193.

Crowe, J. H., Crowe, L. M., Oliver, A. E., Tsvetkova, N., Wolkers, W., and Tablin, F. (2001). The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. *Cryobiology*, *43*(2), 89-105.

Daeschel, M. A., Andersson, R. E., and Fleming, H. P. (1987). Microbial ecology of fermenting plant materials. *FEMS Microbiology Reviews*, *3*(3), 357-367.

Daeschel, M. A., and Fleming, H. P. (1984). Selection of lactic acid bacteria for use in vegetable fermentations. *Food Microbiology*, *1*(4), 303-313.

Daeschel, M. A., and Fleming, H. P. (1987). Achieving pure culture cucumber fermentations: a review. *Society for Industrial Microbiology*, *28*(2), 141-148.

Danon, B., Marcotullio, G., and de Jong, W. (2014). Mechanistic and kinetic aspects of pentose dehydration towards furfural in aqueous media employing homogeneous catalysis. *Green Chemistry*, *16*(1), 39-54.

De Man, J. C., Rogosa, D., and Sharpe, M. E. (1960). A medium for the cultivation of *lactobacilli*. *Journal of Applied Bacteriology*, *23*(1), 130-135.

De Vos W.M., and Vaughan, E.E. (1994). Genetics of lactose utilization in lactic acid bacteria. *FEMS Microbiology Reviews*, *15*(2-3), 217-237.

De Vos, W. M., and Hugenholtz, J. (2004). Engineering metabolic highways in *Lactococci* and other lactic acid bacteria. *Trends in Biotechnology*, *22*(2), 72-79.

Elli, M., Zink, R., Rytz, A., Reniero, R., and Morelli, L. (2000). Iron requirement of *Lactobacillus spp*. in completely chemically defined growth media. *Journal of Applied Microbiology*, *88*(4), 695-703.

Etchells, J. L., and Bell, T. A. (1950a). Film yeasts on commercial cucumber brines. *Food Technology*, *4*(3), 77-83.

Etchells, J. L., and Bell, T. A. (1950b). Classification of yeasts from the fermentation of commercially brined cucumbers. *Farlowia*, *4*(1), 87-112.

Etchells, J. L., Borg, A. F., and Bell, T. A. (1968). Bloater formation by gas-forming lactic acid bacteria in cucumber fermentations. *Applied Microbiology*, *16*(7), 1029-1035.

Etchells, J. L., Bell, T. A., Fleming, H. P., Kelling, R. E., and Thompson, R. L. (1973). Suggested procedure for the controlled fermentation of commercially brined pickling cucumbers—the use of starter cultures and reduction of carbon dioxide accumulation. *Pickle Pak Science*, *3*(4).

Fang, F., Zhang, J., Zhou, J., Zhou, Z., Li, T., Lu, L., Zeng, W., Du, G. and Chen, J. (2018). Accumulation of citrulline by microbial arginine metabolism during alcoholic fermentation of soy sauce. *Journal of Agricultural and Food Chemistry*, *66*(9), 2108-2113.

Fish, W. W. (2012). A reliable methodology for quantitative extraction of fruit and vegetable physiological amino acids and their subsequent analysis with commonly available HPLC systems. *Food and Nutrition Sciences*, *3*(06), 863.

Fish, W. W., and Bruton, B. D. (2014). The expression of citrulline and other members of the arginine metabolic family in developing watermelon fruit. *International Journal of Agriculture Innovations and Research*, *2*(5), 2319-1473.

Fitzsimons, N. A., Cogan, T. M., Condon, S., and Beresford, T. (1999). Phenotypic and genotypic characterization of non-starter lactic acid bacteria in mature cheddar cheese. *Applied and Environmental Microbiology*, *65*(8), 3418-3426.

Fleming, H. P. (1984). Developments in cucumber fermentation. *Journal of Chemical Technology and Biotechnology. Biotechnology*, *34*(4), 241-252.

Fleming, H. P., and McFeeters, R. F. (1985). Residual sugars and fermentation products in raw and finished commercial sauerkraut. *New York State Agricultural Experiment Station Special Report*, *56*, 25-29.

Fleming, H. P., and Pharr, D. M. (1980). Mechanism for bloater formation in brined cucumbers. *Journal of Food Science*, *45*(6), 1595-1600.

Fleming, H. P., Thompson, R. L., Bell, T. A., and Hontz, L. H. (1978). Controlled fermentation of sliced cucumbers. *Journal of Food Science*, *43*(3), 888-891.

Francl, A. L., Thongaram, T., and Miller, M. J. (2010). The PTS transporters of *Lactobacillus gasseri* ATCC 33323. *BMC Microbiology*, *10*(1), 77.

Franco, W., Pérez-Díaz, I. M., Johanningsmeier, S. D., and McFeeters, R. F. (2012). Characteristics of spoilage-associated secondary cucumber fermentation. *Applied and Environmental Microbiology*, *78*(4), 1273-1284.

Franco, W., and Pérez-Díaz, I. M. (2013). Microbial interactions associated with secondary cucumber fermentation. *Journal of Applied Microbiology*, *114*(1), 161-172.

Franco, W., and Pérez-Díaz, I. M. (2012). Role of selected oxidative yeasts and bacteria in cucumber secondary fermentation associated with spoilage of the fermented fruit. *Food Microbiology*, *32*(2), 338-344.

Gänzle, M. G. (2015). Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Current Opinion in Food Science*, *2*, 106-117.

Gänzle, M., and Follador, R. (2012). Metabolism of oligosaccharides and starch in *lactobacilli*: a review. *Frontiers in Microbiology*, *3*, 340.

Gänzle, M. G., Vermeulen, N., and Vogel, R. F. (2007). Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. *Food Microbiology*, *24*(2), 128-138.

Goddijn, O. J., and van Dun, K. (1999). Trehalose metabolism in plants. *Trends in Plant Science*, *4*(8), 315-319.

Goddijn, O.J., Verwoerd, T.C., Voogd, E., Krutwagen, R.W., De Graff, P.T.H.M., Poels, J., van Dun, K., Ponstein, A.S., Damm, B. and Pen, J. (1997). Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. *Plant Physiology*, *113*(1), 181-190.

Hammes, W. P., and Hertel, C. (2015). Lactobacillus. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1-76.

Handley, L. W., Pharr, D. M., and McFeeters, R. F. (1983). Relationship between galactinol synthase activity and sugar composition of leaves and seeds. *Journal of the American Society for Horticultural Science*, *108*, 600-605.

Holzapfel, W. H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology*, *75*(3), 197-212.

Horváth, I. S., Franzén, C. J., Taherzadeh, M. J., Niklasson, C., and Lidén, G. (2003). Effects of furfural on the respiratory metabolism of *Saccharomyces cerevisiae* in glucose-limited chemostats. *Applied and Environmental Microbiology*, *69*(7), 4076-4086.

Hutkins, R.W. (2006). Microbiology and Technology of Fermented Foods. Blackwell Publishing, Ames, IA: IFT Press, 1-473.

Johanningsmeier, S., McFeeters, R. F., Fleming, H. P., and Thompson, R. L. (2007). Effects of *Leuconostoc mesenteroides* starter culture on fermentation of cabbage with reduced salt concentrations. *Journal of Food Science*, *72*(5), M166-M172.

Johanningsmeier, S. D., and McFeeters, R. F. (2015). Metabolic footprinting of *Lactobacillus buchneri* strain LA1147 during anaerobic spoilage of fermented cucumbers. *International Journal of Food Microbiology*, *215*, 40-48.

Johanningsmeier, S. D., and McFeeters, R. F. (2013). Metabolism of lactic acid in fermented cucumbers by *Lactobacillus buchneri* and related species, potential spoilage organisms in reduced salt fermentations. *Food Microbiology*, *35*(2), 129-135.

Johanningsmeier, S. D., and McFeeters, R. F. (2011). Detection of Volatile Spoilage Metabolites in Fermented Cucumbers Using Nontargeted, Comprehensive 2-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry (GC× GC-TOFMS). *Journal of Food Science*, *76*(1), C168-C177.

Jones, I. D., and Etchells, J. L. (1943). Physical and chemical changes in cucumber fermentation. *Food Industries*, *15*, 62-64.

Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek*, *49*(3), 209-224.

Khalid, K. (2011). An overview of lactic acid bacteria. *International Journal of Biosciences*, *1*(3), 1-13.

Kim, J. H., Shoemaker, S. P., and Mills, D. A. (2009). Relaxed control of sugar utilization in *Lactobacillus brevis*. *Microbiology*, *155*(4), 1351-1359.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W. and Stiekema, W. (2003). Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Sciences*, *100*(4), 1990-1995.

Koebmann, B. J., Andersen, H. W., Solem, C., and Jensen, P. R. (2002). Experimental determination of control of glycolysis in *Lactococcus lactis*. In *Lactic Acid Bacteria: Genetics, Metabolism and Applications*, Springer, Dordrecht, 237-248.

Le Guenic, S., Delbecq, F., Ceballos, C., and Len, C. (2015). Microwave-assisted dehydration of D-xylose into furfural by diluted inexpensive inorganic salts solution in a biphasic system. *Journal of Molecular Catalysis A: Chemical*, *410*, 1-7.

Ledesma, O. V., De Ruiz Holgado, A. P., Oliver, G., Giori, G. S. D., Raibaud, P., and Galpin, J. V. (1977). A synthetic medium for comparative nutritional studies of lactobacilli. *Journal of Applied Bacteriology*, *42*(1), 123-133.

Leroy, F., and De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology*, *15*(2), 67-78.

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., and Crowe, L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology*, *61*(10), 3592-3597.

Liu, S. Q., Davis, C. R., and Brooks, J. D. (1995). Growth and metabolism of selected lactic acid bacteria in synthetic wine. *American Journal of Enology and Viticulture*, *46*(2), 166-174.

Liu, S. Q., Pritchard, G. G., Hardman, M. J., and Pilone, G. J. (1996). Arginine catabolism in wine lactic acid bacteria: is it via the arginine deiminase pathway or the arginase-urease pathway?. *Journal of Applied Bacteriology*, *81*(5), 486-492.

Lokman, B. C., Leer, R. J., van Sorge, R., and Pouwels, P. H. (1994). Promotor analysis and transcriptional regulation of *Lactobacillus pentosus* genes involved in xylose catabolism. *Molecular and General Genetics MGG*, *245*(1), 117-125.

Lorca, G.L., Barabote, R.D., Zlotopolski, V., Tran, C., Winnen, B., Hvorup, R.N., Stonestrom, A.J., Nguyen, E., Huang, L.W., Kim, D.S. and Saier Jr, M.H., (2007). Transport capabilities of eleven gram-positive bacteria: comparative genomic analyses. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1768*(6), 1342-1366.

Lu, Z., Fleming, H. P., and McFeeters, R. F. (2002). Effects of fruit size on fresh cucumber composition and the chemical and physical consequences of fermentation. *Journal of Food Science*, *67*(8), 2934-2939.

McLeod, R. A., and Snell, E. E. (1947). Some mineral requirements of the lactic acid bacteria. *Journal of Biological Chemistry*, *170*(1), 351-365.

Mao, Y., Chen, M., & Horvath, P. (2015*). Lactobacillus herbarum* sp. nov., a species related to *Lactobacillus plantarum*. *International Journal of Systematic and Evolutionary Microbiology*, *65*(12), 4682-4688.

McFeeters, R. F., Fleming, H. P., and Daeschel, M. A. (1984). Malic acid degradation and brined cucumber bloating. *Journal of Food Science*, *49*(4), 999-1002.

McFeeters, R. F., Fleming, H. P., and Thompson, R. L. (1982). Malic acid as a source of carbon dioxide in cucumber juice fermentations. *Journal of Food Science*, *47*(6), 1862-1865.

McFeeters, R. F. (1992). Cell wall monosaccharide changes during softening of brined cucumber mesocarp tissue. *Journal of Food Science*, *57*(4), 937-940.

McFeeters, R. F. (1993). Single-injection HPLC analysis of acids, sugars, and alcohols in cucumber fermentations. *Journal of Agricultural and Food Chemistry*, *41*(9), 1439-1443.

McFeeters, R. F., and Barish, A. O. (2003). Sulfite analysis of fruits and vegetables by high-performance liquid chromatography (HPLC) with ultraviolet spectrophotometric detection. *Journal of Agricultural and Food Chemistry*, *51*(6), 1513-1517.

Medina, E., Pérez-Díaz, I. M., Breidt, F., Hayes, J., Franco, W., Butz, N., and Azcarate-Peril, M. A. (2016). Bacterial ecology of fermented cucumber rising pH spoilage as determined by nonculture-based methods. *Journal of Food Science*, *81*(1), M121-M129.

Molenaar, D., Bringel, F., Schuren, F. H., De Vos, W. M., Siezen, R. J., and Kleerebezem, M. (2005). Exploring *Lactobacillus plantarum* genome diversity by using microarrays. *Journal of Bacteriology*, *187*(17), 6119-6127.

Morishita, T., Deguchi, Y., Yajima, M., Sakurai, T., and Yura, T. (1981). Multiple nutritional requirements of *lactobacilli*: genetic lesions affecting amino acid biosynthetic pathways. *Journal of Bacteriology*, *148*(1), 64-71.

Okano, K., Yoshida, S., Yamada, R., Tanaka, T., Ogino, C., Fukuda, H., and Kondo, A. (2009). Improved production of homo-D-lactic acid via xylose fermentation by introduction of xylose assimilation genes and redirection of the phosphoketolase pathway to the pentose

phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, *75*(24), 7858-7861.

Ortakci, F., Broadbent, J. R., Oberg, C. J., and McMahon, D. J. (2015). Growth and gas production of a novel obligatory heterofermentative Cheddar cheese nonstarter *lactobacilli* species on ribose and galactose. *Journal of Dairy Science*, *98*(6), 3645-3654.

Oshiro, M., Shinto, H., Tashiro, Y., Miwa, N., Sekiguchi, T., Okamoto, M., Ishizaki, A. and Sonomoto, K. (2009). Kinetic modeling and sensitivity analysis of xylose metabolism in *Lactococcus lactis* IO-1. *Journal of Bioscience and Bioengineering*, *108*(5), 376-384.

Panagou, E. Z., and Katsaboxakis, C. Z. (2006). Effect of different brining treatments on the fermentation of cv. Conservolea green olives processed by the Spanish-method. *Food Microbiology*, *23*(2), 199-204.

Pederson, C. S., and Albury, M. N. (1956). Variations in bacterial flora of low salt cucumber brines. *Applied Microbiology*, *4*(5), 259.

Pérez-Díaz, I.M., Breidt, F., Buescher, R.W., Arroyo-Lopez, F.N., Jimenez-Diaz, R., Bautista-Gallego, J., Garrido-Fernandez, A., Yoon, S., Johanningsmeier, S.D. (2014). Fermented and acidified vegetables. *Compendium of Methods for the Microbiological Examination of Foods*, Washington, D.C: APHA press, 697-718.

Perna, M. D. S. C., Bastos, R. G., and Ceccato-Antonini, S. R. (2018). Single and combined effects of acetic acid, furfural, and sugars on the growth of the pentose-fermenting yeast *Meyerozyma guilliermondii*. *3 Biotech*, *8*(2), 119.

Pharr, D. M., Sox, H. N., Smart, E. L., Lower, R. L., and Fleming, H. P. (1977). Identification and distribution of soluble saccharides in pickling cucumber plants and their fate in fermentation. *Journal of the American Society for Horticultural Science*.

Pieterse, B., Leer, R. J., Schuren, F. H., and van der Werf, M. J. (2005). Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling. *Microbiology*, *151*(12), 3881-3894.

Plumed-Ferrer, C., Koistinen, K.M., Tolonen, T.L., Lehesranta, S.J., Kärenlampi, S.O., Mäkimattila, E., Joutsjoki, V., Virtanen, V. and Von Wright, A. (2008). Comparative study of sugar fermentation and protein expression patterns of two *Lactobacillus plantarum* strains grown in three different media. *Applied and Environmental Microbiology*, *74*(17), 5349-5358.

Poolman, B., Jensen, P.R. and Gruss, A. (2008). 9th International Symposium on Lactic Acid Bacteria. *Applied and Environmental Microbiology*, *74*(15), 77-101.

Poolman, B. (2002). Transporters and their roles in LAB cell physiology. In *Lactic Acid Bacteria: Genetics, Metabolism and Applications*, Springer, Dordrecht, 147-164.

Pornkamol, U., and Franzen, C. J. (2015). Dynamic flux balancing elucidates NAD (P) H production as limiting response to furfural inhibition in *Saccharomyces cerevisiae*. *Biotechnology Journal*, *10*(8), 1248-1258.

Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993). Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. *Microbiological Reviews*, *57*(3), 543-594.

Ran, H., Zhang, J., Gao, Q., Lin, Z., and Bao, J. (2014). Analysis of biodegradation performance of furfural and 5-hydroxymethylfurfural by *Amorphotheca resinae* ZN1. *Biotechnology for Biofuels*, *7*(1), 51.

Reizer, J., and Saier Jr, M. H. (1997). Modular multidomain phosphoryl transfer proteins of bacteria. *Current Opinion in Structural Biology*, *7*(3), 407-415.

Rimando, A. M., and Perkins-Veazie, P. M. (2005). Determination of citrulline in watermelon rind. *Journal of Chromatography A*, *1078*(1-2), 196-200.

Rogosa, M., Franklin, J. G., & Perry, K. D. (1961). Correlation of the vitamin requirements with cultural and biochemical characters of *Lactobacillus* spp. *Microbiology*, *25*(3), 473-482.

Romano, A. H., Trifone, J. D., and Brustolon, M. (1979). Distribution of the phosphoenolpyruvate: glucose phosphotransferase system in fermentative bacteria. *Journal of Bacteriology*, *139*(1), 93-97.

Romero, C., Bellés, J. M., Vayá, J. L., Serrano, R., and Culiáñez-Macià, F. A. (1997). Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta*, *201*(3), 293-297.

Saier, M. H. (2000). Families of transmembrane sugar transport proteins. *Molecular Microbiology*, *35*(4), 699-710.

Sánchez, J. I., Martínez, B., Guillén, R., Jiménez-Díaz, R., and Rodríguez, A. (2006). Culture conditions determine the balance between two different exopolysaccharides produced by *Lactobacillus pentosus* LPS26. *Applied and Environmental Microbiology*, *72*(12), 7495-7502.

Sanders, J. W., Oomes, S. J. C. M., Membré, J. M., Wegkamp, A., and Wels, M. (2015). Biodiversity of spoilage *lactobacilli*: phenotypic characterisation. *Food Microbiology*, *45*, 34-44.

Schnetz, K., Toloczyki, C., and Rak, B. (1987). Beta-glucoside (bgl) operon of *Escherichia coli* K-12: nucleotide sequence, genetic organization, and possible evolutionary relationship to regulatory components of two *Bacillus subtilis* genes. *Journal of Bacteriology*, *169*(6), 2579-2590.

Siezen, R. J., and van Hylckama Vlieg, J. E. (2011). Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. In: Microbial cell factories. *BioMed Central, 10*(1), S3.

Singh, A. K., and Ramesh, A. (2008). Succession of dominant and antagonistic lactic acid bacteria in fermented cucumber: insights from a PCR-based approach. *Food Microbiology*, *25*(2), 278-287.

Singhvi, M., Joshi, D., Adsul, M., Varma, A., and Gokhale, D. (2010). d-(−)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-2 4. *Green Chemistry*, *12*(6), 1106-1109.

Smokvina, T., Wels, M., Polka, J., Chervaux, C., Brisse, S., Boekhorst, J., van Hylckama Vlieg, J.E. and Siezen, R.J. (2013). Lactobacillus paracasei comparative genomics: towards species pan-genome definition and exploitation of diversity. *PloS One*, *8*(7), e68731.

Soltan Dallal, M.M., Zamaniahari, S., Davoodabadi, A., Hosseini, M., and Rajabi, Z. (2017). Identification and characterization of probiotic lactic acid bacteria isolated from traditional persian pickled vegetables. *GMS Hygiene and Infection Control*, *12*.

Spano, G., Beneduce, L., Tarantino, D., Giammanco, G. M., and Massa, S. (2002). Preliminary characterization of wine *lactobacilli* able to degrade arginine. *World Journal of Microbiology and Biotechnology*, *18*(9), 821-825.

Spector, M.P. (2009). Metabolism, central (intermediary). *Encyclopedia of Microbiology*. Editor(s): 3rd edition Schaechter, M., Mobile, AL: Academic Press, 242-264.

Sterr, Y., Weiss, A., and Schmidt, H. (2009). Evaluation of lactic acid bacteria for sourdough fermentation of amaranth. *International Journal of Food Microbiology*, *136*(1), 75-82.

Takagaki, A., Ohara, M., Nishimura, S., and Ebitani, K. (2010). One-pot formation of furfural from xylose via isomerization and successive dehydration reactions over heterogeneous acid and base catalysts. *Chemistry Letters*, *39*(8), 838-840.

Tamang, B., and Tamang, J. P. (2010). In situ fermentation dynamics during production of gundruk and khalpi, ethnic fermented vegetable products of the Himalayas. *Indian journal of Microbiology*, *50*(1), 93-98.

Tamang, J. P., Tamang, B., Schillinger, U., Franz, C. M., Gores, M., and Holzapfel, W. H. (2005). Identification of predominant lactic acid bacteria isolated from traditionally fermented vegetable products of the Eastern Himalayas. *International journal of food microbiology*, *105*(3), 347-356.

Tamminen, M., Joutsjoki, T., Sjöblom, M., Joutsen, M., Palva, A., Ryhänen, E. L., and Joutsjoki, V. (2004). Screening of lactic acid bacteria from fermented vegetables by carbohydrate profiling and PCR–ELISA. *Letters in Applied Microbiology*, *39*(5), 439-444.

Tanaka, K., Komiyama, A., Sonomoto, K., Ishizaki, A., Hall, S., and Stanbury, P. (2002). Two different pathways for D-xylose metabolism and the effect of xylose concentration on the yield coefficient of L-lactate in mixed-acid fermentation by the lactic acid bacterium *Lactococcus lactis* IO-1. *Applied Microbiology and Biotechnology*, *60*(1-2), 160-167.

Tanganurat, W., Quinquis, B., Leelawatcharamas, V., Bolotin, A. (2009). Genotypic and phenotypic characterization of *Lactobacillus plantarum* strains isolated from Thai fermented fruits and vegetables. *Journal Basic Microbioogyl*, 49(4), 377-85.

Tobisch, S., Glaser, P., Krüger, S., and Hecker, M. (1997). Identification and characterization of a new beta-glucoside utilization system in *Bacillus subtilis*. *Journal of Bacteriology*, *179*(2), 496-506.

Tuteja, J., Nishimura, S., and Ebitani, K. (2012). One-pot synthesis of furans from various saccharides using a combination of solid acid and base catalysts. *Bulletin of the Chemical Society of Japan*, *85*(3), 275-281.

Unrean, P. (2017). Flux control-based design of furfural-resistance strains of *Saccharomyces cerevisiae* for lignocellulosic biorefinery. *Bioprocess and Biosystems Engineering*, *40*(4), 611-623.

Westby, A., Nuraida, L., Owens, J. D., and Gibbs, P. A. (1993). Inability of *Lactobacillus plantarum* and other lactic acid bacteria to grow on D-ribose as sole source of fermentable carbohydrate. *Journal of Applied Bacteriology*, *75*(2), 168-175.

Yan, P. M., Xue, W. T., Tan, S. S., Zhang, H., and Chang, X. H. (2008). Effect of inoculating lactic acid bacteria starter cultures on the nitrite concentration of fermenting Chinese paocai. *Food Control*, *19*(1), 50-55.

Yokota, A., Kawasaki, S., Iwano, M., Nakamura, C., Miyake, C., and Akashi, K. (2002). Citrulline and DRIP-1 protein (ArgE homologue) in drought tolerance of wild watermelon. *Annals of Botany*, *89*(7), 825-832.

Yu, J., Gao, W., Qing, M., Sun, Z., Wang, W., Liu, W., Pan, L., Sun, T., Wang, H., Bai, N. and Zhang, H. (2012). Identification and characterization of lactic acid bacteria isolated from traditional pickles in Sichuan, China. *The Journal of General and Applied Microbiology*, *58*(3), 163-172.

Zhang, Y., Zeng, F., Hohn, K., and Vadlani, P. V. (2016). Metabolic flux analysis of carbon balance in *Lactobacillus* strains. *Biotechnology Progress*, *32*(6), 1397-1403.

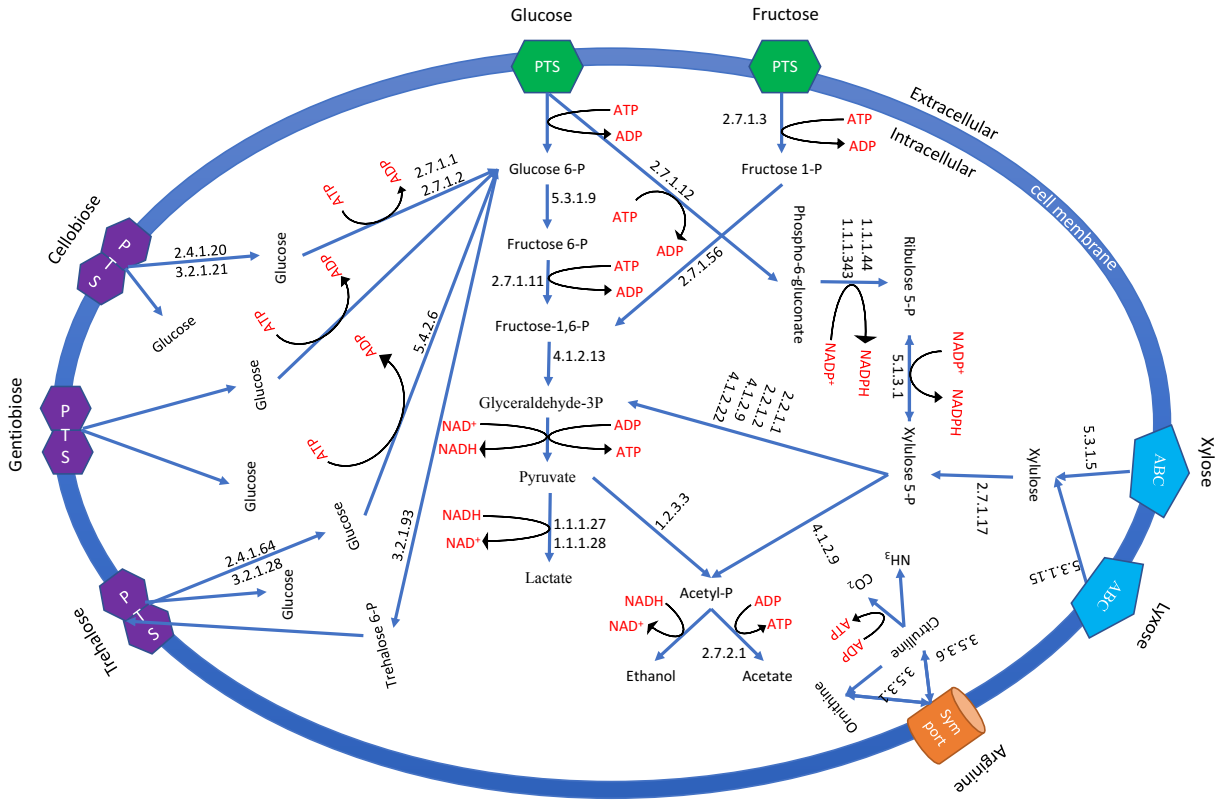

Figure 1.1 Proposed carbohydrate fermentation pathways for cellobiose, gentiobiose, trehalose, glucose, fructose, and citrulline used by selected lactic acid bacteria including *L. plantarum*, *L. pentosus*, *L. buchneri*, *L. brevis*, and *P. pentosaceus*. Drawn according to Refs. (Ganzle 2015; Plummed-Ferrer and others, 2008; Chen and others, 2016). Estimated transporters; ATP-binding cassette (ABC), phosphotransferase system (PTS), symport. EC numbers: alpha-trehalose phosphorylase [EC:2.4.1.64]; alpha-trehalase [EC:3.2.1.28]; cellobiose phosphorylase [EC:2.4.1.20]; beta-glucosidase [EC:3.2.1.21]; ketohexokinase [EC:2.7.1.3]; 1-phosphofructokinase [EC:2.7.1.56]; trehalose-6-phosphate hydrolase [EC:3.2.1.93]; glucose-6-phosphate isomerase [EC:5.3.1.9]; 6-phosphofructokinase 1 [EC:2.7.1.11]; fructose-bisphosphate aldolase, class I [EC:4.1.2.13]; L-lactate dehydrogenase [EC:1.1.1.27]; D-lactate dehydrogenase [EC:1.1.1.28]; gluconokinase [EC:2.7.1.12]; 6-phosphogluconate dehydrogenase [EC:1.1.1.44 1.1.1.343]; ribulose-phosphate 3-epimerase [EC:5.1.3.1]; transketolase [EC:2.2.1.1]; transaldolase [EC:2.2.1.2]; xylulose-5-phosphate/fructose-6-phosphate phosphoketolase [EC:4.1.2.9 4.1.2.22]; D-lyxose ketol-isomerase [EC:5.3.1.15]; xylose isomerase [EC:5.3.1.5]; xylulokinase [EC:2.7.1.17]; pyruvate oxidase [EC:1.2.3.3]; acetate kinase [EC:2.7.2.1]; beta-phosphoglucomutase [EC:5.4.2.6]; hexokinase [EC:2.7.1.1]; glucokinase [EC:2.7.1.2]; arginine deiminase [EC:3.5.3.6]; arginase [EC:3.5.3.1].

CHAPTER 2

Content of xylose, trehalose and L-citrulline in cucumber fermentations and utilization of such compounds by certain lactic acid bacteria

Redife Aslihan Ucar[1], Ilenys M. Pérez-Díaz[2], Lisa Dean[3]

[1]North Carolina State University, Department of Food, Bioprocessing, and Nutrition Sciences, Raleigh, North Carolina, USA; [2]USDA-Agriculture Research Service, Food Science Research Unit, Raleigh, North Carolina, USA; [3]USDA-Agriculture Research Service, Market Quality Research Unit, Raleigh, North Carolina, USA

2.1 Abstract

The utilization of alternate energy sources by the lactic acid bacteria (LAB) prevailing in cucumber fermentations, including *L. pentosus*, *L. plantarum* and *L. brevis*, may hamper the ability of spoilage associated microbes, such as *L. buchneri*, to derive energy for growth and/or metabolic activity after primary fermentation. In this study, the concentration of the alternate energy sources, trehalose, xylose and L-citrulline, freely available in fresh and fermented cucumbers using HPLC was evaluated. The ability of *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus buchneri* to utilize those energy sources was scrutinized using fermented cucumber juice as a model system (FCJM) to simulate conditions post-fermentation. Trehalose was detected in 2 of 3 industrial cucumber fermentation samples that were collected on day 3 of the bioconversion at 15.51 ± 1.68 mM. This dissacharide was not found in the fresh fruit or in fermented cucumbers collected on day 38 of the bioconversion. Most of the LAB tested were able to utilize trehalose in FCJM at pH 4.7, with the excepted of *Lactobacillus brevis*. Xylose was detected in one of the four fresh cucumber samples at 36.05 mM and was utilized by most of the LAB tested, exceptions *L. plantarum*. L-citrulline was detected in all of the fresh and fermented cucumber samples at 1.65 ± 0.63 mM (n=4). This plant derived amino acid was uniquely utilized by *L. buchneri* and was produced by *L. plantarum*, *L. pentosus* and *L. brevis*. L-citrulline (12.43 ± 2.3 mM) was converted to ammonia (14.54 ± 3.60 mM) and the biogenic amine ornithine (14.19 ± 1.07 mM) at pH 4.7 in the presence of 0.5 ± 0.2 mM glucose and was presumably converted to arginine in the presence of excess glucose (16.49 ± 1.06 mM). The utilization of the L-citrulline resulted in an increase in cell density of 0.5 log CFU/mL as compared to FCJM supplemented with glucose alone and a final pH that was 0.48 ± 0.02 units higher than in the absence of L-citrulline. The presence of L-citrulline in FCJM at pH 3.7 enhanced growth in the presence of excess glucose.

To better understand the interactions of the LAB tested in cucumber fermentations, we observed the changes in the biochemistry, pH and colony counts during the fermentation of fresh cucumber juice (FrCJ) medium supplemented with 18.25 ± 0.49 mM trehalose, 51.97 ± 1.30 mM xylose and 4.23 ± 0.61 mM L-citrulline and in unsupplemented FrCJ medium, that were inoculated with mixed cultures. It was found that the use of *L. pentosus*, *L. brevis* and *L. buchneri* in a mixed starter culture aids in the removal of alternate energy sources, including L-citrulline, and the generation of a stable FrCJ fermentation for 55 days under anaerobiosis. Further studies will define if the use of a tripartite starter culture in cucumber fermentation enhances microbial stability during long term bulk storage.

2.2 Introduction

The utilization of glucose and fructose by *Lactobacillus pentosus* and *Lactobacillus plantarum* was the main biochemical conversion monitored using HPLC to determine the progress of cucumber fermentation. However other compounds that could serve as energy sources for microbes have also been found in fresh and fermented cucumbers. Sucrose and xylose have been extracted from cucumber mesocarp and endocarp (Handley and others, 1983; McFeeters, 1992; Pharr and others, 1977). Rhamnose, fucose, arabinose, mannose, galactose, and galacturonic acid have been extracted from cucumber mesocarp at concentrations between 0.1 and 7 mM and found not to change as a function of tissue softening induced by heating or fermentation (McFeeters, 1992). The concentrations of L-citrulline, trehalose, cellobiose, xylose, lyxose, gentiobiose, furfural, and lactic acid were found to change in anaerobic fermented cucumber spoilage by *L. buchneri* using twodimensional time of flight mass spectrometry (TFMS) (Johanningsmeier and McFeeters, 2015). L-citrulline, D-trehalose, and D-cellobiose were utilized by *L. buchneri* prior to lactic acid degradation (Johanningsmeier and McFeeters, 2015). Cellobiose, trehalose, and gentiobiose have also been found in traditional pickles in Sichuan, China (Yu and others, 2012) and in the traditional Indian vegetable fermented products gundruk, sinki, and kalphi (Tamang and others, 2005). It has been postulated that the utilization of alternative energy sources in cucumber fermentation supports the growth and metabolic activity of spoilage associated microbes such as *L. buchneri* (Johanningsmeier and McFeeters, 2015).

The lactobacilli typically prevailing in cucumber fermentations such as *L. pentosus* and *L. plantarum* are notorious for their ability to utilize a variety of carbon sources (Lorca and others, 2007; Kleerebezem and Hugenholtz, 2003; Siezen and van Hylckama Vlieg, 2011). Lactobacilli species have been used as functional starter cultures for fermented food products such as plant-

derived foods, meats, wine, cheese, beer and sourdough. *L. plantarum* NCIMB 8026 is able to utilize ribose, xylose and L-arabinose in the presence of glucose (Westby and others, 1993). A group of 185 *Lactobacillus plantarum* strains was found to utilize D-trehalose (Siezen and others, 2010). A substantial number of *L. casei, L. plantarum*, *L. buchneri* and *L. brevis* strains are able to grow on D-trehalose and D-xylose (Sanders and others, 2015).

We hypothesize that utilization of alternative energy sources by the organisms prevailing in cucumber fermentations, including *L. pentosus*, *L. plantarum* and *L. brevis* hampers the ability of spoilage associated microbes, such as *L. buchneri*, to derive energy for growth and/or metabolic activity post-fermentation. In a previous study, we observed that while the disaccharides cellobiose and gentiobiose can be utilized by several lactic acid bacteria (LAB) in a fermented cucumber juice model system at pH 4.7, the concentration of such disaccharides that is freely available in fresh and fermented cucumbers is less than 0.01 mM (Ucar and others, 2018, *submitted*). This study evaluates the intrinsic concentration of trehalose, xylose and L-citrulline in fresh and fermented cucumbers and determines the ability of certain LAB to utilize such compounds in FCJM to simulate conditions post-primary fermentation.

Trehalose is a disaccharide composed of two glucose units joined by an α-α, (1,1) linkage produced by organisms (Crowe and others, 2001). Although, some non-lactic acid producing microbes such as *Escherichia coli* and *Saccharomyces cerevisiae* can synthesize trehalose, most of the literature records recognize the ability of a wider spectrum of microbes capable of transporting and utilizing the disaccharide (Romero and others, 1997). Trehalose is known to accumulate up to 12 % of the plant dry weight, in cryptobiotic species, and impart stress tolerance, particularly drought (Goddijn and van Dun, 1999). Bacteria that are freeze-dried in the presence of trehalose recover significantly better that those treated with sucrose and retained viability even

after extended exposure to high humidity (Crowe and others, 2001; Leslie and others, 1995). Given that trehalose lacks reducing ends, it is resistant to heat, extreme pH and the Maillard's reaction, and stabilizes biological structures under stress (Goddijn and van Dun, 1999). Trehalose forms a glass-like structure around biological membranes and enzymes after dehydration (Colaço, 1994).

Xylose, a pentose sugar, is metabolized by several LAB via the Phosphoketolase Pathway and in some cases via the Embden-Meyerhof Pathway (Chaillou and others, 1999; Carr and others, 2002; Bustos and others, 2005; Bringel and others, 1996; Lokman and others, 1994; Chaillou and others, 1999; Zhang and others, 2016; Yu and others, 2012). The pentose is transported by *L. pentosus* via a phosphotransferase system involving the enzymatic products of *xylP* and *xylQ* through the EII^{Man} transporter system (Lokman and others, 1994). Proteomic analysis of *L. brevis* suggests the metabolism of xylose alone or concomitantly with glucose proceeds heterofermentatively (Kim and others, 2009; Yu and others, 2012). *L. brevis* cells supplemented with xylose or glucose and xylose express the *xyl* operon (Kim and others, 2009). However, *L. plantarum* strains able to ferment pentoses have been found unable to grow in culture media supplemented with xylose (Westby and others, 1993; Hammes and Hertel, 2015). A group of 185 *L. plantarum* strains were found to utilize D-trehalose, but not xylose (Siezen and others, 2010). Hexoses are known to serve for biosynthesis of peptidoglycan and other building blocks and fructose is required as an electron acceptor rather than as a substrate for fermentation by *L. plantarum* (Westby and others, 1993; Kandler, 1983; Hammes and Hertel, 2015). Xylose utilization by *L. plantarum* has been observed by co-inoculating 2 or more strains in MRS supplemented with the sugar (Siezen and others, 2010). It is speculated that carbohydrates may be co-metabolize by *L. plantarum* with different purposes.

L-citrulline is a non-protein α-amino acid first isolated from watermelon in 1914 by Koga and Ohtake and identified in 1930 by Wada (Fragkos and Forbes, 2011). The physiological level of L-citrulline in cantaloupe, cucumbers and pumpkin is 22.23 ± 3.69 mM (Fish, 2012). L-citrulline is involved in the detoxification of catabolic ammonia, in the production of the vasodilator, nitric oxide, and the precursor of arginine in the kidney of mammalians (Fish, 2014). Thus, although L-citrulline is a non-proteinaceous and non-essential amino acid in mammalian systems, it has a significant physiological role. In plants, L-citrulline has been associated with protection against oxidative stress, particularly during periods of drought (Yokota and others, 2002). L-citrulline can be used as an alternate source for the generation of ATP by bacteria when fermentable carbohydrates are insufficient in the environment (Spano and others, 2002; Johanningsmeier and McFeeters, 2015; Liu and others, 1996). Some LAB produce L-citrulline from the degradation of arginine and the α-amino acid produced can be consumed by other lactobacilli (Arena and Manca de Nadra, 2002). In the conversion of L-arginine and water to L-citrulline and ammonia via arginine deiminase (*arcA*), L-citrulline is phosphorylated to form L-ornithine and carbamyl phosphate via the ornithine transcarbamylase (*arcB*). Carbamyl phosphate and ADP react to form ATP, carbon dioxide and ammonia via the carbamate kinase (*arcC*). Thus, the catabolism of arginine can be used by LAB to derive energy in the form of ATP, particularly in the absence of sugars in acidic environments (Spano and others, 2002; Liu and others, 1996; Gänzle and others, 2007; Gänzle, 2015). The conversion of ornithine into the biogenic amine putrescine and L-citrulline and/or carbamayl-phosphate to ethyl-carbamate, a carcinogen, in the presence of ethanol are two of the undesired consequences of arginine catabolism in fermented foods. However, the production of ammonia induces an increase in the extracellular pH (Bauer and Dicks, 2017; Gänzle, 2015; Liu and others, 1996).

In this study, we determined the concentrations of trehalose, xylose and L-citrulline in fresh cucumbers and cucumber fermentations using HPLC analysis. The ability of *L. plantarum*, *L. pentosus*, *L. brevis*, and *L. buchneri* to utilize trehalose, xylose and L-citrulline was also determined using FCJM to mimic conditions at the end of cucumber fermentation. Furthermore, the ability of *L. buchneri* to utilize L-citrulline in the presence and absence of glucose was conducted. To better understand the interactions of the LAB in cucumber fermentations we observed the changes in the biochemistry, pH and colony counts in co-cultures of LAB inoculated in fresh cucumber juice (FrCJ) medium and FrCJ supplemented with xylose, trehalose and L-citrulline.

2.3 Materials and Methods

Preparation of fresh and fermented cucumber samples for HPLC analysis. Samples of 4-fresh, size 2B, pickling cucumber lots to be fermented in commercial vessels were obtained from a local processor. The corresponding fermented cucumber samples were collected on days 3 and 38 of fermentation together with the fermentation cover brine in a 50:50 ratio. Fresh and fermented cucumbers were sliced using aseptic techniques and blended for 90 s at medium speed using a Waring Commercial Blender 700S (Torrington, CT, USA) equipped with sterilized glass cups. Fermented cucumbers were blended together with the fermentation cover brine in a 50:50 ratio. Cucumber slurries were homogenized using a Seward Stomacher 400 (Bohemia, NY, USA) in 6" x 4.5" filter stomacher bags for 1 min at maximum speed. One mL aliquots of the filtered homogenate were spun at 15,294 rcf for 10 min in an Eppendorf benchtop refrigerated centrifuge 5810R (Hamburg, Germany) to remove residual particulate matter. Supernatants were used for HPLC analyses conducted as described below.

Measurement of trehalose and xylose from experimental samples. Aliquots of 100 µL of fresh cucumber juice, juice extracted from cucumbers fermented in commercial vessels or from experimental media were diluted to 2 mL with water spiked with 50 µL of an internal standard of lactose (Sigma Aldrich, St. Louis, MO). All solutions were filtered through OnGuard-H cartridges (Dionex, Sunnyvale, CA), to remove free amino acids, into autosampler vials. The extracts were analyzed using a BioLC (Dionex Corporation, Sunnyvale, CA) at a controlled temperature of 25 °C. The system consisted of a gradient pump, an autosampler, and a Pulsed Amperometric Detector (PAD). The mobile phase was 50 mM sodium hydroxide (NaOH) (Thermo Fisher Scientific, Fairlawn, NJ) at an isocratic flow rate of 1.0 mL/min. The column used was a PA-1, 250 mm length and 4 mm i.d. (Dionex Corporation), fitted with a PA-1 Guard column (Dionex Corporation). The detector was programmed to run a quadruple waveform as recommended by the manufacturer. The detector sensitivity was set to 500 nCoulombs (nC). The injection volume was 10 µL. Each sugar was quantified by calculating the ratio of the unknown peak height to the internal standard peak height and comparing it with a ratio of sugar standards to the internal standard (lactose). Trehalose and xylose were purchased from Fluka Chemie (Steinheim, Germany) (Pattee and others, 2000).

Measurement of L-citrulline from experimental samples. The concentration of L-citrulline was analyzed in experimental samples using the typical procedure for free amino acids. Samples were filtered through 0.22 µ PVDF syringe filters (EMD Millipore Corp., Darmstadt, Germany). Extracts were analyzed using a Hitachi Model L-8900 Analyzer (Hitachi High Technologies, Dallas, TX, USA). The analyzer was fitted with an Ion Exchange analytical column (Hitachi # 2622SC PF, 40 mm length, 6.0 mm i.d.) connected to a guard column of the same composition. Separation of amino acids was carried out using a gradient of borate buffers (PF type, Hitachi

High Technologies, Dallas, TX) and a temperature gradient of 30 to 70 °C according to the user manual supplied with the instrument with additional changes provided by Hitachi personnel (Otaka, 2013). Post column derivatization was performed by the instrument using ninhydrin (WAKO Chemicals USA, Richmond, VA). Visible detection was used at a wavelength of 570 nm. Standard curves of L-citrulline and ornithine (Sigma Aldrich, St. Louis, MO, USA) were prepared in 0.02 N hydrochloric acid (Thermo Fisher, Fairlawn, NJ, USA) over a range of 0.3 to 30 µg/mL. Standard curves were also prepared using an amino acid standard mix (Pierce H Standard, Thermo Pierce, Rockford, IL). Amonia was determined from the mixture. The range of concentration was 0.5 to 5 µg/mL. Under these conditions, L-citrulline eluted at 30.2 min, ornithine at 97.7 min and ammonia at 87.8 min (Otaka, 2013).

Bioinformatic analysis of the genes coding for enzymes involved in trehalose, xylose and L-citrulline metabolism by certain LAB: The analysis of the enzymes involved in the metabolism of trehalose, xylose and L-citrulline was conducted using the publically available genome sequences for *L. pentosus* (3), *L. plantarum* (107), *L. brevis* (21), *L. buchneri* (6), and *P. pentosaceous* (7) using the Joint Genome Institute -Integrated Microbial Genomes platform (IMG; https://img.jgi.doe.gov/cgi-bin/m/main.cgi), the KEGG Orthology Pathways database (KO; http://www.kegg.jp/kegg/pathway.html) and the Metacyc (http://metacyc.org) and Biocyc (http://biocyc.org) online tools at the IMG platform as described by Ucar and others (*submitted*).

Preparation of fermented cucumber juice media (FCJM): FCJM was prepared as described by Ucar and others (*submitted*). Briefly, size 2B (32-38 mm in diameter) fresh whole pickling cucumbers from two different lots were secured from the local retail (Raleigh, NC) and fermented in a cover brine containing 80 mM calcium chloride ($CaCl_2$) (Brenntag, Durham, NC), 6 mM potassium sorbate (Mtisubishi International Food Ingredients, Atlanta, GA), 10.1 mM

calcium hydroxide ($Ca(OH)_2$) (Sigma-Aldrich, St. Louis, MO) and 44 mM acetic acid, added as 20% vinegar (Fleischmann Vinegar, MO, USA), to adjust the initial pH to 4.7 ± 0.1. At the end of these fermentations the pH was measured at 3.3 ± 0.1 and the media contained 0.5 ± 0.2 mM and 1.69 ± 0.6 mM glucose and fructose, respectively, as determined by HPLC conducted as described below. The cover brine and juice from this fermentation was used to prepare FCJM. The FCJM derived from each cucumber lot were independently used and supplemented with trehalose (USBiological, cat. # T8270, 99.7% purity), xylose (Sigma-Aldrich, ≥ 99% purity) or L-L-citrulline (Sigma-Aldrich, ≥ 98% purity). The pH of the supplemented and un-supplemented FCJM was adjusted to 4.7 ± 0.1 or 3.7 ± 0.1 as indicated in the text using a 5N NaOH solution (Spectrum Chemicals, NJ, USA) and 3N hydrochloric acid (HCl) (Spectrum Chemicals, NJ, USA). pH measurements were taken using an Acumet® Research 25 pH meter (Fisher Scientific, CA, USA) equipped with a Gel-Filled Pencil-Thin pH Combination Electrode (Acumet, Fisher Scientific). The pH-adjusted FCJM were filter sterilized using 0.2-µ filtration units (Nalgene®-Rapid Flow™, Thermo Scientific). 10 mL aliquots of each FCJM were aseptically transferred to 15 mL conical tubes for experimentation.

LAB cultures preparation: The bacterial cultures used for experimentation are described in Table 2.1. The LAB cultures were transferred from frozen stocks, prepared with Lactobacilli MRS broth (Benton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 15% glycerol, to 10 mL of MRS broth. The cultures were incubated at 30 °C for 48 to 72 h prior to inoculating fermentations or FCJM. The FCJM was inoculated to 10^5 CFU/mL. The optical density at 600 nm of the MRS cultures was measured and used to adjust the inoculation level. A sterile 0.85% sodium chloride (NaCl) solution was used to adjust the inocula concentration as needed. Inocula with mixed cultures were prepared by combining the cells

suspension in saline solution so that each strain will be at 10^5 CFU/mL in the experimental medium.

Analysis of the ability of certain LAB to utilize xylose, trehalose and L-citrulline in fermented cucumber juice. The fermented cucumber juice medium prepared as described above was supplemented with 18.13 ± 1.17 mM trehalose (USBiological, cat. # T8270, 99.7% purity), 18.65 ± 0.49 mM xylose (Sigma-aldrich, cas # 58-86-6, ≥99% purity) or 0.56 ± 0.02 mM L-citrulline (Sigma-aldrich, cas # 372-75-8, ≥98% purity) individually. FCJM supplemented with 14.21 ± 5.18 mM glucose (Sigma-Aldrich, cas # 50-99-7, ≥ 99,5% purity) or 20.61 ± 2.52 mM fructose (Sigma-Aldrich, cas # 57-48-7, ≥ 99% purity) were used as positive controls. The supplemented media were filter-sterilized using 0.2 µ filtration units (Nalgene®-Rapid Flow™, Thermo Scientific) after adjusting the pH of the media to 4.7 ± 0.1 (Acumet® Research 25 pH meter, Fisher Scientific, CA, USA) using 5N NaOH (Spectrum Chemicals, NJ, USA). Aliquots of 10 mL of each supplemented FCJM were transferred to 15 mL conical plastic tubes (cat. # 430766; Corning Incorporated, Corning, NY) prior to inoculation with a mixture of the three strains of *L. plantarum*, *L. pentosus*, *L. brevis*, or *L. buchneri* described in Table 2.1. The inocula were prepared as described above. All tubes were incubated at 30 °C for 7 days to enable the utilization of energy sources under conditions simulating post-fermentation. Negative controls were not inoculated. Samples were aseptically collected at the end point and serially diluted in 0.85% saline solution prior to plating which was done as described below. The concentration of xylose, trehalose, and L-citrulline remaining in the media after secondary fermentation were measured by HPLC done as described above. The concentrations of glucose, fructose, lactic acid, acetic acid and ethanol present in the FCJM after incubation for 7 days were measured using HPLC analysis as described below.

Enumeration of LAB from MRS agar plates: Spiral plating was conducted using an Autoplate 400 Eddy Jet 2 spiral plater (IUL, Barcelona, Spain) onto Lactobacilli deMan Rogosa and Sharpe (MRS) agar (Becton, Dickinson and Company; Franklin Lakes, New Jersey, USA) supplemented with 1% cycloheximide (Remel, San Diego, California, USA) for the enumeration of LAB. MRS plates were incubated aerobically at 30 °C for 48 h. Colonies were counted using a Flash & Go Automatic Colony Counter (IUL, Barcelona, Spain). The limit of detection was 4 $\log_{10}$ CFU/mL.

Measurement of glucose, fructose, lactic acid, acetic acid and ethanol concentrations in FCJM using HPLC. The concentrations of glucose, fructose, lactic acid, acetic acid and ethanol in FCJM before and after secondary fermentation were measured using HPLC analysis. 1.5 mL of each fermentation sample was spun at 15,294 x g for 15 min at room temperature using an Eppendorf Centrifuge Model 5810 (Hamburg, Germany). A minimum of 500 µL of the supernatants were transferred into glass HPLC vials. The concentrations of the organic compounds were measured using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and the HPLC method described by McFeeters (1993) with some modifications. The operating conditions of the UFLC Shimadzu HPLC system (Shimadzu Corporation, Canby, OR, USA) included a column temperature of 65 °C and a 0.01 N sulfuric acid (H_2SO_4) eluent at 0.9 mL/min. A diode array detector was set at 210 nm at a rate of 1 Hz to quantify lactic acid. An RID-10A refractive index detector connected in series with the diode array detector was used to measure acetic acid, glucose, fructose and ethanol. External standardization of the detectors was done using at least five concentrations of standard compounds.

Evaluation of the ability of *L. buchneri* to utilize L-citrulline in the presence of glucose in FCJM. Fermented cucumber juice medium prepared as described above was supplemented with 12.61 ± 0.02 mM L-citrulline, 10.60 ± 0.20 mM L-citrulline and 16.49 ± 1.06 mM glucose or

12.71 ± 0.56 mM glucose and the pH adjusted to 4.7 ± 0.1. FCJM was supplemented with 10.98 ± 4.24 mM L-citrulline, 15.24 ± 0.47 mM L-citrulline and 10.83 ± 1.46 mM glucose or 10.20 ± 1.20 mM glucose in a second batch and the pH was adjusted to pH 3.7 ± 0.1. The supplemented FCJM pH was adjusted to 4.7 ± 0.1 or 3.7 ± 0.1 as described above prior to filter sterilization using 0.2 µ filtration units (Nalgene®-Rapid Flow™, Thermo Scientific). The filter sterilized media were aliquotted into 15 mL conical tubes (cat. # 430766; Corning Incorporated, Corning, NY) for experimentation. Positive and negative controls were prepared without L-citrulline supplementation and non-inoculated, respectively. Aliquots of 15 mL of the experimental media were inoculated with *L. buchneri* (LA0030, LA1149, and LA1147) (Table 2.1). All tubes were incubated at 30 °C for 7 to 10 days, depending on the extent of growth observed. Samples for the enumeration of *L. buchneri*, pH measurement and HPLC analyses were aseptically collected and processed as described above.

Observation of the biochemical changes in fresh cucumber juice (FrCJ) medium inoculated with mixed starter cultures of LAB: FrCJ was prepared as described above for FCJM and mixed with cover brine in a 50:50 ratio by volume. The cover brine contained 80 mM $CaCl_2$ (16.87 g), 6 mM potassium sorbate (1.71g), 44 mM acetic acid, added as 20% vinegar (Fleischmann Vinegar, MO, USA), and 0.15% $Ca(OH)_2$ and water. Two different batches of fresh cucumbers were used for the preparation of the FrCJ, that were supplemented with 18.25 ± 0.49 mM trehalose (USBiological, cat. # T8270, 99.7% purity), 51.97 ± 1.30 mM xylose (Sigma-aldrich, cas # 58-86-6, ≥ 99% purity) and 4.23 ± 0.61 mM L-citrulline (Sigma-aldrich, cas # 372-75-8, ≥ 98% purity). The pH of the FrCJ media was adjusted to 5.0 ± 0.1 prior to filter sterilization using 0.2 µ filtration units (Nalgene®-Rapid Flow™, Thermo Scientific). Aliquots of 15 mL were transferred to 50 mL conical tubes (cat. # 430829; Corning Incorporated, Corning,

NY) using aseptic techniques. The experimental tubes were inoculated with *L. pentosus* (LA0445 and 1.8.9) and *L. brevis* (3.2.19), *L. brevis* (3.2.19) alone, and *L. pentosus* (LA0445 and 1.8.9), *L. brevis* (3.2.19), and *L. buchneri* (LA1149 and LA1147) altogether to the levels indicated on Tables 2.6 and 2.7. The inocula were prepared as described above (Table 2.1). The combined inoculation volumes represented less than 10% of the FCJM volume. Un-supplemented FrCJ medium was used as a negative control and inoculated with *L. pentosus* (LA0445 and 1.8.9) and *L. brevis* (3.2.19), *L. brevis* (3.2.19) alone, and *L. pentosus* (LA0445 and 1.8.9), *L. brevis* (3.2.19), and *L. buchneri* (LA1149 and LA1147) altogether (Table 2.1). The inocula were prepared as described above. Samples were collected on days 0, 3, 7, 10, 30, 36 and 60 using aseptic techniques for spiral plating, pH measurements and HPLC analyses conducted as described above.

2.4 Results

Analysis of trehalose, xylose and L-citrulline content in fresh and fermented cucumbers: Concentrations of trehalose, xylose and L- citrulline were determined in fresh cucumbers and commercial cucumber fermentations. Trehalose was only detected in two out of the three samples tested collected from commercial cucumber fermentations that were 3 days old to 15.51 ± 1.68 mM (Table 2.2). Xylose was found in 1 out of 4 fresh cucumber samples to 36.05 mM and was not detected on samples of commercial cucumber fermentations. Decreasing concentrations of L-citrulline were found in fresh cucumbers and samples of commercial cucumber fermentations collected on days 3 and 38 to 1.65 ± 0.63, 0.85 ± 0.36 and 0.65 ± 0.07 mM, respectively (Table 2.2). As expected, glucose and fructose concentrations decreased as a function of the fermentation age (Table 2.2).

Bioinformatic analysis of secondary energy sources: Evaluation of the metabolic potential of certain LAB to utilize trehalose, xylose, and L-citrulline was done using putative metabolic pathway analysis (Table 2.3). Table 2.3 shows the results generated using 98, 3, 7, 21 and 6 genome sequences corresponding to *L. plantarum*, *L. pentosus, P. pentosaceus*, *L. brevis* and *L. buchneri*. Finished genome sequences, permanent drafts and draft sequences were used for the analysis. The metabolic potential analysis suggests that trehalose, a disaccharide, could be converted to D-glucose via the trehalose phosphorylase enzyme (EC 2.4.1.64) found in more than 97% of *L. buchneri* and *L. brevis* strains and some of the *L. plantarum* and *L. pentosus* genomes (Table 2.3). However, the trehalose phosphorylase putative coding gene was missing in all of the *P. pentosaceus* genomes included in the analysis (Table 2.3). It was also found that putatives D-xylose-5-Phosphate 3-Epimerase (EC 5.1.3.1) and Xylulokinase (EC 2.7.1.17) are commonly encoded by the *L. buchneri* genomes and less typically by the *L. plantarum* genomes (Table 2.3). These enzymes are involved in the conversion of D-xylulose to D-xylulose-5-phosphate via the pentose and glucuronate interconversion pathway. The genomes of several strains of *L. pentosus*, *P. pentosaceus*, and *L. brevis* were found to frequently encode for such key putative enzymes. More than 97% and 60% of the *L. plantarum* and *L. pentosus* genome sequences, respectively, were found to encode for two key enzymes in the Pentose Phosphate Pathway, the Transketolase (EC 2.2.1.1) and Transaldolase (EC 2.2.1.2), which convert D-Ribulose-5-Phosphate to D-Glyceraldehyde-3 Phosphate to feed Glycolysis; and are absent in the *L. buchneri* genome sequences (Table 2.3). More than 97% of the *L. plantarum*, *L. pentosus*, and *P. pentosus* genome sequences encode for enzymes involved in Glycolysis, while only 97 to 5% of the *L. brevis* genome sequences encode for the proteins involved in such pathway. The *L.*

buchneri genome sequences do not encode for key enzymes involved in Glycolysis, particularly those converting D-Glyceraldehyde-3-Phosphate to smaller products.

More than 97% of the *L. plantarum*, *L. pentosus*, and *L. buchneri* genome sequences included in the analysis encode for putative genes associated with L-citrulline metabolism. L-citrulline could be converted to fumarate or arginine via L-citrulline-aspartate ligase (EC 6.3.4.5) and arginine-succinate lyase (EC 4.3.2.1) (Table 2.3). Additionally, all the *P. pentosaceus* and *L. buchneri* genomes analyzed encode for a putative arginine deiminase which interconverts L-citrulline to arginine. More than 85% of the *L. brevis* genome sequences encode for a putative arginine deiminase (Table 2.3).

Utilization of trehalose, xylose and L-citrulline by certain LAB: The LAB that are known to homoferment or heteroferment produced mostly lactic acid or a mixture of lactic acid, acetic acid and ethanol, respectively, in FCJM supplemented with glucose or fructose (Table 2.4). Ethanol was not produced by the heterofermenters, *L. brevis* and *L. buchneri*, from fructose (Table 2.4). Substantial differences were observed in the FCJM pH and LAB colony counts by the end point of the second fermentation regardless of the species tested. However, the sum of the amounts of lactic acid, acetic acid and ethanol produced substantially surpasses the theoretical amounts that were expected based on carbon balance (Table 2.4). It is speculated that although the primary fermentation removed more than 99% of the detectable glucose and fructose from the system (42.72 ± 7.4 glucose and 43.15 ± 6.5 mM fructose; Table 2.1), not all of the sugars were converted to lactic acid, acetic acid and ethanol prior to the preparation of the FCJM. An enhanced carbon balance is observed if the amounts of glucose and fructose present in the fresh cucumbers are added to those supplemented in the FCJM and total production is considered. Thus, approximately 57 mM glucose and 43 mM fructose were converted to about 182 and 191

mM lactic acid by *L. plantarum* and *L. pentosus*, respectively, in the FCJM supplemented with glucose and 43 mM glucose and 63 mM fructose were converted to 169 and 167 mM lactic acid by *L. plantarum* and *L. pentosus*, respectively, in the FCJM supplemented with fructose. A similar carbon balance is observed for the other LAB tested if acetic acid and ethanol production is also considered. About 20 to 30 mM of the hexose-derived carbon was still not accounted for, which was presumed to be assimilated by the biomass.

L. brevis (1.73±0.72 mM) and *L. buchneri* (<0.01 mM) utilized nearly all the xylose supplemented in the FCJM (18.65 ± 0.49) while *L. plantarum* and *L. pentosus* did not (Table 2.4). *L. brevis* and *L. buchneri* converted xylose to lactic acid and acetic acid but not ethanol, dropped the pH from 4.7 ± 0.1 to about 4.2 and doubled their cell densities, reaching colony counts above 8 log of CFU/mL (Table 2.4). On the contrary, no increases in cell densities were observed in FCJM inoculated with *L. plantarum* or *L. pentosus*, suggesting such cells were not energized by xylose (Table 2.4).

The opposite pattern from xylose utilization was observed for trehalose catabolism. Trehalose was converted to lactic acid by *L. plantarum* and *L. pentosus* with the consequent decrease in pH to 3.7 and an increase in cell density above 8 log CFU/mL (Table 2.4). *L. brevis* did not utilize trehalose and *L. buchneri* partially utilized trehalose (2.36 ± 1.44) converting it to lactic and acetic acids and deriving sufficient energy for growth and some acidification (Table 2.4).

L. buchneri was unique in utilizing L-citrulline and deriving energy for growth with minimal changes in pH (Table 2.4). An increase in the FCJM L-citrulline concentration was measured from samples inoculated with *L. plantarum*, *L. pentosus* and *L. brevis* with significant decreases in pH and increases in colony counts (Table 2.4). Although, *L. plantarum*, *L. pentosus*

and *L. brevis* were unable to utilize L-citrulline, they produced small of amounts of the amino acid and were able to grow in the FCJM supplemented with it (Table 2.4).

Analysis of the ability of *L. buchneri* to utilize L-citrulline in the presence of excess and limiting glucose: Utilization of L-citrulline by *L. buchneri* in FCJM with an adjusted pH of 4.7 ± 0.1 and supplemented with 12.61 ± 0.02 mM of the amino acid resulted in an increase in colony counts and pH from 4.7 ± 0.1 to 5.18 ± 0.01 and the production of acetic acid, ornithine and ammonia (Table 2.5). A slight decrease in pH was observed when L-citrulline was co-utilized with glucose by *L. buchneri* in FCJM at pH 4.7, supplemented with the amino acid and sugar. Co-metabolism of 10.44 ± 0.9 mM L-citrulline with 16.49 ± 5.3 mM glucose prevented the formation of ornithine and ammonia and resulted in the equimolar production of lactic acid (21.41 ± 9.84 mM) and acetic acid (20.72 ± 12.08 mM) (Table 2.5). Production of ammonia and ornithine was observed in the non-supplemented FCJM in which an increase in colony counts of 2 log of CFU/mL was detected (Table 2.5). Supplementation of the FCJM with 16.49 ± 1.06 mM glucose resulted in some production of acetic and lactic acids within the 10 days of incubation with minimal changes in pH and colony counts (Table 2.5).

Minimal changes in the concentration of L-citrulline, pH and ammonia production were observed after 10 days of incubation in the FCJM at pH 3.7, supplemented with L-citrulline and inoculated with *L. buchneri* (Table 2.5). However, there was a 1 log of CFU/mL increase in colony counts from MRS agar plates. Supplementation of the FCJM with glucose and L-citrulline (pH 3.7 ± 0.1) enabled a 2.5 log of CFU/mL increase in colony counts, production of lactic acid (2.55 ± 4.36), acetic acid (20.96 ± 2.32) and ammonia (3.18 ± 2.90) and an ending pH of 3.74 ± 0.14 (Table 2.5). The absence of L-citrulline in FCJM supplemented with glucose (pH 3.7 ± 0.1) resulted in the utilization of 12.71 ± 1.13 mM of the hexose, which was converted to

6.90 ± 3.70 mM lactic acid and 11.97 ± 2.04 mM acetic acid with an ending pH of 3.62 ± 0.07 after 7 days of incubation (Table 2.5). In the absence of supplements in the FCJM at pH 3.7, *L. buchneri* did not proliferate, maintaining a colony count at 5.2 ± 0.12 (Table 2.5).

Observation of the biochemical changes in fresh cucumber juice (FrCJ) medium supplemented with xylose, trehalose and L-citrulline and inoculated with mixed starter cultures of LAB: Treatments 1, 2 and 3 were inoculated with *L. pentosus* LA0445 and 1.8.9, *L. brevis* 3.2.19 and *L. buchneri* LA1147 and LA1149. The *L. pentosus* and *L. brevis* strains were inoculated to 2, 3 and 4 log CFU/mL and 3, 4 and 5 log CFU/mL, respectively, in the FrCJ medium. *L. buchneri* strains, a spoilage associated LAB for fermented cucumbers, were inoculated to 2 log CFU/mL in all treatments. No substantial differences in substrate utilization trends or fermentation end products were observed as a function of *L. pentosus* or *L. brevis* inoculation level and thus only data for treatment 1 is shown in Table 2.6. Utilization of trehalose, L-citrulline and xylose was observed in treatments 1, 2 and 3 with the disaccharide as the preferred substrate over xylose, but not L-citrulline (Table 2.6). The FrCJ medium pH decreased from 5.00 ± 0.01 to 3.40 ± 0.01 after 36 days of incubation (Table 2.6). Colony counts from MRS agar plates increased from 5.0 log CFU/mL to 8.1 ± 0.32 log CFU/mL by day 3 and were at 7.48 ± 0.47 log CFU/mL on day 10 (Table 2.6). Cell densities decreased to 3.51 ± 0.05 and 2.71 ± 0.28 log CFU/mL by days 30 and 36, respectively. However, a second increment in colony counts from MRS plates was observed on day 60 to 5.26 ± 0.01 log CFU/mL in the FrCJ (Table 2.6). The exclusion of *L. pentosus* from the inocula in treatment 4 resulted in a more complete utilization of the alternate energy sources, xylose, trehalose and L-citrulline after 10 days of incubation, an end fermentation pH of 3.5 ± 0.02, slightly higher than the standard end of fermentation pH which fluctuates between 3.3 and 3.0 and the highest production of acetic and

lactic acids at about 157 mM as compared to 117 to 125 mM in the other treatments (Table 2.6). Xylose utilization was initiated earlier in the fermentation, 27.97 ± 3.27 mM less lactic acid was produced, the amount of acetic acid formed doubled as compared to treatment 1 and a steady drop in colony counts to 5.17 ± 0.20 occurred by 60 days of incubation (Table 2.6). Exclusion of *L. buchneri* from the inocula used in treatment 1, essentially inoculation with *L. pentosus* and *L. brevis* presented trends in substrate utilization and fermentation end product formation that were similar to treatment 1, except for a reduction in acetic acid production by about 15 mM and some fluctuations in colony counts after 30 days of incubation (Table 2.6). Treatment 6 was inoculated with *L. brevis* alone to 4 log CFU/mL. *L. brevis* utilized more xylose than the corresponding amount utilized in treatment 1 but less than in treatment 4, which also contained *L. buchneri* (Table 6). L-citrulline was also removed by *L. brevis* in treatment 6 which presented a final pH of 3.54 ± 0.04 (Table 2.6).

Observation of the biochemical changes in raw fresh cucumber juice (FrCJ) medium inoculated with mixed starter cultures of LAB: As suggested by the data presented in Table 2.1, only L-citrulline was detected in the FrCJ medium utilized for this experiment (Table 2.7). Inoculation of FrCJ medium with *L. pentosus* to 2 log CFU/mL and *L. brevis* to 3 log CFU/mL (treatment 1) resulted in the presence of traces of L-citrulline (0.52 ± 0.29 mM) in the medium after 60 days of incubation, production of 65.66 ± 5.73 mM lactic acid and 33.73 ± 4.22 mM acetic acid and ending pH of 3.51 ± 0.06 with viable colony counts from MRS plates at 4.44 ± 0.40 log CFU/mL (Table 2.7). Inoculation of the FrCJ medium with *L. brevis* alone to 4 log CFU/mL (treatment 2) resulted in an incomplete fermentation with the production of 34.00 ± 17.33 mM lactic acid, 37.06 ± 1.30 mM acetic acid, an ending pH of 4.06 ± 0.18 and viable colony counts from MRS agar plates at 4.71 ± 0.02 (Table 2.7). However, use of a tripartite

starter culture of *L. pentosus* inoculated to 2 log of CFU/mL, *L. brevis* inoculated to 3 log CFU/mL and *L. buchneri* inoculated to 2 log CFU/mL (treatment 3) resulted in the complete removal of L-citrulline, mostly occurring between days 10 and 30, and the production of 58.65 ± 6.63 mM lactic acid and 36.05 ± 3.48 mM acetic acid with an end of fermentation pH around 3.64 ± 0.01 and viable colony counts at 5.02 ± 0.24 log CFU/mL (Table 2.7). No changes in lactic and acetic acids concentrations were observed in the FrCJM inoculated with the three cultures after day 10 (Table 2.7). Colony counts for treatment 3 did not increase after day 10 either (Table 2.7).

2.5 Discussion

This study investigated the utilization of alternative energy sources by the organisms prevailing in cucumber fermentations, including *L. pentosus*, *L. plantarum*, *L. brevis* and *P. pentosaceous*. It was hypothesized that utilization of alternate energy sources by the LAB prevailing in cucumber fermentation would hamper the ability of spoilage associated microbes such as *L. buchneri* to derive energy for growth and/or metabolic activity post-fermentation. The amounts of potential alternate energy sources in commercial cucumber fermentations were determined. It was found that xylose is occasionally present in fresh cucumbers but disappears from fermentations before day 3 (Table 2.1). Trehalose is often produced between day 1 and 3 of commercial cucumber fermentations, suggesting that the indigenous microbiota is responding to the osmotic stress in cover brines containing at least 5.8 % sodium chloride (NaCl) in an effort to retain viability (Crowe and others, 2001; Gänzle and Follador, 2012; Goddijn and van Dun, 1999; Romero and others, 1997). L-citrulline is present in fresh cucumbers and at the end of commercial fermentations (Table 2.1). Thus, the removal of L-citrulline becomes a target in the prevention of growth of spoilage organisms such as *L. buchneri* post-fermentation.

To define the ability of certain LAB to utilize potential alternate energy sources a FCJM was used as a model system to mimic conditions post-fermentation. The LAB tested were able to metabolize glucose and fructose in FCJM and generate an increase in cell densities of about 2.5 to 3 log CFU/mL (Table 2.4) suggesting that there are sufficient essential nutrients for growth of LAB in such medium and that the reduction in pH after a fermentation is apparently completed is the main factor stopping further proliferation and lactic acid production. The lack of carbon balance using the data collected from the fermentation of FCJM supplemented with glucose and fructose suggest an uncoupling with regards to the consumption of the sugars and its conversion to end products such as lactic acid, acetic acid and ethanol (Table 2.4). Such uncoupling highlights the need to consider a cucumber fermentation completed only after all the carbon is accounted for instead of the time when glucose and fructose are no longer detectable. The same imbalance in the carbon consumed vs. produced applied towards the end of the FCJM fermentation, which was still missing between 20 and 30 mM of the carbon consumed as hexoses (Table 2.4). Nevertheless, the fact that LAB can proliferate in FCJM if the pH is adjusted above 3.3 ± 0.1, suggest that fermented cucumbers are more microbiologically unstable than it was presumed.

As expected, trehalose was utilized in FCJM under conditions similar to those present after cucumber fermentations are completed by the LAB that prevail in commercial cucumber fermentations, *L. plantarum* and *L. pentosus* (Pérez-Díaz and others, 2016), and to a lesser extent by *L. buchneri*. Trehalose uptake by lactobacilli and pediococci is facilitated by phosphotransferases and intracellular phosphor-glycosyl hydrolases (Gänzle and Follador, 2012; Andersson and others, 2005; Francl and others, 2010; Carr and others, 2002; Sterr and others, 2009; Mao and others, 2015; Tamang and others, 2005; Hammes and Hertel, 2015). The putative

metabolic potential to utilize trehalose was identified in this study for 97% of the *L. buchneri* and *L. brevis* genome sequences included in the bioinformatic analysis and some of the *L. plantarum* and *L. pentosus* genome sequences, but not for the *P. pentosaceus* genome sequences. In line with observations made by others, *L. brevis* did not utilize trehalose in FCJM even though the putative enzymes involved in its metabolism were found in the genome sequences studied (Table 2.3) (Carr and others, 2002; Sterr and others, 2009; Mao and others, 2015; Tamang and others, 2005; Hammes and Hertel, 2015). The *L. brevis* utilized in this study were isolated from vegetable fermentations and beer and are underrepresented in the pool of genomes that are currently publically available (Table 2.3).

Utilization of the pentose, xylose, by *L. plantarum* and *L. pentosus* has been discussed in the literature given the use of such a trait in the *pentosus* strain to establish the species apart from *L. plantarum* (Fred and others, 1921). In general LAB utilize pentose sugars, including xylose, via the Pentose Phosphate / Glycolytic Pathway or the Phosphoketolase Pathway (Abdel-Rahman and others, 2011; Tanaka and others, 2002; Okano and others, 2009). In this study, the presence of putative genes coding for D-xylose-5-Phosphate 3-Epimerase and Xylulokinase was frequently detected in the *L. buchneri* genome sequences but seldom found in the *L. plantarum* genomes (Table 2.3). The genomes of several strains of *L. pentosus*, *P. pentosaceus*, and *L. brevis* also harbored such putative genes (Table 2.3). Additionally, genes putatively encoding for key enzymes in the Pentose Phosphate Pathway were found in the *L. plantarum* and *L. pentosus* genome sequences studied which convert D-Ribulose-5-Phosphate to D-Glyceraldehyde-3 Phosphate, an important Glycolysis intermediary. In this study, neither *L. plantarum* ATCC 14917, WCSF1 and 3.2.8 nor *L. pentosus* LA0445, ATCC 8041 and 1.8.9 were energized by xylose in FCJM at pH 4.7 ± 0.1 (Table 2.4). However, *L. brevis* ATCC14869, ATCC8287 and

7.2.43 converted xylose to lactic acid and acetic acid deriving energy to double (Table 2.4). It has been reported that strains of *L. brevis* and *L. plantarum* are able to ferment xylose via the Phosphoketolase Pathway (Zhang and others, 2016). Specific strains of *P. pentosaceus* and *L. pentosus* were found to metabolize xylose, but not *L. buchneri* and *L. plantarum* by another group (Carr and others, 2002; Bustos and others, 2005; Bringel and others, 1996; Lokman and others, 1994; Chaillou and others, 1999). The *L. buchneri* genome sequences were found to be severely impaired with regards to putative genes coding for enzymes involved in xylose utilization and the end of glycolysis (Table 2.3). However, *L. buchneri* ATCC4005, LA1147 and LA1149 utilized nearly all the xylose supplemented in the FCJM (18.65 ± 0.49) converting it to lactic acid and acetic acid, dropping the pH from 4.7 ± 0.1 to about 4.2 and deriving energy for growth to 8 log CFU/mL (Table 2.4). Thus, it was apparent that the ability of specific LAB strains to utilize xylose is dependent on their niche and/or culture conditions.

The ability of *L. buchneri* to utilize L-citrulline under conditions similar to those present in commercial cucumber fermentations after glucose and fructose are consumed was confirmed in this study. Genes coding for the L-citrulline-aspartate ligase and the arginine-succinate lyase were commonly found in the *L. plantarum*, *L. pentosus*, and *L. buchneri* genome sequences, but not in the *L. brevis* and *P. pentosaceous* genome sequences (Table 2.3). All the *P. pentosaceus* and *L. buchneri* genome sequences and more than 85 % of the *L. brevis* genomic sequences were unique in encoding for a putative arginine deiminase which interconverts L-citrulline to arginine (Table 2.3). Arginine deiminase is used by LAB to convert arginine into L-citrulline as an intermediate and then to ammonia, ornithine, ATP, and CO_2 (Bauer and Dicks, 2017; Liu and others, 1995, Liu and others, 1996). However, *L. buchneri* was unique in utilizing L-citrulline and deriving energy for growth with minimal changes in pH (Table 2.4). This observation is consistent with those made

by others from wine fermentations (Liu and others, 1995; Liu and others, 1996; Araque and others, 2011). An increase in the FCJM L-citrulline concentration was measured from samples inoculated with *L. plantarum*, *L. pentosus* and *L. brevis* with significant decreases in pH and increases in colony counts, suggesting the conversion of arginine naturally present in the medium to the non-proteinaceous amino acid (Table 2.4). Several studies focusing on wine fermentations showed that a strain of *L. buchneri* (CUC-3) can metabolize either arginine or L-citrulline using the ADI pathway (Liu and others, 1995, Liu and others, 1996). Some strains of *L. buchneri, L. brevis, L. hilgardii,* and *P. pentosaceus* can synthesize L-citrulline from the degradation of arginine in wine fermentations (Araque and others, 2011). Two strains of *L. plantarum* (N8 and N4) have been found capable of utilizing both L-citrulline and arginine in a stressful environment such as orange juice (Arena and others, 1999). On the other hand, L-citrulline accumulation occurs in soy sauce fermentation during the lactic acid production stage and the alcoholic fermentation (Fang and others, 2018). Strains of *Bacillus amyloliquefaciens* are able to metabolize L-citrulline and ethyl carbamate in soy sauce fermentation (Zhang and others, 2016; Fang and others, 2018).

An additional experiment was conducted to confirm the ability of *L. buchneri* to utilize L-citrulline in the presence of limiting and excess sugars at pH 4.7 and 3.7. A mixed *L. buchneri* inocula consisting of three strains, LA0030, LA1149, and LA1147, was used to inoculate FCJM supplemented with L-citrulline, glucose or a combination of the two (Table 2.5). L-citrulline was utilized in the presence of limiting glucose and converted to ammonia and ornithine inducing an increase in pH (Table 2.5). The presence of glucose enable the conversion of L-citrulline into an unidentified product, which is presumed to be arginine that had been incorporated into biomass or other metabolic activity, but not to ammonia or ornithine (Table 2.5) (Bauer and Dicks, 2017; Gänzle, 2015; Liu and others, 1996). Production of ammonia and ornithine by *L. buchneri* was

also observed in the unsupplemented FCJM suggesting that arginine had been utilized as a source of L-citrulline (Table 2.5). The presence of L-citrulline in the FCJM enhanced growth of *L. buchneri* as compared to the unsupplemented control and proliferation of the LAB at the lower pH (3.7 ± 0.1) (Table 2.5).

Table 2.6 demonstrates inoculation of a mixed starter culture of *L. brevis* and *L. buchneri* enables a more complete fermentation in FrCJ medium supplemented with xylose, trehalose and L-citrulline as compared to the used of mixed starter cultures containing *L. pentosus*. *L. brevis* was able to utilize about 50% of the xylose supplemented in FrCJ medium and a substantial portion of the trehalose and L-citrulline supplemented. Co-inoculation of *L. brevis* and *L. buchneri* in the FrCJ medium resulted in the removal of all the three alternate energy sources supplemented, the highest production of lactic and acetic acids and an ending pH higher than that observed when *L. pentosus* was inoculated. These observations suggest that the utilization of L-citrulline could have raised the pH enabling more acid production and a higher end of fermentation pH. Additionally, diversion of the sugars to acetic acid instead of lactic acid could have contributed to a higher final pH. Acetic acid has a higher dissociation constant as compared to lactic acid. Interestingly, no changes in pH were observed after the primary fermentation was completed by *L. brevis* and *L. buchneri* suggesting microbial stability for about 50 days under anaerobiosis (Table 2.6).

Utilization of a mixed starter culture of *L. pentosus*, *L. brevis* and *L. buchneri* in FrCJ medium resulted in the removal of L-citrulline immediately after the conversion of sugars to lactic acid and acetic acid, a slightly higher pH as compared to cultures inoculated with *L. brevis* and *L. pentosus* and stable colony counts from MRS agar plates (Table 2.7). The use of a mixed culture of *L. pentosus* and *L. brevis* resulted in the presence of L-citrulline in the FrCJ medium after the primary fermentation was concluded (Table 2.7). Utilization of *L. brevis* alone resulted

in the partial utilization of xylose and an incomplete fermentation. The use of a tripartite starter culture for the fermentation of cucumber is a viable strategy to prevent spoilage of fermented cucumbers during bulk storage that merits further investigation.

2.6 Conclusion

The occasional presence of trehalose and xylose in commercial cucumber fermentations does not represents a steady alternate energy source for spoilage organisms given that the sugars can be metabolized by the LAB prevailing in the system, *L. pentosus*, *L. plantarum* and *L. brevis*. The presence of L-citrulline in commercial cucumber fermentations could propel the development of spoilage during long term bulk storage given that it is not utilized by *L. pentosus*, *L. plantarum* and *L. brevis* during primary fermentation. Utilization of a combination of *L. pentosus*, *L. brevis* and *L. buchneri* as a starter culture in FrCJ medium resulted in the early removal of alternate energy sources such as xylose, trehalose and L-citrulline. Further studies are needed to determine if the application of the tripartite starter culture proposed here could complete a cucumber fermentation and generate a microbiologically stable fermented product for bulk storage.

2.7 Acknowledgments

The authors thank Ms. Sandra Parker and Ms. Janet Hayes and Mr. Robert Price with the USDA-ARS Food Science Research Unit located in Raleigh, NC for administrative and technical support, respectively. The authors also thank the Turkish Government for the fellowship support for Redife Aslihan Ucar. No conflict of interests is declared.

2.8 References

Abdel-Rahman, M. A., Tashiro, Y., Zendo, T., Shibata, K., and Sonomoto, K. (2011). Isolation and characterisation of lactic acid bacterium for effective fermentation of cellobiose into optically pure homo L-(+)-lactic acid. *Applied Microbiology and Biotechnology*, *89*(4), 1039-1049.

Andersson, U., Molenaar, D., Rådström, P., and de Vos, W. M. (2005). Unity in organisation and regulation of catabolic operons in *Lactobacillus plantarum*, *Lactococcus lactis* and *Listeria monocytogenes*. *Systematic and Applied Microbiology*, *28*(3), 187-195.

Araque, I., Reguant, C., Rozès, N., and Bordons, A. (2011). Influence of wine-like conditions on arginine utilization by lactic acid bacteria. *International Microbioogyl*, *14*, 225-233.

Arena, M. E., Saguir, F. M., and de Nadra, M. M. (1999). Arginine dihydrolase pathway in *Lactobacillus plantarum* from orange. *International Journal of Food Microbiology*, *47*(3), 203-209.

Arena, M. E., and Manca de Nadra, M. C. (2002). Comparative survey in *Lactobacillus plantarum* of the growth and metabolism of arginine and citrulline in different media. *Journal of Agricultural and Food Chemistry*, *50*(22), 6497-6500.

Bauer, R., and Dicks, L. M. (2017). Control of malolactic fermentation in wine. A review. *South African Journal of Enology and Viticulture*, *25*(2), 74-88.

Bringel, F., Curk, M. C., and Hubert, J. C. (1996). Characterization of lactobacilli by Southern-type hybridization with a *Lactobacillus plantarum* pyrDFE probe. *International Journal of Systematic and Evolutionary Microbiology*, *46*(2), 588-594.

Bustos, G., Moldes, A. B., Cruz, J. M., and Domínguez, J. M. (2005). Influence of the metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots

hydrolyzates using *Lactobacillus pentosus*. *Biotechnology Progress*, *21*(3), 793-798.

Carr, F. J., Chill, D., and Maida, N. (2002). The lactic acid bacteria: a literature survey. *Critical Reviews in Microbiology*, *28*(4), 281-370.

Chaillou, S., Pouwels, P. H., and Postma, P. W. (1999). Transport of d-Xylose in *Lactobacillus pentosus*, *Lactobacillus casei*, and *Lactobacillus plantarum*: Evidence for a Mechanism of Facilitated Diffusion via the Phosphoenolpyruvate: Mannose Phosphotransferase System. *Journal of Bacteriology*, *181*(16), 4768-4773.

Colaço, C. A. L. S., Smith, C. J. S., Sen, S., Roser, D. H., Newman, Y., Ring, S., and Roser, B. J. (1994). Chemistry of protein stabilization by trehalose. In *ACS symposium series*, 222-240. DOI: 10.1021/bk-1994-0567.ch014.

Crowe, J. H., Crowe, L. M., Oliver, A. E., Tsvetkova, N., Wolkers, W., and Tablin, F. (2001). The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. *Cryobiology*, *43*(2), 89-105.

Dunn, M. S., Shankman, S., Camien, M. N., and Block, H. (1947). The amino acid requirements of twenty-three lactic acid bacteria. *Journal of Biological Chemistry*, *168*(1), 1-22.

Fang, F., Zhang, J., Zhou, J., Zhou, Z., Li, T., Lu, L., Zeng, W., Du, G. and Chen, J. (2018). Accumulation of citrulline by microbial arginine metabolism during alcoholic fermentation of soy sauce. *Journal of Agricultural and Food Chemistry*, *66*(9), 2108-2113.

Fish, W. W. (2012). A reliable methodology for quantitative extraction of fruit and vegetable physiological amino acids and their subsequent analysis with commonly available HPLC systems. *Food and Nutrition Sciences*, *3*(06), 863.

Fish, W. W., and Bruton, B. D. (2014). The expression of citrulline and other members of the arginine metabolic family in developing watermelon fruit. *International Journal of Agriculture Innovations and Research*, *2*(5), 2319-1473.

Fleming, H. P., McFeeters, R. F., Daeschel, M. A., Humphries, E. G., and Thompson, R. L. (1988). Fermentation of cucumbers in anaerobic tanks. *Journal of Food Science*, *53*(1), 127-133.

Fragkos, K., and Forbess, A. (2011). Was citrulline first a laxative substance? The truth about modern citrulline and its isolation. *Nihon ishigaku zasshi/Journal of Japanese History of Medicine*, *57*(3), 275-292.

Francl, A. L., Thongaram, T., and Miller, M. J. (2010). The PTS transporters of *Lactobacillus gasseri* ATCC 33323. *BMC Microbiology*, *10*(1), 77.

Franco, W., Pérez-Díaz, I. M., Johanningsmeier, S. D., and McFeeters, R. F. (2012). Characteristics of spoilage-associated secondary cucumber fermentation. *Applied and Environmental Microbiology*, *78*(4), 1273-1284.

Fred, E. B., Peterson, W. H., and Anderson, J. A. (1921). The characteristics of certain pentose destroying bacteria, especially as concerns their action on arabinose and xylose. *Journal of Biological Chemistry*, *48*(2), 385-412.

Gänzle, M. G. (2015). Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Current Opinion in Food Science*, *2*, 106-117.

Gänzle, M., and Follador, R. (2012). Metabolism of oligosaccharides and starch in *lactobacilli*: a review. *Frontiers in Microbiology*, *3*, 340.

Gänzle, M. G., Vermeulen, N., and Vogel, R. F. (2007). Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. *Food Microbiology*, *24*(2), 128-138.

Goddijn, O. J., and van Dun, K. (1999). Trehalose metabolism in plants. *Trends in Plant Science*, *4*(8), 315-319.

Hammes, W. P., and Hertel, C. (2015). *Lactobacillus*. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1-76.

Handley, L. W., Pharr, D. M., and McFeeters, R. F. (1983). Relationship between galactinol synthase activity and sugar composition of leaves and seeds. *Journal of the American Society for Horticultural Science*, *108*, 600-605.

Hols, P., Slos, P., Dutot, P., Reymund, J., Chabot, P., Delplace, B., Delcour, J. and Mercenier, A. (1997). Efficient secretion of the model antigen M6-gp41E in *Lactobacillus plantarum* NCIMB 8826. *Microbiology*, *143*(8), 2733-2741.

Johanningsmeier, S. D., and McFeeters, R. F. (2015). Metabolic footprinting of *Lactobacillus buchneri* strain LA1147 during anaerobic spoilage of fermented cucumbers. *International journal of Food Microbiology*, *215*, 40-48.

Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek*, *49*(3), 209-224.

Kim, J. H., Shoemaker, S. P., and Mills, D. A. (2009). Relaxed control of sugar utilization in *Lactobacillus brevis*. *Microbiology*, *155*(4), 1351-1359.

Kleerebezem, M., and Hugenholtz, J. (2003). Metabolic pathway engineering in lactic acid bacteria. *Current Opinion in Biotechnology*, *14*(2), 232-237.

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., and Crowe, L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and environmental microbiology*, *61*(10), 3592-3597.

Liu, S. Q., Davis, C. R., and Brooks, J. D. (1995). Growth and metabolism of selected lactic acid bacteria in synthetic wine. *American Journal of Enology and Viticulture*, *46*(2), 166-174.

Liu, S. Q., Pritchard, G. G., Hardman, M. J., and Pilone, G. J. (1996). Arginine catabolism in wine lactic acid bacteria: is it via the arginine deiminase pathway or the arginase-urease pathway?. *Journal of Applied Bacteriology*, *81*(5), 486-492.

Lokman, B. C., Leer, R. J., van Sorge, R., and Pouwels, P. H. (1994). Promotor analysis and transcriptional regulation of *Lactobacillus pentosus* genes involved in xylose catabolism. *Molecular and General Genetics MGG*, *245*(1), 117-125.

Lorca, G.L., Barabote, R.D., Zlotopolski, V., Tran, C., Winnen, B., Hvorup, R.N., Stonestrom, A.J., Nguyen, E., Huang, L.W., Kim, D.S. and Saier Jr, M.H., (2007). Transport capabilities of eleven gram-positive bacteria: comparative genomic analyses. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1768*(6), 1342-1366.

Mao, Y., Chen, M., and Horvath, P. (2015*). Lactobacillus herbarum* sp. nov., a species related to *Lactobacillus plantarum*. *International Journal of Systematic and Evolutionary Microbiology*, *65*(12), 4682-4688.

McFeeters, R. F. (1992). Cell wall monosaccharide changes during softening of brined cucumber mesocarp tissue. *Journal of Food Science*, *57*(4), 937-940.

McFeeters, R. F., and Barish, A. O. (2003). Sulfite analysis of fruits and vegetables by high-performance liquid chromatography (HPLC) with ultraviolet spectrophotometric detection. *Journal of Agricultural and Food Chemistry*, *51*(6), 1513-1517.

Okano, K., Yoshida, S., Yamada, R., Tanaka, T., Ogino, C., Fukuda, H., and Kondo, A. (2009). Improved production of homo-D-lactic acid via xylose fermentation by introduction of xylose assimilation genes and redirection of the phosphoketolase pathway to the pentose

phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, *75*(24), 7858-7861.

Otaka, S. 2013. Hitachi High Technologies, Dallas, TX. Personal Communication.

Pattee, H. E., Isleib, T. G., Giesbrecht, F. G., and McFeeters, R. F. (2000). Investigations into genotypic variations of peanut carbohydrates. *Journal of Agricultural and Food Chemistry*, *48*(3), 750-756.

Pérez-Díaz, I.M., Breidt, F., Buescher, R.W., Arroyo-López, F.N., Jiménez-Dıaz, R., Fernandez, A.G., Gallego, J.B., Yoon, S.S., Johanningsmeier, S.D. (2016). Fermented and acidified vegetables. *Compendium of methods for the microbiological examination of foods,* 4th edn. Washington, D.C.: American Public Health Association, 521-32.

Pharr, D. M., Sox, H. N., Smart, E. L., Lower, R. L., and Fleming, H. P. (1977). Identification and distribution of soluble saccharides in pickling cucumber plants and their fate in fermentation. *Journal of the American Society for Horticultural Science*.

Rogosa, M., Franklin, J. G., & Perry, K. D. (1961). Correlation of the vitamin requirements with cultural and biochemical characters of *Lactobacillus* spp. *Microbiology*, *25*(3), 473-482.

Romero, C., Bellés, J. M., Vayá, J. L., Serrano, R., and Culiáñez-Macià, F. A. (1997). Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta*, *201*(3), 293-297.

Sanders, J. W., Oomes, S. J. C. M., Membré, J. M., Wegkamp, A., and Wels, M. (2015). Biodiversity of spoilage *lactobacilli*: phenotypic characterisation. *Food Microbiology*, *45*, 34-44.

Siezen, R.J., Tzeneva, V.A., Castioni, A., Wels, M., Phan, H.T., Rademaker, J.L., Starrenburg, M.J., Kleerebezem, M., Molenaar, D. and Van Hylckama Vlieg, J.E. (2010). Phenotypic and

genomic diversity of Lactobacillus plantarum strains isolated from various environmental niches. *Environmental Microbiology*, *12*(3), 758-773.

Siezen, R. J., and van Hylckama Vlieg, J. E. (2011). Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. In: Microbial cell factories. *BioMed Central, 10*(1), S3.

Spano, G., Beneduce, L., Tarantino, D., Giammanco, G. M., and Massa, S. (2002). Preliminary characterization of wine *lactobacilli* able to degrade arginine. *World Journal of Microbiology and Biotechnology*, *18*(9), 821-825.

Sterr, Y., Weiss, A., and Schmidt, H. (2009). Evaluation of lactic acid bacteria for sourdough fermentation of amaranth. *International Journal of Food Microbiology*, *136*(1), 75-82.

Tamang, J. P., Tamang, B., Schillinger, U., Franz, C. M., Gores, M., and Holzapfel, W. H. (2005). Identification of predominant lactic acid bacteria isolated from traditionally fermented vegetable products of the Eastern Himalayas. *International Journal of Food Microbiology*, *105*(3), 347-356.

Tamminen, M., Joutsjoki, T., Sjöblom, M., Joutsen, M., Palva, A., Ryhänen, E. L., and Joutsjoki, V. (2004). Screening of lactic acid bacteria from fermented vegetables by carbohydrate profiling and PCR–ELISA. *Letters in Applied Microbiology*, *39*(5), 439-444.

Tanaka, K., Komiyama, A., Sonomoto, K., Ishizaki, A., Hall, S., and Stanbury, P. (2002). Two different pathways for D-xylose metabolism and the effect of xylose concentration on the yield coefficient of L-lactate in mixed-acid fermentation by the lactic acid bacterium *Lactococcus lactis* IO-1. *Applied Microbiology and Biotechnology*, *60*(1-2), 160-167.

Westby, A., Nuraida, L., Owens, J. D., and Gibbs, P. A. (1993). Inability of *Lactobacillus plantarum* and other lactic acid bacteria to grow on D-ribose as sole source of fermentable carbohydrate. *Journal of Applied Bacteriology*, *75*(2), 168-175.

Yokota, A., Kawasaki, S., Iwano, M., Nakamura, C., Miyake, C., and Akashi, K. (2002). Citrulline and DRIP-1 protein (ArgE homologue) in drought tolerance of wild watermelon. *Annals of Botany*, *89*(7), 825-832.

Yu, J., Gao, W., Qing, M., Sun, Z., Wang, W., Liu, W., Pan, L., Sun, T., Wang, H., Bai, N. and Zhang, H. (2012). Identification and characterization of lactic acid bacteria isolated from traditional pickles in Sichuan, China. *The Journal of General and Applied Microbiology*, *58*(3), 163-172.

Zhang, Y., Zeng, F., Hohn, K., and Vadlani, P. V. (2016). Metabolic flux analysis of carbon balance in *Lactobacillus* strains. *Biotechnology Progress*, *32*(6), 1397-1403.

Table 2.1. Description of the lactic acid bacteria strains used in this study.

Genus	Species	ID Number(s)	Sources	Reference
Lactobacillus	*plantarum*	LA0070; ATCC 14917	Pickle cabbage	ATCC < PA Hansen < Roy Techn. Coll., Copenhagen < S. Orla-Jensen (*Streptobacterium plantarum*) https://www.atcc.org/products/all/14917.aspx#history
Lactobacillus	*plantarum*	LA1196; ATCC BAA-793; NCIMB 8826; WCFS1	Saliva	Hols and others, 1997
Lactobacillus	*plantarum*	3.2.8	Commercial cucumber fermentation	Pérez-Díaz and others, 2016
Lactobacillus	*pentosus*	LA0233; ATCC 8041	Sauerkraut	Fred and others, 1921
Lactobacillus	*pentosus*	LA0445; BI0007, MOP3	Commercial cucumber fermentation	Fleming and others, 1988
Lactobacillus	*pentosus*	1.8.9	Commercial cucumber fermentation	Pérez-Díaz and others, 2016
Lactobacillus	*brevis*	LA0200; ATCC 8287	Green Sevillano fermenting olives	RH Vaughn 269Y; Dunn and others, 1947
Lactobacillus	*brevis*	LA0036; ATCC 14869, NRRL B-4527	Human feces	ATCC < PA Hansen < Roy. Techn. Coll., Copenhagen < S. Orla-Jensen 14 (*Betabacterium breve*); Rogosa and Hansen, 1971
Lactobacillus	*brevis*	7.2.43	Commercial cucumber fermentation	Pérez-Díaz and others, 2016
Lactobacillus	*buchneri*	LA0030; ATCC 4005, NRRL B1837	Tomato pulp	Rogosa and Hansen, 1971
Lactobacillus	*buchneri*	LA1149	Commercial cucumber fermentation	Franco and others, 2012
Lactobacillus	*buchneri*	LA1147; E-33-07	Commercial cucumber fermentation	Franco and others, 2012

Table 2.2. Measurements of trehalose, xylose and L-citrulline in fresh and fermented cucumbers by HPLC. The limit of detection for the sugars and L-citrulline were 0.01 mM and 1.71 µM, respectively. Numbers provided in parenthesis next to the concentration values represent the number of samples analyzed in which compounds were found.

Primary and Alternative Energy Sources	Concentration (mM)		
	Fresh Cucumbers (n[!] = 4)	**Day 3 of Fermentation (n = 3)**	**Day 38 of Fermentation (n = 4)**
Glucose	42.72 ± 7.4 (4)	39.29 ± 4.07 (3)	BDL (4)
Fructose	43.15 ± 6.5 (4)	38.25 ± 5.13 (3)	BDL (4)
Trehalose	BDL (4)	15.51 ± 1.68 (2)	BDL (4)
Xylose	36.05 (1)	BDL (4)	BDL (4)
L-citrulline	1.65 ± 0.63 (4)	0.85 ± 0.36 (3)	0.65 ± 0.07 (4)

[!]n= number of samples analyzed

Table 2.3. Bioinformatic analysis of the putative pathways involved in the utilization of alternative energy sources by selected lactic acid bacteria (*L. plantarum, L. pentosus, P. pentasaceus, L. brevis,* and *L. buchneri*). Each row represents the percentage of a specific lactic acid bacterium genomes that encode for the corresponding putative enzyme described on the fifth column in a metabolic pathway. All types of genome sequences were included in the analysis (finished, draft, and permanent drafts). LAB species are listed at the top of the table and the number of strains per species is listed at the bottom. The enzyme name & Enzyme Classification (EC) number, the type of pathway, target, organic compound utilized, and expected products are listed in the right four rows, respectively.

L. plantarum	*L. pentosus*	*P. pentosaceous*	*L. brevis*	*L. buchneri*	**Enzyme Name [EC No.]**		**Energy Generating Pathways**	**Target**	**Expected Product**
					Trehalose Phosphorylase	2.4.1.64	Starch & Sucrose	Trehalose	D-Glucose
					β-Phosphoglucomutase	5.4.2.6			β-D- Glucose-1-P*
					Phosphoglycerate Mutase	5.4.2.12			
					Phosphopyruvate Hydratase	4.2.1.11			
					Pyruvate Kinase	2.7.1.40			
					Lactic Acid Dehydrogenase	1.1.1.27			
					Glyceraldehyde-3-P Dehydrogenase	1.2.1.12	Glycolysis	Xylose	Lactic Acid
					Phosphoglycerate Kinase	2.7.2.3			
					Phosphoglycerate Mutase	5.4.2.11			
					Phosphoglycerate Mutase	5.4.2.12			
					Phosphopyruvate Hydratase	4.2.1.11			
					Pyruvate Kinase	2.7.1.40			

Table 2.3 (continued)

					Lactic Acid Dehydrogenase	1.1.1.27		
					Xylulokinase	2.7.1.17	Pentose Phosphate	D-Glyceraldehyde-3-P
					D-Xylulose-5-P 3-Epimerase	5.1.3.1		
					Ribose-5-P Isomerase	5.3.1.6		
					Transketolase	2.2.1.1		
					Transaldolase	2.2.1.2		
					3-Hexulose-6-P Synthase	4.1.2.43		
					6-Phospho-3-Hexuloisomerase	5.3.1.27		
					6-Phosphofructokinase	2.7.1.11		
					Fructoaldolase	4.1.2.13		
					6-Phosphogluconic Dehydrogenase	1.1.1.44		
					Phosphogluconate Dehydrogenase	1.1.1.343		
					6-Phosphogluconolactonase	3.1.1.31		
					Glucose-6-phosphate Dehydrogenase	1.1.1.49		
					Glucose-6-phosphate Dehydrogenase	1.1.1.363		
					Glucose-6-phosphate Isomerase	5.3.1.9		
					Citrulline-Aspartate Ligase	6.3.4.5	Arginine Biosynthesis	Citrulline	Fumarate or Arginine
					Arginine-Succinate Lyase	4.3.2.1			
					Arginine Deiminase	3.5.3.6			Arginine
98	3	7	21	6	**No. of Strains** (to the left)		*Phosphate		

more than 97% of the strains
between 97 and 5% of the strains
less than 5% of the strains
Missing

Table 2.4. Utilization of trehalose, xylose, and L-citrulline by selected lactic acid bacteria in fermented cucumber juice (FCJM) at pH 4.7. Trehalose, xylose and L-citrulline were supplemented to 18.13 ± 1.17, 18.65 ± 0.49 and 0.56 ± 0.02 mM, respectively. The control group glucose and fructose were supplemented to 14.21 ± 5.18 and 20.61 ± 2.52 mM, respectively. Organic compound concentrations are provided in the table below in mM. The amount of lactic acid, acetic acid and ethanol produced were calculated from the amounts measured in the FCJM after 7 days of incubation minus the amounts detected in the non-inoculated FCJM. Minimal detection limit was 0.01 mM for the sugars (BDL= 0.01 mM) and 1.71 μM for L-citrulline. Ethanol was not produced from fructose, xylose or trehalose.

Lactic Acid Bacteria Tested ➤	***L. plantarum***	***L. pentosus***	***L. brevis***	***L. buchneri***	**No Inocula (Negative Control)**
Glucose					
Glucose Utilized	14.71 ± 5.38[a]	14.24 ± 5.22[a]	12.66 ± 4.50[b]	14.71 ± 5.38[a]	NA[*]
Lactic Acid Produced	79.01 ± 6.51[a]	88.43 ± 6.62[b]	43.05 ± 9.45[c]	33.39 ± 0.97[d]	103.02 ± 3.71
Acetic Acid Produced	BDL[a]	BDL[a]	7.06 ± 2.31[b]	7.54 ± 0.25[b]	35.90 ± 0.96
Ethanol Produced	BDL[a]	BDL[a]	8.33 ± 0.03[b]	9.10 ± 2.07[b]	9.73 ± 0.03
Final pH	3.91 ± 0.00[a]	3.89 ± 0.01[a]	4.32 ± 0.04[b]	4.24 ± 0.04[b]	4.55 ± 0.03
Growth (Log CFU/mL)	≥ 8	≥ 8	≥ 8	8.83 ± 0.20	NA
Fructose					
Fructose Utilized	22.30 ± 3.12[a]	22.30 ± 3.12[a]	21.36 ± 2.74[a]	21.98 ± 2.98[a]	NA
Lactic Acid Produced	70.34 ± 9.38[a]	67.90 ± 5.83[a]	35.58 ± 13.01[b]	5.06 ± 1.30[c]	99.34 ± 0.55
Acetic Acid Produced	BDL[a]	BDL[a]	9.49 ± 0.41[b]	8.03 ± 3.64[b]	35.41 ± 0.00
Final pH	3.9 ± 0.05[a]	3.89 ± 0.04[a]	4.35 ± 0.04[b]	4.58 ± 0.04[b]	4.55 ± 0.03
Growth (Log CFU/mL)	≥ 8	≥ 8	≥ 8	8.78 ± 0.29	NA
Xylose					
Xylose Utilized	BDL[a]	3.14 ± 0.37[b]	16.92 ± 0.23[c]	18.65 ± 0.49[c]	NA

Table 2.4 (continued)

Lactic Acid Produced	BDL[a]	29.54 ± 18.70[b]	30.77 ± 4.85[b]	9.74 ± 12.16[c]	98.96 ± 1.20
Acetic Acid Produced	BDL[a]	BDL[a]	6.83 ± 4.42[b]	13.51 ± 2.18[c]	39.99 ± 0.01
Final pH	4.26 ± 0.01[a]	4.21 ± 0.01[a]	4.19 ± 0.04[a]	4.24 ± 0.05[a]	4.55 ± 0.03
Growth (Log CFU/mL)	< 4	< 4	≥ 8	8.53 ± 0.02	NA
Trehalose					
Trehalose Utilized	18.13 ± 1.17[a]	13.11 ± 1.04[b]	BDL[c]	2.36 ± 1.44[d]	NA
Lactic Acid Produced	94.05 ± 3.14[a]	63.28 ± 22.02[b]	BDL[c]	3.72 ± 5.22[d]	98.96 ± 0.01
Acetic Acid Produced	BDL[a]	BDL[a]	BDL[a]	10.99 ± 4.77[b]	22.09 ± 0.56
Final pH	3.7 ± 0.07[a]	3.87 ± 0.06[a]	4.5 ± 0.01[b]	4.56 ± 0.01[b]	4.55 ± 0.03
Growth (Log CFU/mL)	≥ 8	≥ 8	< 4	8.47 ± 0.03	NA
L-citrulline					
Remaining Citrulline	0.73 ± 0.01[a]	0.72 ± 0.04[a]	0.76 ± 0.18[a]	0.08 ± 0.01[b]	0.59 ± 0.07
Final pH	4.3 ± 0.01[a]	4.31 ± 0.01[a]	4.15 ± 0.19[b]	4.49 ± 0.02[c]	4.51 ± 0.01
Growth (Log CFU/mL)	≥ 8	≥ 8	≥ 8	8.39 ± 0.08	NA

[*] Not Applicable

** The results in the rows within blocks followed by the same alphabets (a, b, c, and d) indicate that they are not significantly different ($p>0.05$) from each other and the treatments with the different alphabets indicate that they are significantly different ($p<0.05$).

Table 2.5. Utilization of L-citrulline by *Lactobacillus buchneri* in the presence of glucose. The initial fermented cucumber juice medium pH was adjusted to 4.7 or 3.7 ± 0.1. Values for metabolic end products (mM), pH and colony counts determined from Lactobacilli MRS Agar plates are shown. The amount of lactic acid and acetic acid produced were calculated from the total amounts measured in the FCJM from samples collected after seven days of incubation minus the concentrations detected in the Non-Inoculated FCJM. While no significant difference was determined between the treatments and control values for cultures with an initial pH of 3.7 ± 0.1 ($p>0.05$), a significant difference was observed in the pH values measured from treatments and control corresponding to cultures with an initial pH of 4.7 ± 0.1 ($p<0.05$) using an ANOVA test. Minimal limit of detection for metabolites was < 0.01 mM.

Energy Sources Supplemented ►	**L-citrulline**	**Glucose & L-citrulline**	**Glucose**	**Non-Supplemented**	**Not-Inoculated**
Initial pH of 4.7 ± 0.1					
L-citrulline Utilized	12.43 ± 2.3	10.44 ± 0.9	BDL	BDL	NA*
Glucose Utilized	BDL	16.49 ± 5.3	12.71 ± 1.13	BDL	NA
Lactic Acid Produced	BDL	21.41 ± 9.84	8.23 ± 6.53	BDL	102.47 ± 4.86
Acetic Acid Produced	23.67 ± 1.41	20.72 ± 12.08	11.97 ± 2.04	6.23 ± 1.41	51.28 ± 1.41
Arginine Produced	BDL	BDL	BDL	BDL	NA
Ornithine Produced	14.19 ± 1.07	BDL	BDL	4.31 ± 4.93	NA
Ammonia Produced	14.54 ± 3.6	BDL	BDL	2.77 ± 2.57	NA
Final pH	5.18 ± 0.007	4.65 ± 0.07	4.5 ± 0.07	4.7 ± 0.03	4.7 ± 0.01
Colony Counts (Log CFU/mL)	7.55 ± 0.04	7.4 ± 0.09	7.08 ± 0.07	7.11 ± 0.18	NA
Initial pH of 3.7 ± 0.1					
L-citrulline Utilized	2.41 ± 0.5	15.64 ± 0.00	0.59 ± 0.13	0.36 ± 0.03	NA
Glucose Utilized	BDL	10.83 ± 3.29	10.2 ± 2.32	BDL	NA
Lactic Acid Produced	BDL	2.55 ± 4.36	6.90 ± 3.70	BDL	102.26 ± 5.30
Acetic acid Produced	BDL	20.96 ± 2.32	16.08 ± 4.87	BDL	51.81 ± 0.76
Arginine Produced	BDL	BDL	BDL	BDL	NA
Ornithine Produced	BDL	BDL	BDL	BDL	NA
Ammonia Produced	1.61 ± 0.85	3.18 ± 2.90	BDL	BDL	NA
Final pH	3.69 ± 0.02	3.74 ± 0.14	3.62 ± 0.07	3.68 ± 0.02	3.68 ± 0.01
Colony Counts (Log CFU/mL)	6.20 ± 0.16	7.55 ± 0.6	6.91 ± 0.03	5.2 ± 0.15	NA

* Not Applicable

Table 2.6. Fermentation by certain LAB of fresh cucumber juice supplemented with 18.25 ± 0.49 mM trehalose, 51.97 ± 1.30 mM xylose and 4.23 ± 0.61 mM L-citrulline. The starter cultures were inoculated to variable levels. *L. pentosus* (LA0455 and 1.8.9), *L. brevis* (3.2.19) and *L. buchneri* (LA1149 and LA1147) were used for inoculation. The FCJ medium initial pH was 5.0 ± 0.1. Minimal detection limits for the fermentation metabolites using HPLC was 0.01 mM. There were 10.30 ± 3.56 mM glucose and 12.16 ± 5.35 mM fructose present in this FrCJ medium derived from the fresh cucumber juice used to prepare the culture medium.

Time (Day)	Remaining Substrate Concentration (mM)			Fermentation Products (mM)		pH	Growth (Log CFU/mL)
	Xylose	Trehalose	L-citrulline	Lactic Acid	Acetic Acid		
Treatment 1: Inoculation with *L. pentosus* (2 log CFU/mL), *L. brevis* (3 log CFU/mL) and *L. buchneri* (2 log CFU/mL)							
0	51.97 ± 1.30^{a}	18.25 ± 0.49^{a}	4.23 ± 0.61^{a}	BDL*	36.63 ± 2.05^{a}	5.00 ± 0.01^{a}	Not Available
3	46.14 ± 3.53^{b}	4.33 ± 1.00^{b}	2.51 ± 1.90^{b}	79.44 ± 9.49^{a}	32.25 ± 2.19^{a}	3.51 ± 0.07^{b}	8.1 ± 0.32^{a}
7	52.15 ± 7.31^{a}	5.25 ± 0.84^{c}	2.84 ± 1.22^{b}	94.76 ± 2.19^{b}	31.53 ± 2.24^{a}	3.46 ± 0.01^{b}	7.46 ± 0.58^{b}
10	47.85 ± 1.48^{b}	5.38 ± 1.78^{c}	2.00 ± 0.00^{c}	101.58 ± 5.77^{c}	35.49 ± 2.85^{a}	3.43 ± 0.01^{b}	7.48 ± 0.47^{b}
30	38.11 ± 4.5^{c}	6.17 ± 1.83^{c}	1.21 ± 1.01^{d}	106.12 ± 1.67^{c}	51.82 ± 9.71^{b}	3.34 ± 0.01^{c}	3.51 ± 0.05^{c}
36	37.08 ± 1.59^{c}	6.17 ± 0.99^{c}	0.91 ± 0.67^{e}	92.95 ± 1.00^{b}	53.80 ± 5.36^{b}	3.40 ± 0.01^{b}	2.71 ± 0.28^{d}
60	41.18 ± 0.95^{d}	7.60 ± 2.08^{d}	1.05 ± 0.73^{e}	106.28 ± 8.22^{c}	56.68 ± 2.48^{b}	3.40 ± 0.01^{b}	5.26 ± 0.01^{e}
Treatment 2: Inoculation with *L. brevis* (4 log CFU/mL) and *L. buchneri* (2 log CFU/mL)							

Table 2.6 (continued)

0	51.97 ± 1.30[a]	18.25 ± 0.49[a]	4.23 ± 0.61[a]	BDL[a]	36.63 ± 2.05[a]	5.00 ± 0.01[a]	Not Available
3	50.68 ± 2.63[a]	3.05 ± 1.20[b]	2.52 ± 1.47[b]	51.81 ± 4.99[b]	53.96 ± 1.38[b]	3.90 ± 0.02[b]	8.68 ± 0.39[a]
7	33.71 ± 4.46[b]	1.80 ± 0.54[c]	1.78 ± 0.57[c]	70.69 ± 15.04[c]	66.01 ± 3.99[c]	3.67 ± 0.08[c]	8.64 ± 0.42[a]
10	19.66 ± 2.07[c]	1.93 ± 0.56[c]	0.66 ± 0.19[d]	76.08 ± 8.62[d]	82.89 ± 0.28[d]	3.61 ± 0.04[c]	7.86 ± 0.41[b]
30	0.51 ± 0.20[d]	1.86 ± 0.74[c]	< 1.71 μM[e]	77.03 ± 5.23[d]	104.44 ± 5.02[e]	3.49 ± 0.02[d]	6.65 ± 0.08[c]
36	0.39 ± 0.15[e]	2.57 ± 0.42[b]	< 1.71 μM[e]	70.72 ± 3.33[c]	110.67 ± 1.74[e]	3.57 ± 0.05[c]	5.71 ± 0.71[d]
60	0.34 ± 0.10[e]	2.02 ± 0.90[c]	< 1.71 μM[e]	78.31 ± 4.37[d]	109.00 ± 0.58[e]	3.52 ± 0.02[c]	5.17 ± 0.20[e]
Treatment 3: Inoculation with *L. pentosus* (2 log CFU/mL) and *L. brevis* (3 log CFU/mL)							
0	51.97 ± 1.30[a]	18.25 ± 0.49[a]	4.23 ± 0.61[a]	BDL	36.63 ± 2.05[a]	5.00 ± 0.01[a]	Not Available
3	45.91 ± 3.62[b]	4.34 ± 0.87[b]	2.49 ± 1.42[b]	74.31 ± 2.04[a]	31.81 ± 2.05[b]	3.54 ± 0.03[b]	8.22 ± 0.01[a]
7	42.10 ± 12.57[b]	4.19 ± 0.18[c]	2.59 ± 1.14[b]	90.84 ± 1.60[b]	32.91 ± 3.27[b]	3.46 ± 0.04[c]	6.55 ± 0.25[b]
10	52.26 ± 5.57[a]	6.28 ± 0.18[d]	2.39 ± 1.30[b]	98.07 ± 0.73[c]	32.17 ± 3.47[b]	3.42 ± 0.04[c]	5.47 ± 0.57[c]
30	49.42 ± 1.87[a]	5.23 ± 1.74[e]	1.76 ± 0.85[c]	103.61 ± 4.32[c]	32.05 ± 3.00[b]	3.30 ± 0.05[d]	6.92 ± 0.00[b]
36	23.49 ± 32.83[c]	4.54 ± 4.48[e]	2.12 ± 1.70[d]	93.98 ± 7.09[b]	35.03 ± 6.59[a]	3.39 ± 0.01[d]	4.44 ± 4.43[d]
60	36.88 ± 13.98[d]	4.73 ± 2.37[e]	2.52 ± 1.73[b]	106.58 ± 5.64[c]	41.02 ± 9.21[c]	3.3 ± 0.06[d]	6.90 ± 0.00[b]
Treatment 4: Inoculation with *L. brevis* (4 log CFU/mL)							

Table 2.6 (continued)

0	51.97 ± 1.30[a]	18.25 ± 0.49[a]	4.23 ± 0.61[a]	BDL	36.63 ± 2.05[a]	5.00 ± 0.01[a]	Not Available
3	53.92 ± 0.41[a]	3.24 ± 1.18[b]	2.70 ± 0.53[b]	44.07 ± 7.11[a]	53.25 ± 4.52[b]	3.96 ± 0.07[b]	8.78 ± 0.03[a]
7	41.72 ± 10.70[b]	1.69 ± 0.96[c]	2.64 ± 1.35[b]	60.48 ± 1.42[b]	64.97 ± 2.86[c]	3.73 ± 0.02[c]	8.31 ± 0.17[b]
10	36.23 ± 12.85[b]	1.82 ± 0.84[c]	1.88 ± 0.78[c]	69.39 ± 1.98[c]	67.16 ± 1.16[c]	3.68 ± 0.01[c]	7.60 ± 0.03[c]
30	22.31 ± 1.67[c]	2.19 ± 1.22[d]	1.18 ± 0.69[d]	77.27 ± 4.88[d]	74.12 ± 11.56[d]	3.51 ± 0.04[d]	6.66 ± 0.23[d]
36	20.40 ± 12.95[c]	2.15 ± 0.81[d]	1.19 ± 0.11[d]	72.23 ± 2.89[e]	80.10 ± 10.45[e]	3.59 ± 0.03[d]	3.53 ± 2.72[e]
60	17.65 ± 15.55[c]	2.07 ± 0.74[d]	1.30 ± 0.23[d]	74.62 ± 6.61[e]	81.83 ± 21.31[e]	3.54 ± 0.04[d]	5.75 ± 0.00[f]

[!] Estimated inoculation level. [*] BDL: below detection level

** The results in the columns within blocks followed by the same alphabets (a, b, c, d, e, and f) indicate that they are not significantly different ($p>0.05$) from each other and the treatments with the different alphabets indicate that they are significantly different ($p<0.05$) as statistically.

Table 2.7. Fermentation by certain LAB of raw fresh cucumber juice medium inoculated with mixed starter cultures: The starter cultures were inoculated to variable levels. *L. pentosus* (LA0455 and 1.8.9), *L. brevis* (3.2.19) and *L. buchneri* (LA1149 and LA1147) were used for inoculation. The FCJ medium initial pH was 5.0 ± 0.1. There were 10.30 ± 3.56 mM glucose and 12.16 ± 5.35 mM fructose present in this FrCJ medium.

Time (day)	Remaining Concentration (mM)			Products Concentration (mM)		pH	Growth (log CFU/mL)
	Xylose	Trehalose	L-citrulline	Lactic Acid	Acetic Acid		
Treatment 1: Inoculation with *L. pentosus* (2 log CFU/mL) and *L. brevis* (3 log CFU/mL)							
0	< 0.01	< 0.01	0.39 ± 0.11^{a}	BDL*	34.01 ± 2.83^{a}	5.00 ± 0.01^{a}	Not Available
3	1.90 ± 1.14	0.08 ± 0.04	0.38 ± 0.18^{a}	59.66 ± 4.01^{a}	33.65 ± 2.23^{a}	3.60 ± 0.01^{b}	8.03 ± 0.49^{a}
7	< 0.01	< 0.01	0.56 ± 0.10^{b}	62.45 ± 0.08^{a}	33.77 ± 2.32^{a}	3.59 ± 0.03^{b}	7.24 ± 0.00^{b}
10	< 0.01	< 0.01	0.45 ± 0.09^{c}	64.23 ± 4.30^{a}	33.67 ± 3.24^{a}	3.60 ± 0.02^{b}	5.28 ± 0.08^{c}
30	< 0.01	< 0.01	0.41 ± 0.15^{c}	65.82 ± 0.61^{a}	32.08 ± 3.13^{a}	3.48 ± 0.04^{b}	5.71 ± 0.78^{d}
36	< 0.01	< 0.01	0.38 ± 0.03^{a}	65.00 ± 7.04^{a}	33.43 ± 1.50^{a}	3.52 ± 0.01^{b}	4.11 ± 1.48^{e}
60	0.05 ± 0.01	< 0.01	0.52 ± 0.29^{b}	65.66 ± 5.73^{a}	33.73 ± 4.22^{a}	3.51 ± 0.06^{b}	4.44 ± 0.40^{f}

Table 2.7 (continued)

Treatment 2: Inoculation with *L. brevis* (4 log CFU/mL)							
0	< 0.01	< 0.01	0.39 ± 0.11[a]	BDL	34.01 ± 2.83[a]	5.00 ± 0.01[a]	Not Available
3	2.33 ± 0.31	< 0.01	0.35 ± 0.18[a]	24.37 ± 3.49[a]	38.78 ± 1.16[b]	4.26 ± 0.01[b]	8.42 ± 0.18[a]
7	0.54 ± 0.07	< 0.01	0.33 ± 0.11[a]	25.84 ± 2.34[a]	39.32 ± 2.76[b]	4.33 ± 0.00[c]	7.23 ± 0.03[b]
10	< 0.01	< 0.01	0.34 ± 0.07[a]	26.65 ± 3.01[a]	38.20 ± 3.69[b]	4.25 ± 0.06[b]	7.00 ± 0.09[b]
30	< 0.01	< 0.01	< 1.71 μM	37.98 ± 20.02[b]	40.63 ± 0.93[b]	3.97 ± 0.18[d]	5.99 ± 0.82[c]
36	< 0.01	< 0.01	< 1.71 μM	37.52 ± 15.64[b]	41.76 ± 2.60[b]	4.11 ± 0.28[e]	5.11 ± 0.17[d]
60	< 0.01	< 0.01	< 1.71 μM	34.00 ± 17.33[b]	37.06 ± 1.30[b]	4.06 ± 0.18[e]	4.71 ± 0.02[e]
Treatment 3: Inoculation with *L. pentosus* (2 log CFU/mL), *L. brevis* (3 log CFU/mL) and *L. buchneri* (2 log CFU/mL)							
0	< 0.01	< 0.01	0.39 ± 0.11[a]	BDL	34.01 ± 2.83[a]	5.00 ± 0.01[a]	Not Available
3	2.73 ± 1.38	0.09 ± 0.05	0.44 ± 0.07[b]	58.16 ± 1.76[a]	33.02 ± 1.58[a]	3.63 ± 0.03[b]	7.91 ± 0.40[a]
7	< 0.01	< 0.01	0.48 ± 0.09[b]	64.02 ± 1.79[b]	32.90 ± 0.90[a]	3.61 ± 0.04[b]	6.68 ± 0.10[b]
10	0.04 ± 0.01	< 0.01	0.33 ± 0.18[a]	63.32 ± 6.36[b]	33.25 ± 2.79[a]	3.61 ± 0.01[b]	7.06 ± 0.09[c]
30	< 0.01	< 0.01	< 1.71 μM	59.78 ± 3.50[a]	33.32 ± 3.96[a]	3.57 ± 0.01[b]	5.48 ± 0.18[d]
36	< 0.01	< 0.01	< 1.71 μM	56.80 ± 7.31[a]	36.03 ± 3.07[a]	3.57 ± 0.01[b]	5.26 ± 0.04[e]
60	< 0.01	< 0.01	< 1.71 μM	58.65 ± 6.63[a]	36.05 ± 3.48[a]	3.64 ± 0.01[b]	5.02 ± 0.24[f]

[!] Estimated inoculation level. [*] BDL: below detection level. ** The results in the columns within blocks followed by the same alphabets (a, b, c, d, e, and f) indicate that they are not significantly different ($p>0.05$) from each other and the treatments with the different alphabets indicate that they are significantly different ($p<0.05$) as statistically.

CHAPTER 3

Gentiobiose and cellobiose content in fresh and fermenting cucumbers and utilization of such disaccharides by lactic acid bacteria in fermented cucumber juice medium

Redife Aslihan Ucar[1], Ilenys M. Pérez-Díaz[2], Lisa Dean[3]

[1]North Carolina State University, Department of Food, Bioprocessing, and Nutrition Sciences, Raleigh, North Carolina, USA; [2]USDA-Agriculture Research Service, Food Science Research Unit, Raleigh, North Carolina, USA; [3]USDA-Agriculture Research Service, Market Quality Research Unit, Raleigh, North Carolina, USA

3.1 Abstract

This study determined the cellobiose and gentiobiose content in fresh and fermented cucumbers using HPLC and evaluated the ability of selected lactic acid bacteria (LAB) relevant in cucumber fermentations to utilize such sugars. The ability of *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus buchneri*, *Lactobacillus brevis*, and *Pediococcus pentosaceus* to utilize cellobiose and gentiobiose was studied in fermented cucumber juice medium (FCJM) to simulate conditions post-fermentation. Microbial growth and fermentation of cellobiose and gentiobiose was monitored by colony counts, pH and metabolite concentration measurements using MRS agar plates, a probe and HPLC analysis, respectively. The disaccharide concentrations in fresh and fermented cucumbers were below 0.01 mM. Cellobiose and gentiobiose were utilized by *L. plantarum*, *L. pentosus* and *L. buchneri* to varying extents in FCJM at pH 4.7. Cellobiose was homofermentatively utilized by the three LAB at pH 4.7, but not at 3.7. While *L. brevis* and *P. pentosaceous* were unable to utilize cellobiose efficiently in FCJM (pH 4.7), they were able to removed gentiobiose. Supplementation of gentiobiose to FCJM at a pH of 4.7 to about 8 and 18 mM resulted in homo- and heterofermentation, respectively. Some differences were observed with regards to cellobiose utilization as a function of the lactobacilli strain used, but not in the conversion of gentiobiose. *L. plantarum* and *L. pentosus* were found able to proliferate in the unsupplemented FCJM at pH 4.7 and produced between 15 and 46 mM lactic acid. Incases where the disaccharides be present in industrial cucumber fermentations due to factors other than those studied here, the indigenous LAB could be expected to remove them prior to the development of spoilage.

3.2 Introduction

Completion of cucumber fermentation by lactic acid bacteria (LAB) is typically monitored by measuring the sugars naturally present in the fruits, glucose and fructose, and the lactic acid, acetic acid, and ethanol that derive from them, using high performance liquid chromatography (HPLC). The disappearance of all the glucose and fructose in fermentation vessels signals the end-point of a cucumber fermentation. Industrially, measurements of pH with time is used to follow the progress of a cucumber fermentation. Commercial production has made use of reductive sugars strips, typically used for urinalysis, to routinely monitor the completion of fermentation in batches prior to processing. Despite the fact that HPLC analysis enables the concomitant detection of glucose and fructose to organic acids and ethanol and expands the sensitivity threshold and ability to determine specific yields in cucumber fermentations; a comprehensive understanding of the metabolic conversions occurring in a fermentation impart certain attribute to specific batches is lacking (McFeeters, 1993; Lu and others, 2002).

Metabolic profiling of anaerobically fermented cucumbers that were spoiled by *Lactobacillus buchneri* revealed changes in 92 compounds including citrulline, trehalose, cellobiose, xylose, lyxose, gentiobiose and lactic acid (Johanningsmeier and McFeeters, 2015). Incremental concentrations were observed in alcohols and butanoic and pentanoic acids and were accompanied by decreases in the concentration of monosaccharides, disaccharides, amino acids, nucleosides, long chain fatty acids and ketones (Johanningsmeier and McFeeters, 2015). It was additionally observed that citrulline, D-trehalose, and D-cellobiose were particularly utilized by *Lactobacillus buchneri* prior to lactic acid degradation (Johanningsmeier and McFeeters, 2015).

This study evaluated the function of disaccharides, particularly cellobiose and gentiobiose, as potential secondary energy sources in cucumber fermentations for selected LAB. It was

theorized that a greater understanding of the role of energy sources specifically in cucumber fermentations for LAB would enable the design of functional starter or adjunct cultures that are able to complete the bioconversion and remove secondary energy sources. In doing so, a starter/adjunct culture would be able to prevent the growth of spoilage microbes during long term storage.

Cellobiose and gentiobiose are disaccharides composed of two glucose units joined by a β-(1-4) or β-(1->6) glycosidic linkage, respectively. Both disaccharides are plant metabolites found in some food products such as vegetables, fruits, corn syrups and others (Buckenhüskes, 1997). Cellobiose is the product of cellulose or a plant β-glucan degradation.

A number of microbes can breakdown cellobiose to the glucose subunits via β-glucosidases (Singhvi and others, 2010; Abdel-Rahman and others, 2011). The metabolism of disaccharides by LAB is regulated via the PTS systems and/or intracellular phospho-glycosyl hydrolases (Andersen and others, 2012; Barrangou and others, 2006; Francl and others, 2010). Various β-glucosides are translocated by LAB via the phosphotransferase systems (PTS), and subsequently cleaved by phospho-β-glucosidase to produce phosphorylated glucose and the respective glycon (Bardowski and others, 1994; Bardowski and others, 1995; Schnetz and others, 1987; Tobisch and others, 1997; Aleksandrzak-Piekarczyk and others, 2011). Specific PTS permeases and the 6-phospho-β-glucosidase are regulated by cellobiose and gentiobiose in *Lactobacillus acidophilus* at the transcriptional level (Andersen and others, 2012). The cellobiose PTS permease coding genes in *L. acidophilus* is homologous to the functionally characterized system in *Lactobacillus gasseri* (Andersen and others, 2012). The cellobiose and gentiobiose induced PTSs form a unique phylogenetic cluster among the *L. acidophilus* PTSs (Andersen and others, 2012). However, a gentiobiose specific PTS has not been functionally characterized in *L. gasseri* and/or other LAB

(Andersen and others, 2012; Francl and others, 2010). A putative cellobiose PTS was found in *Lactobacillus paracasei* by comparative genomics (Smokvina and others, 2013). Such gene casettes varied among *L. paracasei* strains (Smokvina and others, 2013). In *Lactococcus lactis* the cellobiose-specific PTS system is comprised of CelB, PtcB and PtcA, is CcpA-dependent and able to transport lactose (Aleksandrzak-Piekarczyk and others, 2011).

While a number of lactobacilli, including *Lactobacillus plantarum*, *Lactobacillus johnsonii*, *Lactobacillus casei*, *L. gasseri, L. paracasei* and *L. acidophilus*, have a diversity of disaccharide hydrolases or disaccharide-phosphate hydrolases, some, such as *Lactobacillus brevis*, *Lactobacillus reuteri*, *Lactobacillus buchneri*, *Lactobacillus spicheri* and *Lactobacillus delbrueckii* are restricted in this regard (Fitzsimons and others, 1999; Yu and others, 2012; Rogosa and others, 1961; Gänzle and Follador, 2012; Andersen and others, 2012; Barrangou and others, 2006; Francl and others, 2010). Differences in disaccharides utilization have also been observed at the strain level. 90% of a group of 72 *L. plantarum* isolates from vegetables and fruits were able to use D-cellobiose, while less than 50% were capable of fermenting gentiobiose. Variability in the ability to utilize cellobiose and gentiobiose by 10 *L. plantarum* isolated from Thai fermented vegetables and fruits was also observed by Tanganurat and others (2009). Lactobacilli able to utilize cellobiose and convert it to L- and D-lactic acid (Carr and others, 2002; Gänzle, 2015; Fitzsimons and others, 1999; Tamang and others, 2005; Mao and others, 2015; Soltan Dallal and others, 2017; Yu and others, 2012).

This study confirmed the concentration of cellobiose and gentiobiose naturally found in fresh and fermented cucumbers using HPLC analysis for quantification and evaluated the ability of selected LAB relevant to cucumber fermentations to utilize these sugars. A fermented cucumber juice (FCJ) model system was used to evaluate the ability of *L. plantarum*, *L.*

pentosus, *L. buchneri*, *L. brevis*, and *P. pentosaceus* to utilize cellobiose and gentiobiose to simulate conditions post-fermentation.

3.3 Materials and Methods

Measurement of cellobiose and gentiobiose concentration in fresh and fermented cucumbers: Samples of 4-fresh, size 2B, pickling cucumber lots were obtained from a local processor. The corresponding fermented cucumber samples were collected on days 3 and 38 of fermentation. Fresh and fermented cucumbers were sliced using aseptic techniques and blended using a Waring Commercial Blender 700S (Torrington, CT, USA) equipped with a sterilized glass cups for 90 s at medium speed. Cucumber slurries were homogenized using a Seward Stomacher 400 (Bohemia, NY, USA) in 6" x 4.5" filter stomacher bags for 1 min at maximum speed. One mL aliquots of the filtered homogenate were spun at 15,294 rcf for 10 min in an Eppendorf benchtop refrigerated centrifuge 5810R (Hamburg, Germany) to remove residual particulate matter. Supernatants were used for HPLC analysis to determine the cellobiose and gentiobiose concentrations.

To perform HPLC analysis, 100 µL of the fresh and fermented cucumber juice supernatants was diluted to 2 mL with water spiked with 50 µL of lactose (Sigma-Aldrich, St. Louis, MO) as an internal standard. All solutions were filtered through Dionex OnGuard-H cartridges (Sunnyvale, CA) to remove the free amino acids into autosampler vials. The extracts were analyzed using a BioLC (Dionex Corporation, Sunnyvale, CA) at a controlled temperature of 25 °C. The system consisted of a gradient pump, an autosampler, and a Pulsed Amperometric Detector (PAD). The mobile phase was 50 mM sodium hydroxide (NaOH) (Thermo-Fisher Scientific, Fairlawn, NJ) at an isocratic flow rate of 1.0 mL/min. The column used was a PA-1, 250 mm length and 4 mm i.d. (Dionex Corporation), fitted with a PA-1 Guard column (Dionex

Corporation). The detector was programmed to run a quadruple waveform as recommended by the manufacturer. The injection volume was 10 μL. Each sugar was quantified by calculating a ratio of the unknown peak height to the lactose (internal standard) peak height and comparing it with a ratio of a known concentration of cellobiose and gentiobiose (Sigma-Aldrich and Fluka Chemie, Steinheim, Germany, respectively) (Pattee and others, 2000).

Bioinformatic analysis of the genes coding for enzymes involved in cellobiose and gentiobiose metabolism by certain LAB: The analysis of the putative enzymes involve in the metabolism of gentiobiose and cellobiose was conducted using the publically available genome sequences for *L. pentosus* (3), *L. plantarum* (107), *L. brevis* (21), *L. buchneri* (6), *P. pentosaceous* (7). The Joint Genome Institute -Integrated Microbial Genomes (Chen and others, 2016) was used to develop informative metabolic maps with regards to the abundance of certain genes in the genomes described above. The KEGG Orthology Pathways (KO) tool was specifically used to populate Table 3.2. The Metacyc (Caspi and others, 2017) and Biocyc (Karp and others, 2017) online tools at the IMG platform were used to define and/or confirmed the reference pathways for glycolysis and β-glucosidases involved in cellobiose and gentiobiose catabolism.

Preparation of fermented cucumber juice media (FCJM): Size 2B (32-38 mm in diameter) fresh whole pickling cucumbers from two different lots were secured from a local retail outlet (Raleigh, NC). Fresh pickling cucumbers in good condition and free of mechanical damage were selected, washed with plain water and packed into four one-gallon glass jars. 2 jars for each cucumber lot were packed using a 50:50 (w/v, cucumbers/cover brine) pack-out ratio. The cover brine was prepared so that it equilibrated with the cucumbers at 80 mM $CaCl_2$ (Brenntag, Durham, NC), 6 mM potassium sorbate (Mtisubishi International Food Ingredients, Atlanta,

GA), 10.1 mM $Ca(OH)_2$ (Sigma-Aldrich, St. Louis, MO) and 44 mM acetic acid, added as 20% vinegar (Fleischmann Vinegar, MO, USA), to adjust the initial pH to 4.7. A mixed starter culture of *Lactobacillus plantarum* 3.8.2 and *Lactobacillus pentosus* LA 0445 (Table 3.1) was prepared as described below and supplemented to 10^5 CFU/mL. Jars were closed with commercial metal lug caps that were heated in boiling water for 10 second to soften the plastisol liner. Each lid was equipped with a rubber septum in its center to allow for sampling of cover brine using a 10 mL syringe attached to a 18G X 1 1/2" needle (Becton Dickinson Co., Franklin Lakes, NY). The jars were incubated at 30 °C for 10 days. The pH was measured using a Fisher Accumet pH meter (Model AR25, Fisher Scientific, Pittsburgh, PA, USA) combined with a Gel-Filled Pencil-Thin pH Combination Electrode (Acumet, Fisher Scientific). The completion of the fermentation was confirmed by measuring sugars, organic acids and ethanol in cover brine samples using the HPLC analysis conducted as described below. At the end of these fermentations, the pH was determined to be 3.3 ± 0.1 and the media contained 0.5 ± 0.2 mM and 1.69 ± 0.6 mM glucose and fructose, respectively.

The cover brine and juice from fermented cucumbers was used to prepare FCJM. Fermentation cover brines were decanted from the jars into 2 L beakers. The fermented cucumbers were passed through an automatic juice extractor (Juiceman Jr. Model JM-1, Beachwood, Ohio, USA) to separate the pulp from the liquid content on a jar volume basis. The pulp remaining in the fermented cucumber juice was removed by straining with a 100% cotton cheesecloth (grade #90, 44 x 36 threads/inch, Cartridge Plus, Inc., Riva, MD) and a subsequent centrifugation at 3,750 x g for 15 min at ambient temperature using a bucket rotor (Eppendorf Centrifuge Model 5810, Hamburg, Germany). The respective clear fermented cucumber juices and fermentation cover brines were mixed to the same ratio as in the fermentation jars to make-

up the FCJM. The FCJM derived from each cucumber lot were independently used and supplemented with gentiobiose (Sigma-Aldrich G-3000, 85% purity) or cellobiose (98% purity) as needed. The pH of the supplemented and un-supplemented FCJM was adjusted to 4.7 ± 0.1 or 3.7 ± 0.1 as needed using a 5N NaOH solution (Spectrum Chemicals, NJ, USA) and 3N HCl (Spectrum Chemicals, NJ, USA). pH measurements were taken. The pH-adjusted FCJM were filter sterilized using 0.2-μ filtration units (Nalgene®-Rapid Flow™, Thermo Scientific). 10 mL aliquots of each FCJM were aseptically transferred to 15 mL conical tubes for experimentation.

Lactic acid bacteria cultures preparation: The bacterial cultures used for experimentation are described in Table 3.1. The lactic acid bacteria cultures were transferred from frozen stocks, prepared with Lactobacilli MRS broth (Becton Dickinson Co.) supplemented with 15% glycerol (Sigma-Aldrich), to 10 mL of MRS broth. The cultures were incubated at 30 °C for 48 to 72 h prior to inoculating fermentations or FCJM. The *Lactobacillus plantarum* and *Lactobacillus pentosus* cultures were transferred to fresh cucumber juice after growing in MRS broth and prior to inoculating the FCJM for the experiment designed to evaluate their ability to use cellobiose and gentiobiose under aerobiosis and anaerobiosis at different initial pH values. Fresh cucumber juice was prepared in the same way as the fermented cucumber juice was prepared (as described above). The fresh cucumber juice was also filtered-sterilized prior to inoculation. The FCJM was inoculated to 10^5 CFU/mL. The optical density at 600 nm of the MRS or fresh cucumber juice cultures was measured using a Novaspec II, (Pharmacia, Stockholm, Sweden) and used to adjust the inoculation level. A sterile 0.85% NaCl (Sigma-Aldrich) solution was used to adjust the inocula concentration as needed. Inocula with mixed cultures was prepared by combining the cells suspension in saline solution so that each strain will be at 10^5 CFU/mL in the FCJM.

Evaluation of the ability of certain lactic acid bacteria to utilize gentiobiose and cellobiose in FCJM: The experimental design tested the ability of three strains of *L. pentosus*, *L. plantarum*, *L. brevis*, *L. buchneri* and *Pediococcus pentosaceous* to utilize cellobiose and gentiobiose in FCJM under aerobiosis and at an initial pH of 4.7 (Table 3.1). There were two treatments for each mixed culture tested with technical and independent duplicates for a combined total of 4 replicates per species and treatment. Additionally, the mixed cultures were inoculated in the un-supplemented FCJM. All FCJM cultures were incubated at 30 ^{0}C for 7 days to enable secondary fermentation.

Samples were aseptically collected at the end point of incubation and were serially diluted in a sterile 0.85% NaCl solution prior to spiral plating on Lactobacilli MRS media using an Autoplate 4000 Eddy Jet 2 spiral plater (IUL, Barcelona, Spain). MRS agar plates were incubated at 30 ^{0}C aerobically for 48 h. Colony counts from MRS agar plates were obtained using a Flash & Go Automatic Colony Counter (IUL, Barcelona, Spain). The detection limit for colony counts was 2.4 log CFU/mL.

The pH and fermentation biochemistry was monitored at the end point only. Changes in pH as a function of fermentation age were monitored as described above. The fermentation biochemistry was monitored by HPLC analysis of FCJM samples collected aseptically from the 15 mL conical tubes containing the cultures. 1.5 mL of each fermentation sample was centrifuge at 15,294 x g for 15 min at room temperature. A minimum of 500 µL of the supernatants were transferred into glass HPLC vials. Organic acids and carbohydrate concentrations were measured using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and the HPLC method described by McFeeters (1993) with some modifications. The operating conditions of the UFLC Shimadzu HPLC (Shimadzu Corporation, Canby, OR, USA) system were a column temperature

of 65 °C and a 0.01 N H_2SO_4 eluent at 0.9 mL/min. The diode array detector was set at 210 nm at a rate of 1 Hz to quantify malic, lactic, succinic, propionic and butyric acids. An RID-10A refractive index detector (Shimadzu Corporation) connected in series with the diode array detector was used to measure acetic acid, lactic acid, glucose, fructose and ethanol. External standard curves were also run using at least five concentrations of standard compounds for quantification purposes.

Evaluation of the ability of *Lactobacillus pentosus* and *Lactobacillus plantarum* to utilize gentiobiose and cellobiose in FCJM: The experimental design included the testing of the ability of three strains of *L. pentosus*, including ATCC8041, LA0445 and 1.8.9 and three strains of *L. plantarum*, particularly ATCC14917, WCSF1 and 3.2.8, to utilize cellobiose and gentiobiose in FCJM under aerobiosis and anaerobiosis at an initial pH of 4.7 or 3.7 (Table 3.1). Thus, there were 4 treatments for each strain tested with technical and independent duplicates for a combined total of 4 replicates per strain and treatment. This experimental design generated a total of 92 independent experimental tests plus twelve non-inoculated FCJM controls (2 per media type (3) for each lot (2). Additionally, the cultures were inoculated in the un-supplemented FCJM. All FCJM cultures were incubated at 30 ^{0}C for 7 days to enable secondary fermentation. Samples were aseptically collected at the end point of incubation and plated on Lactobacilli MRS agar plates to determined colony counts as described above. The pH and fermentation biochemistry was monitored at the end point as described above.

3.4 Results

Cellobiose and gentiobiose concentration in fresh and fermented cucumber samples: Initially, the four fresh cucumber samples and 7 fermented cucumber samples did not contain measurable quantites of gentiobiose and cellobiose. The 7 fermented cucumber samples

consisted of triplicates from 3 days old fermentations and quadruplets from 38 days old fermentations.

Bioinformatic Analysis: The enzymes coding for cellobiase was found by gene homology in most of the lactic acid bacteria genomes scrutinized, except for *Pediococcus pentosaceous* (Table 3.2). The presence of putative cellobiase in the *L. plantarum* genomes was strain dependent. *L. brevis* and *L. buchneri* had some glycolysis pathway related enzymes missing (Table 3.2).

Ability of certain lactic acid bacteria to utilize cellobiose and gentiobiose under aerobiosis at an initial pH of 4.7: Table 3.3 shows the ability of certain lactic acid bacteria of relevance in cucumber fermentations to utilize the disaccharides cellobiose and gentiobiose in FCJM with an adjusted pH of 4.7 ± 0.1. As expected and in agreement with previous publications *L. brevis* and *P. pentosaceous* were unable to utilize cellobiose to a significant extent. *L. plantarum*, *L. pentosus* and *L. buchneri* utilized most of the cellobiose added to the FCJM homofermentatively. *L. plantarum* produced an excess of lactic acid, which is speculated to have been derived from the residual energy sources in the FCJM such as glucose, fructose and malic acid, among others. The *L. plantarum* strains produced 46.53 ± 16.95 mM lactic acid in the un-supplemented FCJM after the 7 day incubation under aerobiosis. *L. pentosus* converted 15.81 ± 0.23 mM cellobiose to 59.20 ± 4.16 mM lactic acid after 7 days of incubation. The ability of *L. plantarum* and *L. pentosus* to utilize cellobiose resulted in a 3 log of CFU/g increase in colony counts and a final pH of 3.8 ± 0.3, a 1 pH unit decrease. The conversion of cellobiose to lactic acid by *L. buchneri* was incomplete, presumably due to a slower acid production rate or the diversion of the carbons to other products, such as propanediol, that were not measured (Johanningsmeier and McFeeters,

2013). A slower acid production was also evidenced by the higher pH (4.53 ± 0.04) after 7 days of incubation.

All the strains tested were able to utilize gentiobiose. Except for *L. plantarum*, the lactobacilli converted gentiobiose into lactic acid, acetic acid and ethanol in close to a heterofermentative ratio of 2:1:0.5 (Table 3.3). *L. plantarum* converted 17.81 ± 3.14 mM gentiobiose to 102.07 ± 14.07 mM lactic acid (Table 3.3). The ability to utilize the disaccharides resulted into microbial growth in the FCJM as determined by plating on MRS agar plates (Table 3.3). Changes in pH at the end point fluctuated between 0.4 and 0.7 pH units (Table 3.3).

Ability of three strains of *L. pentosus* and *L. plantarum* to utilize cellobiose and gentiobiose under aerobiosis and anaerobiosis at an initial pH of 4.7 and 3.7: None of the disaccharides were utilized by the six strains of *L. plantarum* and *L. pentosus* evaluated when the initial pH was adjusted to 3.7 ± 0.1, as evidenced by lack of significant differences in pH (ANOVA test at a $p > 0.05$), a reduction in colony counts below detection levels and no changes in the concentrations of cellobiose and gentiobiose supplemented in the FCJM was found (data not shown). On the contrary both bacterial species utilized the disaccharides under aerobiosis or anaerobiosis when the pH of the FCJM was adjusted to 4.7 ± 0.1 (Tables 3.4 and 3.5).

While the strains isolated from cucumber or cabbage fermentations were able to utilize cellobiose under aerobiosis or anaerobiosis in FCJM with an adjusted pH of 4.7 ± 0.1; strains of *L. plantarum* (WCSF1) isolated from saliva and *L. pentosus* (ATCC8041) obtained from sauerkraut (EB Fred, 1921) were not able to utilize cellobiose in FCJM with an adjusted pH of 4.7 ± 0.1 under either condition of oxygen availability (Tables 3.4 and 3.5). Some variations in the ability to utilize the disaccharides under aerobiosis and anaerobiosis were observed among the strains isolated from cabbage or cucumber fermentations (Table 3.4). *L. plantarum* 3.2.8 and

L. pentosus MOP3, both robust strains in cucumber fermentations (Pérez-Díaz, 2016, submitted), utilized cellobiose in FCJM with an adjusted pH of 4.7 ± 0.1 under aerobiosis and anaerobiosis. However, *L. pentosus* 1.8.9 isolated from commercial cucumber fermentations and *L. plantarum* ATCC14917 obtained from pickled cabbage were not able to utilize cellobiose in FCJM with an adjusted pH of 4.7 ± 0.1 under anaerobiosis and aerobiosis, respectively (Table 3.3).

Cellobiose and gentiobiose were converted homofermentatively and heterofermentaively, respectively, by certain *L. plantarum* and *L. pentosus* strains. The FCJM supplemented with cellobiose and gentiobiose, respectively, produced 98.00 ± 7.81 and 101.35 ± 9.07 mM lactic acid from the primary fermentation. Additionally, there were 43.11 ± 3.89 mM acetic acid in the FCJM that was added to adjust the initial fresh cucumber fermentation pH to 4.7 ± 0.1. Residual ethanol in the amount of 11.73 ± 4.20 and 8.10 ± 2.71 mM was also present in the FCJM prior to the supplementation with the disaccharides. It was found that *L. plantarum* converted 21.01 ± 0.08 mM cellobiose to 66.18 ± 1.88 mM lactic acid in the FCJM at pH 4.7 ± 0.1 (Table 3.4), suggesting the partial conversion of the cellobiose-derived-carbons to lactic acid. Similarly, *L. pentosus* converted cellobiose to 62.54 ± 1.95 mM lactic acid in FCJM at pH 4.7 (Table 3.4). Residual fructose was not removed from FCJM when it was supplemented with cellobiose (data not shown). Changes in the FCJM pH of 0.86 log units resulted in pH values of 3.84 ± 0.02. Utilization of cellobiose resulted in at least a 2 log increase in cell density (Table 3.4). A reduction in the cell concentration of the strains unable to utilize cellobiose was observed in FCJM at pH 4.7 ± 0.1 (Table 3.4).

Table 3.5 provides evidence for gentiobiose utilization by *L. plantarum* and *L. pentosus* in FCJM with an adjusted pH of 4.7 ± 0.1. Decreases in the gentiobiose content in FCJM with an adjusted pH of 4.7 ± 0.1 were observed at 5.25 ± 0.59 and 4.45 ± 1.98 mM for *L. plantarum* and

L. pentosus, respectively, regardless of oxygen availability and strain level differences. The *L. plantarum* and *L. pentosus* cultures produced 70.07 ± 7.56 and 55.69 ± 14.56 mM lactic acid (Table 3.5), suggesting a strict homofermentation when gentiobiose was supplemented to lower amounts in the FCJM as compared to the first experiment with 17.84 ± 3.14 mM (Table 3.3). It was expected that approximately 36 mM would be produced from the 8 mM gentiobiose supplemented, including the ~2 mM of the anomeric impurity in the sugar source, melibiose. *L. plantarum* and *L. pentosus* produced 22.25 ± 13.40 and 14.6 ± 9.0 mM lactic acid, respectively in the un-supplemented FCJM, raising the expected levels of lactic acid to 58 and 51 mM, respectively. As observed from the metabolism of cellobiose by *L. pentosus* and *L. plantarum* in FCJM at pH 4.7 ± 0.1, significant changes in pH to 3.82 ± 0.18 ($p<0.05$) were observed after gentiobiose was utilized (Table 3.5). The increases in cell densities ranged between 2 to 3 log CFU/mL as the result of gentiobiose utilization in FCJM with an adjusted pH of 4.7 ± 0.1 and were 1 log CFU/mL higher than those observed for cellobiose utilization under the same conditions (Tables 3.4 and 3.5).

3.5 Discussion

The goal of this study was to define the potential roles of cellobiose and gentiobiose as energy sources in cucumber fermentations for certain lactic acid bacteria. It was found that although cellobiose and gentiobiose had been detected in cucumber fermentation samples using two-dimensional gas chromatography-time-of-flight-mass spectrometry (GC x GC-TFMS) from cucumber fermentation samples (Johanningsmeier and McFeeters, 2011), no concentrations above the limit of detection were found in fresh and fermented cucumber samples using HPLC analysis here. The marked differences in the results is explained by either the sensitivity of the two techniques or fluctuations in the disaccharides content as a function of the fresh cucumber

physiological state, which was not considered in either study. Our results suggest that the lack of the disaccharides in the fresh fruits resulted in their absence in the fermentation and is thus not formed as the result of the bioconversion.

This study also showed that should the disaccharides be present in a cucumber fermentation as the result of factors not considered here, such as cucumber tissue degradation as a function of spoilage or the enzymatic hydrolysis of cellulose, they could be removed as a function of time by LAB. Despite some differences at the strain level, both *L. pentosus* and *L. plantarum*, which are the leading microbes in cucumber fermentations (Pérez-Díaz and others, 2016), are capable of utilizing cellobiose and gentiobiose as an energy source at a growth permissive pH as a function of time. This observation is in line with previous reports in the literature as the ability of both of these bacterial species to utilize cellobiose and gentiobiose with some variations in the strain level (Sterr and others, 2009; Soltan Dallal and others, 2017; Fitzsimons and others, 1999; Yu and others, 2012; Tamang and others, 2005; Siezen and Vlieg, 2011). Variations in cellobiose utilization by *L. plantarum* strains was predicted by the bioinformatics analysis (Table 3.2). The inability to predict the same for the *L. pentosus* species may be associated with the fact that 107 *L. plantarum* genomes were used for the analysis instead of 3 genomes for the former.

Although, it may still be possible that the disaccharides are utilized by *L. plantarum* and *L. pentosus* in FCJM with an adjusted pH of 3.7 ± 0.1 should an incubation period beyond seven days is provided, it seems unlikely. Growth of *L. plantarum* is known to stop at a pH of 3.3 with the cessation of acid production at a pH of 3.0 (McDonald and others, 1990). While β-glucosidases occur in many organisms, the activity of the enzymes derived from thermophilic bacteria and lactobacilli is known to be severely compromised at a pH of 4.0 with a 20% enzyme

stability, as compared to a 40% stability at pH 5.0 (Takase and Horikoshi, 1988; Kim and others, 2017; Zhong and others, 2016; Yeoman and others, 2010). Thus, a more reasonable interpretation is that *L. pentosus* and *L. plantarum* failed to utilize cellobiose and gentiobiose in FCJM at pH 3.7 given the lack of a β-glucosidase activity.

Only 75% of the cellobiose derived carbons were converted to lactic acid in the second experiment (Table 3.4), resulting in a slower utilization of cellobiose by LAB as compared to gentiobiose. This observation suggests that different mechanisms are used by LAB to ferment the disaccharides or is a reflection of differences in the PTS enzyme or the β-glucosidase affinity for the substrates. Indeed, LAB utilized gentiobiose as a substrate for heterofermentation when supplemented at the higher concentration in FCJM, which was not the case for cellobiose (Table 3.3). Higher cell densities were reached by most LAB as the result of gentiobiose utilization in a 7-day period as compared to those observed from the partial conversion of the cellobiose. However, the partial conversion of the cellobiose-derived-carbons to lactic acid generated enough energy for the cells to increase their concentration by at least 2-log CFU/mL (Table 3.4).

Inclusion of the gentiobiose anomeric form, melibiose, in this study was an unintended consequence associated with the use of the particular commercial preparation. The gentiobiose commercial preparation contained 15 % melibiose as an impurity (~1.5 to 3 mM in the FCJM). Given the magnitude of the products concentration as the result of gentiobiose utilization, it is speculated that melibiose was also utilized by the LAB tested. Melibiose is rarely present in nature (Gänzle and Follador, 2012). However, transport systems for melibiose are suspected in *L. plantarum* (Tamura and Matsushita, 1992).

While the utilization of gentiobiose by the LAB of interest in cucumber fermentations, such as *L. brevis*, *L. buchneri* and *P. pentosaceous*, was observed in FCJM with an adjusted pH

of 4.7 ± 0.1; *L. brevis* and *P. pentosaceous* were unable to utilize cellobiose as an energy source (Table 3.5). *L. brevis* and *P. pentosaceous* are competitors of *L. pentosus* and *L. plantarum* in cucumber fermentations but grow relatively slower (Pérez-Díaz and others, 2016). Perhaps the lack of competitiveness of *L. brevis* and *P. pentosaceous* in cucumber fermentations is associated with a restricted energy source utilization profile. These observations are in agreement with those made by others documenting the inability of *L. brevis*, *L. buchneri*, *L. spicheri*, some leuconostocs and *Enterococcus thailandicus* to utilize cellobiose (Carr and others, 2002; Sterr and others, 2009; Mao and others, 2015; Tamang and others, 2005; Hammes and Hertel, 2015). Contrary to *L. brevis* and *P. pentosaceous*, *L. buchneri* has been associated with spoilage of fermented cucumbers (Franco and others, 2012; Johanningsmeier and McFeeters, 2013). The fact that *L. buchneri*, a spoilage microbe in cucumber fermentations, is able to utilize both of the disaccharides tested confirms the need to design starter or adjunct cultures that are able to remove potential energy sources.

The LAB used in this study were able to utilize gentiobiose and cellobiose in FCJM suggesting that there are enough of other growth factors, such as amino acids, nucleosides, minerals, etc., in the fermented cucumber juice to sustain microbial proliferation. Furthermore, growth of *L. plantarum* and *L. pentosus* in the FCJM at pH 4.7 ± 0.1 was observed in the absence of added substrates. Two parameters were modified in the FCJM to enable microbial growth which were pH and the supplementation with an energy source. These observations confirm that the potential for spoilage in a given cucumber fermentation batch could be assessed by inoculating certain spoilage organisms in the corresponding FCJM as proposed by Fleming and others (1983). This approach represents a tool that could prevent important economic losses at the industrial scale production. More relevant is the understanding that batches of fermented

cucumbers are primarily stable during long term storage due to the development of an extremely acidic pH and to a lesser extent due to the lack of readily available energy sources. Both of these parameters can change as a function of microbial metabolism or enzymatic activities.

3.6 Conclusion

The magnitude of the natural content of gentibiose and cellobiose in fresh and fermented cucumbers and the utilization of the two disaccharides by certain LAB was determined. The plant-derived disaccharides were utilized by *L. plantarum*, *L. pentosus* and *L. buchneri* to variable extents in FCJM. *L. brevis* and *P. pentosaceous* were unable to utilize cellobiose efficiently in FCJM. Cellobiose was homofermentatively utilized by LAB at pH 4.7 ± 0.1 but not at 3.7 ± 0.1. Supplementation of gentiobiose to FCJM at pH 4.7 to 8 and 18 mM resulted in homo- and heterofermentations, respectively. Some strain level differences were observed with regards to cellobiose utilization, but not in the conversion of gentiobiose. *L. plantarum* and *L. pentosus* were able to proliferate in FCJM at pH 4.7 in the absence of added energy sources and produced between 15 and 46 mM lactic acid. The ability of the LAB of relevance to cucumber fermentations to utilize the disaccharides is not of industrial concern, given that the fruits are not a source in the fresh and fermented forms.

3.7 Acknowledgements

The authors thank Ms. Sandra Parker and Ms. Janet Hayes and Mr. Robert Price with the USDA-ARS Food Science Research Unit located in Raleigh, NC for administrative and technical support, respectively. The authors also thank the Turkish Government for the fellowship support for Redife Aslihan Ucar. No conflict of interests is declared.

3.8 References

Abdel-Rahman, M. A., Tashiro, Y., Zendo, T., Shibata, K., and Sonomoto, K. (2011). Isolation and characterisation of lactic acid bacterium for effective fermentation of cellobiose into optically pure homo L-(+)-lactic acid. *Applied Microbiology and Biotechnology*, *89*(4), 1039-1049.

Aleksandrzak-Piekarczyk, T., Polak, J., Jezierska, B., Renault, P., and Bardowski, J. (2011). Genetic characterization of the CcpA-dependent, cellobiose-specific PTS system comprising CelB, PtcB and PtcA that transports lactose in *Lactococcus lactis* IL1403. *International Journal of Food Microbiology*, *145*(1), 186-194.

Andersen, J. M., Barrangou, R., Hachem, M. A., Lahtinen, S. J., Goh, Y. J., Svensson, B., and Klaenhammer, T. R. (2012). Transcriptional analysis of prebiotic uptake and catabolism by *Lactobacillus acidophilus* NCFM. *PLoS One*, *7*(9), e44409.

Bardowski, J., Ehrlich, S. D., and Chopin, A. (1994). BglR protein, which belongs to the BglG family of transcriptional antiterminators, is involved in beta-glucoside utilization in *Lactococcus lactis*. *Journal of Bacteriology*, *176*(18), 5681-5685.

Bardowski, J., Ehrlich, S. D., and Chopin, A. (1995). A protein, belonging to a family of RNA-binding transcriptional anti-terminators, controls beta-glucoside assimilation in *Lactococcus lactis*. *Developments in Biological Standardization*, *85*, 555.

Barrangou, R., Azcarate-Peril, M. A., Duong, T., Conners, S. B., Kelly, R. M., and Klaenhammer, T. R. (2006). Global analysis of carbohydrate utilization by *Lactobacillus acidophilus* using cDNA microarrays. *Proceedings of the National Academy of Sciences*, *103*(10), 3816-3821.

Brenner, D.J., Staley, J.T., and Krieg, N.R. (2006). Bergey's Manual® of Systematic

Bacteriology. The *Proteobacteria*. 2, 27-32.

Buckenhüskes, H.J. (1997) Fermented vegetables. *In Food Microbiology: Fundamentals and Frontiers*, 2nd edition Doyle, P.D. Beuchat, L.R. and Montville, T.J., Washington, D.C.: ASM Press, 595–609.

Carr, F. J., Chill, D., and Maida, N. (2002). The lactic acid bacteria: a literature survey. *Critical Reviews in Microbiology*, *28*(4), 281-370.

Caspi, R., Billington, R., Fulcher, C.A., Keseler, I.M., Kothari, A., Krummenacker, M., Latendresse, M., Midford, P.E., Ong, Q., Ong, W.K. and Paley, S. (2017). The MetaCyc database of metabolic pathways and enzymes. *Nucleic Acids Research*, *46*(D1), D633-D639.

Chen, I.M.A., Markowitz, V.M., Chu, K., Palaniappan, K., Szeto, E., Pillay, M., Ratner, A., Huang, J., Andersen, E., Huntemann, M. and Varghese, N. (2016). IMG/M: integrated genome and metagenome comparative data analysis system. *Nucleic Acids Research*, gkw929.

Dunn, M. S., Shankman, S., Camien, M. N., and Block, H. (1947). The amino acid requirements of twenty-three lactic acid bacteria. *Journal of Biological Chemistry*, *168*(1), 1-22.

Fitzsimons, N. A., Cogan, T. M., Condon, S., and Beresford, T. (1999). Phenotypic and genotypic characterization of non-starter lactic acid bacteria in mature cheddar cheese. *Applied and Environmental Microbiology*, *65*(8), 3418-3426.

Fleming, H. P., McFeeters, R. F., and Thompson, R. L. (1983). Test for susceptibility of fermented vegetables to secondary fermentation. *Journal of Food Science*, *48*(3), 982-983.

Fleming, H. P., McFeeters, R. F., Daeschel, M. A., Humphries, E. G., and Thompson, R. L. (1988). Fermentation of cucumbers in anaerobic tanks. *Journal of Food Science*, *53*(1), 127-133.

Francl, A. L., Thongaram, T., and Miller, M. J. (2010). The PTS transporters of *Lactobacillus gasseri* ATCC 33323. *BMC Microbiology*, *10*(1), 77.

Franco, W., Pérez-Díaz, I. M., Johanningsmeier, S. D., and McFeeters, R. F. (2012). Characteristics of spoilage-associated secondary cucumber fermentation. *Applied and Environmental Microbiology*, *78*(4), 1273-1284.

Franco, W., and Pérez-Díaz, I. M. (2012). Role of selected oxidative yeasts and bacteria in cucumber secondary fermentation associated with spoilage of the fermented fruit. *Food Microbiology*, *32*(2), 338-344.

Fred, E. B., Peterson, W. H., and Anderson, J. A. (1921). The characteristics of certain pentose destroying bacteria, especially as concerns their action on arabinose and xylose. *Journal of Biological Chemistry*, *48*(2), 385-412.

Gänzle, M. G. (2015). Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Current Opinion in Food Science*, *2*, 106-117.

Gänzle, M., and Follador, R. (2012). Metabolism of oligosaccharides and starch in *lactobacilli*: a review. *Frontiers in Microbiology*, *3*, 340.

Hammes, W. P., and Hertel, C. (2015). *Lactobacillus*. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1-76.

Hols, P., Slos, P., Dutot, P., Reymund, J., Chabot, P., Delplace, B., Delcour, J. and Mercenier, A. (1997). Efficient secretion of the model antigen M6-gp41E in *Lactobacillus plantarum* NCIMB 8826. *Microbiology*, *143*(8), 2733-2741.

Johanningsmeier, S. D., and McFeeters, R. F. (2011). Detection of volatile spoilage metabolites in fermented cucumbers using nontargeted, comprehensive 2-dimensional gas chromatography-time-of-flight mass spectrometry (GC× GC-TOFMS). *Journal of Food Science*, *76*(1), C168-C177.

Johanningsmeier, S. D., and McFeeters, R. F. (2013). Metabolism of lactic acid in fermented cucumbers by *Lactobacillus buchneri* and related species, potential spoilage organisms in reduced salt fermentations. *Food Microbiology*, *35*(2), 129-135.

Johanningsmeier, S. D., and McFeeters, R. F. (2015). Metabolic footprinting of *Lactobacillus buchneri* strain LA1147 during anaerobic spoilage of fermented cucumbers. *International Journal of Food Microbiology*, *215*, 40-48.

Karp, P.D., Billington, R., Caspi, R., Fulcher, C.A., Latendresse, M., Kothari, A., Keseler, I.M., Krummenacker, M., Midford, P.E., Ong, Q. and Ong, W.K. (2017). The BioCyc collection of microbial genomes and metabolic pathways. *Briefings in Bioinformatics*.

Kim, Y. S., Lee, C. J., and Ma, J. Y. (2017). Enhancement of active compound, genipin, from Gardeniae Fructus using immobilized glycosyl hydrolase family 3 β-glucosidase from Lactobacillus antri. *Amb Express*, *7*(1), 64.

Lu, Z., Fleming, H. P., and McFeeters, R. F. (2002). Effects of fruit size on fresh cucumber composition and the chemical and physical consequences of fermentation. *Journal of Food Science*, *67*(8), 2934-2939.

Mao, Y., Chen, M., and Horvath, P. (2015*). Lactobacillus herbarum* sp. nov., a species related to *Lactobacillus plantarum*. *International Journal of Systematic and Evolutionary Microbiology*, *65*(12), 4682-4688.

McDonald, L. C., Fleming, H. P., and Hassan, H. M. (1990). Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, *56*(7), 2120-2124.

McFeeters, R. F. (1993). Single-injection HPLC analysis of acids, sugars, and alcohols in cucumber fermentations. *Journal of Agricultural and Food Chemistry*, *41*(9), 1439-1443.

McFeeters, R. F., and Barish, A. O. (2003). Sulfite analysis of fruits and vegetables by high-performance liquid chromatography (HPLC) with ultraviolet spectrophotometric detection. *Journal of Agricultural and Food Chemistry*, *51*(6), 1513-1517.

Mundt, J. O., Beattie, W. G., and Wieland, F. R. (1969). *Pediococci* residing on plants. *Journal of Bacteriology*, *98*(3), 938-942.

Pattee, H. E., Isleib, T. G., Giesbrecht, F. G., and McFeeters, R. F. (2000). Investigations into genotypic variations of peanut carbohydrates. *Journal of Agricultural and Food Chemistry*, *48*(3), 750-756.

Pérez-Díaz, I.M., Breidt, F., Buescher, R.W., Arroyo-López, F.N., Jiménez-Dıaz, R., Fernandez, A.G., Gallego, J.B., Yoon, S.S., Johanningsmeier, S.D. (2016). Fermented and acidified vegetables. *Compendium of Methods for the Microbiological Examination of Foods,* 4th edn. Washington, D.C.: American Public Health Association, 521-32.

Rogosa, M., Franklin, J. G., & Perry, K. D. (1961). Correlation of the vitamin requirements with cultural and biochemical characters of *Lactobacillus* spp. *Microbiology*, *25*(3), 473-482.

Rogosa, M., and Hansen, P. A. (1971). Nomenclatural considerations of certain species of *Lactobacillus Beijerinck*. *International Journal of Systematic and Evolutionary Microbiology*, *21*(2), 177-186.

Schnetz, K., Toloczyki, C., and Rak, B. (1987). Beta-glucoside (bgl) operon of *Escherichia coli* K-12: nucleotide sequence, genetic organization, and possible evolutionary relationship to regulatory components of two *Bacillus subtilis* genes. *Journal of Bacteriology*, *169*(6), 2579-2590.

Siezen, R. J., and van Hylckama Vlieg, J. E. (2011). Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. In: Microbial cell factories. *BioMed Central, 10*(1), S3.

Singhvi, M., Joshi, D., Adsul, M., Varma, A., and Gokhale, D. (2010). d-(−)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-2 4. *Green Chemistry*, *12*(6), 1106-1109.

Smokvina, T., Wels, M., Polka, J., Chervaux, C., Brisse, S., Boekhorst, J., van Hylckama Vlieg, J.E. and Siezen, R.J. (2013). Lactobacillus paracasei comparative genomics: towards species pan-genome definition and exploitation of diversity. *PloS One*, *8*(7), e68731.

Soltan Dallal, M.M., Zamaniahari, S., Davoodabadi, A., Hosseini, M., and Rajabi, Z. (2017). Identification and characterization of probiotic lactic acid bacteria isolated from traditional persian pickled vegetables. *GMS hygiene and infection control*, *12*.

Sterr, Y., Weiss, A., and Schmidt, H. (2009). Evaluation of lactic acid bacteria for sourdough fermentation of amaranth. *International Journal of Food Microbiology*, *136*(1), 75-82.

Takase, M., and Horikoshi, K. (1988). A thermostable β-glucosidase isolated from a bacterial species of the genus *Thermus*. *Applied Microbiology and Biotechnology*, *29*(1), 55-60.

Tamang, J. P., Tamang, B., Schillinger, U., Franz, C. M., Gores, M., and Holzapfel, W. H. (2005). Identification of predominant lactic acid bacteria isolated from traditionally

fermented vegetable products of the Eastern Himalayas. *International Journal of Food Microbiology, 105*(3), 347-356.

Tamura, C., Matsushita, O. (1992). Melibiose transport system in *Lactobacillus plantarum*. *Microbiology and Immunology, 36*(11):1119-28.

Tanganurat, W., Quinquis, B., Leelawatcharamas, V., Bolotin, A. (2009). Genotypic and phenotypic characterization of *Lactobacillus plantarum* strains isolated from Thai fermented fruits and vegetables. *Journal of Basic Microbiology*, 49(4), 377-85.

Tobisch, S., Glaser, P., Krüger, S., and Hecker, M. (1997). Identification and characterization of a new beta-glucoside utilization system in *Bacillus subtilis*. *Journal of Bacteriology*, *179*(2), 496-506.

Yeoman, C. J., Han, Y., Dodd, D., Schroeder, C. M., Mackie, R. I., and Cann, I. K. (2010). Thermostable enzymes as biocatalysts in the biofuel industry. In *Advances in Applied Microbiology*, *70*, 1-55.

Yu, J., Gao, W., Qing, M., Sun, Z., Wang, W., Liu, W., Pan, L., Sun, T., Wang, H., Bai, N. and Zhang, H. (2012). Identification and characterization of lactic acid bacteria isolated from traditional pickles in Sichuan, China. *The Journal of General and Applied Microbiology*, *58*(3), 163-172.

Zhong, F.L., Ma, R., Jiang, M., Dong, W.W., Jiang, J., Wu, S., Li, D. and Quan, L.H. (2016). Cloning and characterization of ginsenoside-hydrolyzing β-glucosidase from *Lactobacillus brevis* that transforms ginsenosides Rb1 and F2 into ginsenoside Rd and compound K. *Journal of Microbiology and Biotechnology*, *26*(10), 1661-1667.

Table 3.1. Description of the lactic acid bacteria strains used in this study.

Genus	Species	ID Number(s)	Sources	Reference
Lactobacillus	*plantarum*	LA0070; ATCC 14917	Pickle cabbage	ATCC < PA Hansen < Roy Techn. Coll., Copenhagen < S. Orla-Jensen (*Streptobacterium plantarum*) https://www.atcc.org/products/all/14917.aspx#history
Lactobacillus	*plantarum*	LA1196; ATCC BAA-793; NCIMB 8826; WCFS1	Saliva	Hols and others, 1997
Lactobacillus	*plantarum*	3.2.8	Commercial cucumber fermentation	Pérez-Díaz and others, 2016
Lactobacillus	*pentosus*	LA0233; ATCC 8041	Sauerkraut	Fred and others, 1921
Lactobacillus	*pentosus*	LA0445; BI0007, MOP3	Commercial cucumber fermentation	Fleming and others, 1988
Lactobacillus	*pentosus*	1.8.9	Commercial cucumber fermentation	Pérez-Díaz and others, 2016
Lactobacillus	*brevis*	LA0200; ATCC 8287	Green Sevillano fermenting olives	RH Vaughn 269Y; Dunn and others, 1947
Lactobacillus	*brevis*	LA0036; ATCC 14869, NRRL B-4527	Human feces	ATCC < PA Hansen < Roy. Techn. Coll., Copenhagen < S. Orla-Jensen 14 (*Betabacterium breve*); Rogosa and Hansen, 1971
Lactobacillus	*brevis*	7.2.43	Commercial cucumber fermentation	Pérez-Díaz and others, 2016
Lactobacillus	*buchneri*	LA0030; ATCC 4005, NRRL B1837	Tomato pulp	Rogosa and Hansen, 1971
Lactobacillus	*buchneri*	LA1149	Commercial cucumber fermentation	Franco and others, 2012
Lactobacillus	*buchneri*	LA1147; E-33-07	Commercial cucumber fermentation	Franco and others, 2012

Table 3.1 (continued)

Pediococcus	*pentosaceus*	LA0073; ATCC 25745	Plant material	Mundt and others, 1969
Pediococcus	*pentosaceus*	LA0076; ATCC 33316	Dried beer yeast	NCDO; Skerman and others, 1980
Pediococcus	*pentosaceus*	3.2.19	Commercial cucumber fermentation	Pérez-Díaz and others, 2016

Table 3.2 Bioinformatic analysis of the cellobiose and gentiobiose putative pathways in certain lactic acid bacteria of relevance to cucumber fermentations. The colored boxes mark the number of strains coding for a specific putative enzyme where red, blue, green and yellow represent more than 97%, between 97 and 5%, less than 5% and missing genes, respectively.

L. plantarum	*L. pentosus*	*P. pentosaceous*	*L. brevis*	*L. buchneri*	Enzyme Name	EC No.	Metabolic Pathway	Target	Expected Product
Species Analyzed									
					Cellobiase	3.2.1.21	Starch & Sucrose	Cellobiose	B-D- glucose
					Phosphotransferase	2.7.1.69	Glycolysis	Cellobiose & Glucose	Lactic Acid
					Phosphoglucomutase	5.4.2.2			
					Glucokinase	2.7.1.2			
					Aldose 1-Epimerase	5.1.3.3			
					Glucose-6-Phosphate Isomerase	5.3.1.9			
					6-Phosphofructokinase	2.7.1.11			
					Hexose Diphosphatase	3.1.3.11			
					Aldolase	4.1.2.13			
					Triose-Phosphate Isomerase	5.3.1.1			
					Glyceraldehyde-3-Phosphatase	1.2.1.12			
					Phosphoglycerate Kinase	2.7.2.3			
					Phosphoglycerate Mutase	5.4.2.11			
					Phosphoglycerate Mutase	5.4.2.12			
					Phosphopyruvate Hydratase	4.2.1.11			
					Pyruvate Kinase	2.7.1.40			

Table 3.3. Utilization of cellobiose and gentiobiose by certain lactic acid bacteria in Fermented Cucumber Juice Medium (FCJM) with an adjusted pH of 4.7 ± 0.1. Cellobiose and gentiobiose were supplemented in FCJM to 15.81 ± 0.23 and 17.81 ± 3.14 mM, respectively. It is estimated that the gentiobiose source utilized contained at least 3 mM of the α-anomer, melibiose.

Cellobiose					
	L. plantarum	***L. pentosus***	***L. brevis***	***L. buchneri***	***P. pentosaceous & L. brevis***
Remaining Cellobiose (mM)	BDL	BDL	14.50 ± 1.15^{a}	BDL	13.97 ± 1.00^{a}
Lactic Acid Produced (mM)	118.36 ± 14.83^{a}	59.20 ± 4.16^{b}	18.25 ± 3.59^{c}	14.35 ± 5.33^{c}	38.54 ± 12.15^{d}
Acetic Acid Produced (mM)	BDL	BDL	BDL	BDL	BDL
Final pH	3.68 ± 0.01^{a}	3.95 ± 0.09^{b}	3.95 ± 0.04^{b}	4.53 ± 0.04^{c}	4.15 ± 0.05^{d}
Growth (Log CFU/mL)	≥ 8	≥ 8	< 4	8.63 ± 0.01	< 4
Gentiobiose					
Remaining Gentiobiose (mM)	BDL	BDL	BDL	BDL	2.06
Lactic Acid Produced (mM)	102.07 ± 14.07^{a}	88.43 ± 6.02^{b}	65.64 ± 4.66^{c}	66.86 ± 5.13^{c}	67.54 ± 6.73^{c}
Acetic Acid Produced (mM)	BDL	31.54 ± 2.48^{a}	33.59 ± 2.79^{a}	40.85 ± 2.22^{b}	24.13 ± 6.65^{c}
Ethanol Produced (mM)	BDL	8.39 ± 0.39^{a}	9.27 ± 0.42^{a}	3.92 ± 0.14^{b}	BDL
Final pH	3.98 ± 0.00^{a}	4.08 ± 0.01^{a}	4.37 ± 0.01^{b}	4.39 ± 0.01^{b}	4.36 ± 0.03^{b}
Growth (Log CFU/mL)	≥ 8	≥ 8	≥ 8	8.69 ± 0.06	8.4 ± 0.05

*The results in the columns within blocks followed by the same alphabets (a, b, c, and d) indicate that they are not significantly different ($p>0.05$) from each other and the treatments with the different alphabets indicate that they are significantly different ($p<0.05$) as statistically.

Table 3.4. Utilization of cellobiose by *Lactobacillus pentosus* and *Lactobacillus plantarum* in Fermented Cucumber Juice Medium with an adjusted pH of 4.7. The amount of cellobiose supplemented was 21.01 ± 0.08 mM.

Lactobacillus plantarum						
	Anaerobic Incubation			**Aerobic Incubation**		
Strain Identification	**ATCC14917**	**WCFS1**	**3.2.8**	**ATCC14917**	**WCFS1**	**3.2.8**
Cellobiose Utilized (mM)	20.45 ± 0.3^{a}	0.15 ± 0.07^{b}	20.45 ± 0.023^{a}	1.14 ± 0.03^{b}	0.04 ± 0.3^{b}	21.09 ± 0.31^{a}
Lactic Acid Produced (mM)	44.17 ± 27.3^{a}	BDL	66.47 ± 4.61^{b}	BDL	BDL	67.89 ± 1.84^{b}
Final pH	3.85 ± 0.03^{a}	4.7 ± 0.00^{b}	3.82 ± 0.08^{a}	4.69 ± 0.007^{b}	4.69 ± 0.014^{b}	3.81 ± 0.007^{a}
Colony Counts from MRS agar (Log CFU/mL)	7.46 ± 0.09^{a}	BDL	7.57 ± 0.09^{b}	BDL	BDL	7.86 ± 0.08^{c}
Lactobacillus pentosus						
	Anaerobic Incubation			**Aerobic Incubation**		
Strain Identification	**ATCC8041**	**MOP3**	**1.8.9**	**ATCC8041**	**MOP3**	**1.8.9**
Cellobiose Utilized (mM)	0.66 ± 0.31^{a}	19.38 ± 0.85^{b}	BDL	BDL	21.09 ± 0.01^{b}	19.80 ± 0.9^{b}
Lactic Acid Produced (mM)	BDL	32.72 ± 5.2^{a}	BDL	BDL	64.40 ± 3.22^{b}	60.51 ± 2.19^{b}
Final pH	4.68 ± 0.03^{a}	3.86 ± 0.04^{b}	4.68 ± 0.01^{a}	4.7 ± 0.03^{a}	3.85 ± 0.01^{b}	4.28 ± 0.01^{c}
Colony Counts from MRS agar (Log CFU/mL)	BDL	7.2 ± 0.11^{a}	BDL	BDL	7.6 ± 0.09^{b}	7.21 ± 0.04^{a}

*****The results in the rows within blocks followed by the same alphabets (a, b, and c) indicate that they are not significantly different ($p>0.05$) from each other and the treatments with the different alphabets indicate that they are significantly different ($p<0.05$) as statistically.

Table 3.5. Utilization of gentiobiose by *Lactobacillus pentosus* and *Lactobacillus plantarum* in Fermented Cucumber Juice Medium with an adjusted pH of 4.7 ± 0.1. Gentiobiose was supplemented into FCJM to 8.05 ± 0.1 mM. It is estimated that the gentiobiose source utilized contained at least 1.5 mM of the α-anomer, melibiose.

*The results in the rows within blocks followed by the same alphabets (a, b, and c) indicate that they are not significantly different ($p>0.05$) from each other and the treatments with the different alphabets indicate that they are significantly different ($p<0.05$) as statistically.

Lactobacillus plantarum						
	Anaerobic Incubation			**Aerobic Incubation**		
Strain Identification	ATCC14917	WCFS1	3.2.8	ATCC14917	WCFS1	3.2.8
Gentiobiose Utilized (mM)	5.15 ± 2.70^{a}	4.25 ± 3.11^{b}	4.95 ± 2.42^{a}	5.73 ± 2.18^{b}	5.62 ± 1.77^{c}	5.77 ± 2.07^{b}
Lactic Acid Produced (mM)	72.95 ± 3.53^{a}	78.09 ± 20.41^{b}	71.66 ± 10.30^{a}	62.12 ± 6.27^{c}	60.37 ± 6.59^{c}	66.25 ± 4.38^{c}
Final pH	3.76 ± 0.05^{a}	3.83 ± 0.06^{a}	3.86 ± 0.04^{a}	3.77 ± 0.00^{a}	3.8 ± 0.08^{a}	3.7 ± 0.06^{a}
Colony Counts from MRS Agar (Log CFU/mL)	8.58 ± 0.12^{a}	8.33 ± 0.12^{a}	8.48 ± 0.08^{a}	8.53 ± 0.07^{a}	8.64 ± 0.06^{a}	8.69 ± 0.04^{a}
Lactobacillus pentosus						
	Anaerobic Incubation			**Aerobic Incubation**		
Strain Identification	ATCC8041	MOP3	1.8.9	ATCC8041	MOP3	1.8.9
Gentiobiose Utilized (mM)	3.24 ± 1.83^{a}	1.24 ± 0.67^{b}	4.85 ± 2.42^{c}	5.00 ± 2.45^{c}	7.00 ± 0.67^{d}	5.38 ± 2.04^{c}
Lactic Acid Produced (mM)	38.15 ± 22.82^{a}	51.39 ± 8.50^{b}	71.54 ± 14.24^{c}	55.47 ± 3.38^{b}	58.36 ± 6.75^{b}	59.12 ± 2.78^{b}
Final pH	4.02 ± 0.06^{a}	4.3 ± 0.007^{b}	3.81 ± 0.007^{c}	3.67 ± 0.014^{d}	3.65 ± 0.00^{d}	3.66 ± 0.007^{d}
Colony Counts from MRS Agar (Log CFU/mL)	8.73 ± 0.13^{a}	7.27 ± 0.14^{b}	8.28 ± 0.14^{c}	8.69 ± 0.05^{a}	8.32 ± 0.07^{c}	8.59 ± 0.03^{a}

CHAPTER 4

Conclusions and Future Directions

Utilization of organic compounds is the main energy generating metabolic activity in lactic acid bacteria (LAB). Significant variability in the ability to utilize various carbohydrate sources has been reported at the strain level for this group of industrially relevant microbes (Yildiz and Wiley, 2017). It was the objective of this exploratory research to evaluate the carbohydrate utilization ability of a selected group of LAB including *Lactobacillus plantarum, L. pentosus, L. brevis, L. buchneri* and *Pediococcus pentosaceous*, isolated from commercial cucumber fermentations. The investigation of the carbohydrate utilization profile has a crucial role in selecting starter cultures that can prevent the development of spoilage in industrial cucumber fermentations. Organic compounds found in cucumber fermentations including glucose, fructose, xylose, trehalose, cellobiose, furfural, gentiobiose, lyxose, and citrulline were targeted in this study (Johanningsmeier and McFeeters, 2015). The presence or absence of those potential alternate energy sources in the fresh cucumber and cucumber fermentation was confirmed using HPLC analysis. Only trehalose, xylose, and citrulline were found to exist in fresh cucumbers and/or commercial cucumber fermentations during the first 3 days. Utilization of such alternate energy sources by LAB was explored. The utilization patterns experimentally observed were compared to the putative pathways present in selected LAB. Results indicate that some of the species contained almost all genes encoding the enzymes involved in the multiple pathways, whereas some species lack genes for the same pathways. It was found that the metabolic regulation of the potential secondary energy sources is strain dependent.

The effect of the initial pH different culture medium on the utilization of xylose, trehalose and citrulline by selected LAB was investigated. Based on the results of the HPLC analysis, final pH, and colony counts from MRS plates, it was concluded that *L. plantarum* and *L. pentosus* are unable to utilize xylose. Trehalose was utilized by all the LAB tested (*L.*

plantarum, L. pentosus and *P. pentosaceous & L. brevis*), except *L. buchneri* and that *L. buchneri* was unique in utilizing citrulline when the initial medium pH was adjusted to 4.7. Citrulline utilization by *L. buchneri* in the presence of limiting and excess glucose was studied in fermented cucumber juice medium (FCJM) as well. The results suggested that when excess glucose was present in the medium, *L. buchneri* was able to derive more energy than when citrulline was present alone in FCJM at a pH of 3.7.

Removal of trehalose, xylose, and citrulline from a fresh cucumber juice model system by *L. pentosus*, *L. brevis*, and *L. buchneri* was studied. It was hypothesized that the activity of such potential energy sources early in the fermentation could repress the growth of spoilage associated microorganisms during long term bulk storage of fermented cucumbers. One of the more significant findings was that *L. brevis* is partially able to utilize trehalose, xylose, and citrulline in a fresh cucumber juice medium concomitantly with glucose and fructose within a 60 days period. However, a combination of *L. brevis* and *L. buchneri* results in a more complete removal of the energy sources. It was also found that the inoculation of un-supplemented fresh cucumber juice medium with *L. pentosus*, *L. brevis* and *L. buchneri* altogether resulted in a complete fermentation free of citrulline after about 15 days that was stable for up to 60 days.

Although cellobiose and gentiobiose were not detected in fresh or fermented cucumbers, the ability of selected LAB to utilize such alternate energy sources was studied. Cellobiose and gentiobiose were utilized by *L. plantarum*, *L. pentosus*, and *L. buchneri* to variable extents, however, *L. brevis* and *P. pentosaceous* were unable to utilize cellobiose efficiently in FCJM at an initial medium pH of 4.7. Utilization of the plant-derived disaccharides by the potential starter culture under aerobic and anaerobic conditions at an initial medium pH of 4.7 or 3.7 was also studied. Three strains of *L. plantarum* and *L. pentosus* were included in this laboratory scale

study. The gentiobiose and cellobiose utilization ability by three different strains of *L. plantarum* and *L. pentosus* was obtained at variable rates at pH 4.7 and seemed to be independent of the presence of air. No utilization of the substrates was observed at a pH of 3.7.

In addition, the ability of selected LAB to utilize furfural and lyxose was investigated although none of these two compounds were found in fresh or fermented cucumbers (Tables 4.1 and 4.2). Experiment 1 was designed to evaluate the ability of selected LAB to utilized furfural in FCJM at pH of 4.7 suggested that *L. brevis* and *L. buchneri* were able to remove furfural (Table 4.1). However, substantial changes in lactic acid, ethanol or acetic acid produced were not observed by *L. brevis* nor *L. buchneri* (Table 4.1). Experiment 2 was designed to evaluate the ability of specific strains of *L. plantarum* and *L. pentosus* to utilize furfural supplemented in FCJM at pH 4.7 under anaerobiosis and aerobiosis and showed that none of the strains removed furfural from the FCJM. However, the LAB were able to produce some lactic acid and acetic acid, slightly decrease the pH and proliferate (Tables 4.2 and 4.3). The measurements of furfural from FCJM were inconsistent and variable among independent replicates (Tables 4.1, 4.2 and 4.3). Furfural may be produced from xylose and other pentoses (Agirrezabal-Tellaria and others, 2014; Takagaki and others, 2010). Thus, it is possible that the xylose naturally present in the FCJM was converted to furfural inducing inconsistencies in the data derived from the HPLC analysis performed to measure such a compound.

Samples were analyzed as described in Li and others (2009). Three milliliters of broth were loaded onto a PEP-SPE column (500 mg/6 mL, Thermo Scientific, Rockwood, TN). The column was pre-conditioned with 5 mL of acetonitrile (Thermo Scientific, Fair Lawn, NJ) followed by 5 mL of water. Samples were allowed to load onto the column with gravity. Columns were then washed with 5 mL of water and the eluent discarded. The column was then

eluted with 3 mL of acetonitrile. The solvent containing the analyte was then analyzed by HPLC against a standard curve of authentic furfural (Sigma Aldrich, St. Louis, MO). The HPLC was a Thermo Surveyor (Thermo Finnigan, San Jose, CA) equipped with a photo diode array detector. The column was a Supelco LiChrosorb C18 (250 mm length, 4.6 mm i.d., 5 micron, Supelco, St. Louis, MO). The column oven was maintained at 30°C and the detector was set to 280 nm. The flow rate was 1.0 mL/min. Mobile phase A was 0.01% trifluoroacetic acid (TFA; Sigma Aldrich) in water and mobile phase B was 100% acetonitrile. The gradient was 95% A and 5% B for 16 min and then increased to 60% B in 1.0 min and held for 3.0 min. B was then decreased to 5% and the system was re-equilibrated for 8 min. The injection volume was 10 microliters. Data was collected and analyzed using the Xcalibur software (Thermo Finnegan) (Li and others, 2009).

Although, the volatilization of furfural could have introduced error in the analysis, the changes observed in furfural concentrations were above those supplemented in the FCJM not below (Bustos and others, 2005). Furfural could also react chemically (Harada and others, 2017). The fact that strains of *L. plantarum* and *L. pentosus* are able to proliferate in the presence of furfural in FCJM at an initial pH of 4.7 under aerobic and anaerobic conditions without removing the compound suggests it is not inhibitory of growth (Tables 4.1 and 4.2). No growth was observed in FCJM supplemented with furfural at pH 3.7 (data not shown). Together these observations suggest that furfural does not serves as an energy source for the LAB included in this test. Similarly the presence of lyxose did not enhance growth of LAB in FCJM.

This research presented an opportunity to explore the prevention of fermented cucumber spoilage by the utilization of starter cultures able to remove citrulline, trehalose and xylose from commercial fermentations. Future research should be undertaken to explore how the potential

energy sources for spoilage associated microorganisms can be removed efficiently from the media. Further experiments, using a broader range of strains or environmental conditions, could shed more light on the reasons of the spoilage in the cucumber fermentation.

4.1 References

Agirrezabal-Telleria, I., Gandarias, I., and Arias, P. L. (2014). Heterogeneous acid-catalysts for the production of furan-derived compounds (furfural and hydroxymethylfurfural) from renewable carbohydrates: A review. *Catalysis Today*, *234*, 42-58.

Bustos, G., Moldes, A. B., Cruz, J. M., and Domínguez, J. M. (2005). Influence of the metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots hydrolyzates using *Lactobacillus pentosus*. *Biotechnology Progress*, *21*(3), 793-798.

Harada, R., Yuzuki, M., Ito, K., Shiga, K., Bamba, T., and Fukusaki, E. (2017). Influence of yeast and lactic acid bacterium on the constituent profile of soy sauce during fermentation. *Journal of Bioscience and Bioengineering*, *123*(2), 203-208.

Johanningsmeier, S. D., and McFeeters, R. F. (2015). Metabolic footprinting of *Lactobacillus buchneri* strain LA1147 during anaerobic spoilage of fermented cucumbers. *International Journal of Food Microbiology*, *215*, 40-48.

Li, M., Yang, Z., Yang, M., Shan, L., and Dong, J. (2009). Determination of furfural in beer by high-performance liquid chromatography with solid-phase extraction. *Journal of the Institute of Brewing*, *115*(3), 226-231.

Takagaki, A., Ohara, M., Nishimura, S., and Ebitani, K. (2010). One-pot formation of furfural from xylose via isomerization and successive dehydration reactions over heterogeneous acid and base catalysts. *Chemistry Letters*, *39*(8), 838-840.

Yildiz, F., and Wiley, R. C. (Eds.). (2017). *Minimally processed refrigerated fruits and vegetables*, New York, NY: Springer US, 537-587. DOI 10.1007/978-1-4939-7018-6.

Table 4.1. Utilization of furfural by selected lactic acid bacteria in fermented cucumber juice medium (FCJM) with an initial pH of 4.7 ± 0.1. Furfural was not present in FCJM and was supplemented to 15.48 ± 0.12 mM. The FCJM pH was adjusted to 4.71 ± 0.01 and the cultures were inoculated to 5 log CFU/mL. Minimal limit of detection for furfural was < 0.001 mM.

LAB Tested ►	***L. plantarum***	***L. pentosus***	***L. brevis***	***L. buchneri***	***P. pentosaceous & L. brevis***
Remaining Concentrations (mM)					
Furfural	13.65 ± 0.06	12.56 ± 2.04	0.83 ± 1.07	BDL	BDL
Glucose	-----	-----	-----	-----	-----
Fructose	1.84 ± 0.02	1.40 ± 0.07	2.00 ± 0.07	1.70 ± 0.01	3.22 ± 2.10
Total Concentrations Produced (mM)					
Lactic Acid	136.29 ± 6.62	117.46 ± 7.90	132.22 ± 2.89	124.31 ± 13.51	123.58 ± 0.21
Acetic Acid	32.30 ± 6.91	28.23 ± 1.86	40.24 ± 6.65	34.02 ± 4.88	29.73 ± 9.08
Ethanol	5.93 ± 0.76	5.65 ± 1.25	7.92 ± 0.18	5.24 ± 1.41	4.93 ± 2.04
pH and Growth					
Final pH	4.30	4.32	4.475	4.51	4.415
Growth (Log CFU/mL)	≥ 8	≥ 8	≥ 8	8.49 ± 0.01	7.46 ± 0.05

* BDL: below detection level

Table 4.2. Utilization of furfural by strains of *Lactobacillus plantarum* under anaerobiosis (top panel) and aerobiosis (bottom panel) in fermented cucumber juice medium (FCJM) with an initial pH of 4.7 ± 0.1. Furfural was not present in FCJM and was supplemented to 15.48 ± 0.12 mM. The FCJM pH was adjusted to 4.71 ± 0.01 and the cultures were inoculated to 5 log CFU/mL. Cultures were incubated under anaerobic conditions for 7 days. Changes in ethanol concentration were not observed. Initial glucose and fructose concentrations were 0.86 ± 0.04 and 7.78 ± 0.47, respectively. Glucose was completely utilized after the 7 days of incubation by all the strains tested (data not shown). Less than 2.23 ± 1.11 fructose remained in the inoculated FCJM after 7 days of incubation (data not shown). Data presented is the average of independent replicates. Minimal limit of detection for furfural was < 0.001 mM.

Strains Tested ►		***L. plantarum LA0070***	***L. plantarum 3.2.8***	***L. plantarum LA1196***	**Non-Inoculated Control**
Anaerobiosis					
Furfural	INITIAL	7.16 ± 0.76	7.16 ± 0.76	7.16 ± 0.76	7.16 ± 0.76
	LAST	15.03 ± 8.25	8.68 ± 0.85	18.83 ± 14.48	1.29 ± 0.46
Lactic Acid	INITIAL	96.15 ± 16.05	96.15 ± 16.05	96.15 ± 16.05	96.15 ± 16.05
	LAST	137.46 ± 9.30	127.81 ± 13.29	131.79 ± 8.05	120.84 ± 3.38
Acetic Acid	INITIAL	28.27 ± 5.13	28.27 ± 5.13	28.27 ± 5.13	28.27 ± 5.13
	LAST	35.01 ± 6.71	40.38 ± 1.33	39.62 ± 1.05	40.56 ± 5.08
Final pH		4.47	4.5	4.46	4.71
Growth (Log CFU/mL)		8.40 ± 0.11	8.33 ± 0.35	8.70 ± 0.79	Not Applicable

Table 4.2 (continued)

Aerobiosis					
Furfural	INITIAL	7.16 ± 0.76	7.16 ± 0.76	7.16 ± 0.76	7.16 ± 0.76
	LAST	10.26 ± 5.02	4.56 ± 0.05	40.73 ± 56.06	1.52 ± 0.13
Lactic Acid	INITIAL	96.15 ± 16.05	96.15 ± 16.05	96.15 ± 16.05	96.15 ± 16.05
	LAST	129.00 ± 11.19	127.26 ± 11.74	128.47 ± 11.17	120.84 ± 3.38
Acetic Acid	INITIAL	28.27 ± 5.13	28.27 ± 5.13	28.27 ± 5.13	28.27 ± 5.13
	LAST	33.96 ± 7.19	36.65 ± 7.55	35.48 ± 6.42	40.56 ± 5.08
Final pH		4.5	4.37	4.56	4.7
Growth (Log CFU/mL)		8.83 ± 0.05	8.37 ± 0.05	8.88 ± 0.01	Not Applicable

Table 4.3. Utilization of furfural by strains of *Lactobacillus pentosus* under anaerobiosis (top panel) and aerobiosis (bottom panel) in fermented cucumber juice medium (FCJM) with an initial pH of 4.7 ± 0.1. Furfural was not present in FCJM and was supplemented to 15.48 ± 0.12 mM. The FCJM pH was adjusted to 4.71 ± 0.01 and the cultures were inoculated to 5 log CFU/mL. Cultures were incubated under anaerobic conditions for 7 days. Changes in ethanol concentration were not observed. Initial glucose and fructose concentrations were 0.86 ± 0.04 and 7.78 ± 0.47, respectively. Glucose was completely utilized after the 7 days of incubation by all the strains tested (data not shown). Less than 2.23 ± 1.11 fructose remained in the inoculated FCJM after 7 days of incubation (data not shown). Data presented is the average of independent replicates. Minimal limit of detection for furfural was < 0.001 mM.

Strains Tested ►		***L. pentosus* LA0233**	***L. pentosus* 1.8.9**	***L. pentosus* LA445**
Anaerobiosis				
Furfural	INITIAL	7.16±0.76	7.16±0.76	7.16±0.76
	FINAL	9.93±6.23	12.88±1.61	11.16±6.94
Lactic Acid	INITIAL	96.15±16.05	96.15±16.05	96.15±16.05
	FINAL	127.01±0.68	126.3505	126.15±20.48
Acetic Acid	INITIAL	28.27±5.13	28.27±5.13	28.27±5.13
	FINAL	43.10±1.25	38.22±12.83	31.83±7.42
Final pH		4.54	4.5	4.48
Growth (Log CFU/mL)		8.43±0.59	7.25±0.19	8.33±1.08
Aerobiosis				
Furfural	INITIAL	7.16 ± 0.76	7.16 ± 0.76	7.16 ± 0.76
	FINAL	7.66 ± 5.32	9.52 ± 4.09	8.77 ± 4.46
Lactic Acid	INITIAL	96.15 ± 16.05	96.15 ± 16.05	96.15 ± 16.05
	FINAL	122.99 ± 7.64	123.22 ± 5.14	122.81 ± 4.34
Acetic Acid	INITIAL	28.27 ± 5.13	28.27 ± 5.13	28.27 ± 5.13

Table 4.3 (continued)

	FINAL	42.58 ± 4.89	39.03 ± 3.56	31.13 ± 4.87
Final pH		4.67	4.48	4.5
Growth (Log CFU/mL)		9.06 ± 0.11	8.38 ± 0.01	8.33 ± 0.05

APPENDIX

Table A.1 Selected genomes for KEGG pathway analysis with the targeted substrate Sequencing status: F, all finished; P, permanent draft; D, draft.

DOMAIN: Bacteria	selected genomes	sequencing status
Lactobacillus brevis	15f	D
Lactobacillus brevis	47f	D
Lactobacillus brevis	AG48	P
Lactobacillus brevis	ATCC 14869	D
Lactobacillus brevis	ATCC 367	F
Lactobacillus brevis	BM-LB13908	D
Lactobacillus brevis	BSO 464	D
Lactobacillus brevis	D6	D
Lactobacillus brevis	DmCS_003	D
Lactobacillus brevis	DSM 20054	D
Lactobacillus brevis	EW	P
Lactobacillus brevis gravesensis	ATCC 27305	P
Lactobacillus brevis	KB290	F
Lactobacillus brevis	Lb1595	D
Lactobacillus brevis	NPS-QW-145	D
Lactobacillus brevis	TMW 1.313	D
Lactobacillus brevis	TMW 1.465	D
Lactobacillus brevis	TMW 1.6	D
Lactobacillus brevis	VBLLa 15-17	P
Lactobacillus brevis	VBLLa 23-09	P
Lactobacillus brevis	WK12	D
Lactobacillus buchneri	ATCC 11577	P
Lactobacillus buchneri	CD034	F
Lactobacillus buchneri	DSM 20057	D
Lactobacillus buchneri	NRRL B-30929	F
Lactobacillus buchneri	VBLLa 18-02	P
Lactobacillus buchneri	VBLLa 18-17	P
Lactobacillus pentosus	DSM 20314	D
Lactobacillus pentosus	FL0421	D
Lactobacillus pentosus	KCA1	P
Lactobacillus plantarum	16	F
Lactobacillus plantarum	19.1	D
Lactobacillus plantarum	19L3	P
Lactobacillus plantarum	2025	D
Lactobacillus plantarum	2165	D

Table A.1 (continued)

Lactobacillus plantarum	38	D
Lactobacillus plantarum	43-3	D
Lactobacillus plantarum	4_3	P
Lactobacillus plantarum	5-2	D
Lactobacillus plantarum	8 RA-3	D
Lactobacillus plantarum	80	D
Lactobacillus plantarum	90sk	D
Lactobacillus plantarum	AG30	P
Lactobacillus plantarum	ATCC 14917	P
Lactobacillus plantarum	AY01	P
Lactobacillus plantarum	B21	D
Lactobacillus plantarum	B67	D
Lactobacillus plantarum	B7	D
Lactobacillus plantarum	C410L1	D
Lactobacillus plantarum	CAUH2	D
Lactobacillus plantarum	CIP104448	D
Lactobacillus plantarum	CMPG5300	D
Lactobacillus plantarum	CNW10	D
Lactobacillus plantarum	CRL 1506	D
Lactobacillus plantarum	DF	D
Lactobacillus plantarum	DmCS_001	P
Lactobacillus plantarum	E2C5	D
Lactobacillus plantarum	EGD-AQ4	P
Lactobacillus plantarum	ER	D
Lactobacillus plantarum	FMNP01	D
Lactobacillus plantarum	HFC8	D
Lactobacillus plantarum	IPLA88	P
Lactobacillus plantarum	JBE245	D
Lactobacillus plantarum	JDM1	F
Lactobacillus plantarum	KP	D
Lactobacillus plantarum	L31-1	D
Lactobacillus plantarum	Lp1610	D
Lactobacillus plantarum	Lp1612	D
Lactobacillus plantarum	Lp90	P
Lactobacillus plantarum	LP91	P
Lactobacillus plantarum	LY-78	D
Lactobacillus plantarum	LZ206	D
Lactobacillus plantarum	LZ227	D

Table A.1 (continued)

Lactobacillus plantarum	MF1298	D
Lactobacillus plantarum	NAB1	D
Lactobacillus plantarum	NAB2	D
Lactobacillus plantarum	NCU116	D
Lactobacillus plantarum	Nizo1837	D
Lactobacillus plantarum	Nizo1839	D
Lactobacillus plantarum	Nizo2029	D
Lactobacillus plantarum	Nizo2256	D
Lactobacillus plantarum	Nizo2257	D
Lactobacillus plantarum	Nizo2258	D
Lactobacillus plantarum	Nizo2259	D
Lactobacillus plantarum	Nizo2260	D
Lactobacillus plantarum	Nizo2262	D
Lactobacillus plantarum	Nizo2264	D
Lactobacillus plantarum	Nizo2457	D
Lactobacillus plantarum	Nizo2484	D
Lactobacillus plantarum	Nizo2485	D
Lactobacillus plantarum	Nizo2494	D
Lactobacillus plantarum	Nizo2535	D
Lactobacillus plantarum	Nizo2726	D
Lactobacillus plantarum	Nio2741	D
Lactobacillus plantarum	Nizo2757	D
Lactobacillus plantarum	Nizo2766	D
Lactobacillus plantarum	Nizo2776	D
Lactobacillus plantarum	Nizo2801	D
Lactobacillus plantarum	Nizo2802	D
Lactobacillus plantarum	Nizo2806	D
Lactobacillus plantarum	Nizo2814	D
Lactobacillus plantarum	Nizo2831	D
Lactobacillus plantarum	Nizo2855	D
Lactobacillus plantarum	Nizo2877	D
Lactobacillus plantarum	Nizo2889	D
Lactobacillus plantarum	Nizo2891	D
Lactobacillus plantarum	Nizo3400	D
Lactobacillus plantarum	Nizo3892	D
Lactobacillus plantarum	Nizo3893	D
Lactobacillus plantarum	Nizo3894	D
Lactobacillus plantarum	NL42	D

Table A.1 (continued)

Lactobacillus plantarum	PS128	D
Lactobacillus plantarum	SF2A35B	D
Lactobacillus plantarum	SNU.Lp177	D
Lactobacillus plantarum	TIFN101	D
Lactobacillus plantarum	UC8491	D
Lactobacillus plantarum	UCMA 3037	P
Lactobacillus plantarum	VBLLa 11-47	P
Lactobacillus plantarum	WCFS1	F
Lactobacillus plantarum	WHE 92	P
Lactobacillus plantarum	WJL	D
Lactobacillus plantarum	WJL	P
Lactobacillus plantarum	WLPL04	D
Lactobacillus plantarum	XB7	D
Lactobacillus plantarum	Zhang-LL	D
Lactobacillus plantarum	ZJ316	F
Lactobacillus plantarum	ZJ95	D
Lactobacillus plantarum	ZS2058	D
Pediococcus pentosaceus	ATCC 25745	F
Pediococcus pentosaceus	CGMCC 7049	P
Pediococcus pentosaceus	DSM 20336	D
Pediococcus pentosaceus	FBL2	D
Pediococcus pentosaceus	IE-3	P
Pediococcus pentosaceus	NKYL15	D
Pediococcus pentosaceus	SL4	F