

ABSTRACT

ANEKELLA, KARTHEEK. Characterization of *L. plantarum/pentosus* for Starter Cultures in Cucumber Fermentation and Conjugative Transferability of Antibiotic Resistance in Lactic Acid Bacteria (under the direction of Drs. Sophia Kathariou and Ilenys Perez-Diaz)

Starter cultures are not widely used in cucumber fermentation industries due to a lack of comprehensive study on ideal starter culture properties and emerging requirements for cucumber fermentation. Thus, the objective of this study was to determine the predominant and robust autochthonous bacteria from commercial cucumber fermentations, applicability of starter cultures in CaCl₂ brined fermentation without NaCl and characterize their relevant metabolic, physiological and safety characteristics. Fermentation brine samples were collected from a commercial cucumber fermentation tank from MN on various days. Additionally, isolates that were previously isolated from a NC fermentation tank were included in this study. *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus brevis* were predominantly found among other lactic acid bacteria (LAB) based on tentative identification by 16S rRNA sequencing. *L. plantarum* isolates were screened for their ability to rapidly ferment and possess antimicrobial activity against *Lactobacillus buchneri* but not *L. brevis*. Eight tentative *L. plantarum* isolates were chosen for further characterization. Multi locus sequence typing and other housekeeping genes revealed that seven of these isolates were *L. pentosus* except one which was *L. plantarum*. Polymerized chain reaction product of *recA* confirmed that *L. pentosus* accounted for more than 95% and 70% of the isolates from NC and MN fermentation tanks, respectively. Starter culture properties including carbohydrate utilization, amount of D- and L- lactic acid production, malic acid decarboxylation and production of biogenic amines, exopolysaccharides and bacteriocins were studied phenotypically. DNA-based detection of genes associated with D-xylose metabolism, production of biogenic amines, exopolysaccharides and bacteriocins was also

conducted. The minimum inhibitory concentrations towards six antibiotics are well within the range of the proposed cut-off values. In order to determine their starter culture potential in NaCl-free brine, cucumber fermentation were conducted with CaCl₂ brine in 1) laboratory scale one gallon jars at 30 °C and 15 °C and 2) in industrial scale fermentation tanks at ambient conditions in NC during summer. The isolates displayed rapid sugar utilization and varying acid production trends at 30 °C and 15 °C. Reduction of pH and increase of lactobacilli counts were rapid in starter culture inoculated jars and industrial tanks suggesting a faster fermentation in the CaCl₂ brine. A manufacturing defect referred to as “bloating” was observed in all the pickles after 14 days in the jars.

To understand the antimicrobial resistances and horizontal gene transfer of antibiotic resistance in plant originated LAB, isolates from fresh cantaloupe rinds (n=85) and cucumber fermentation (n=8) were studied. Along with antibiotic resistance (erythromycin and tetracycline), co-resistance of LAB towards heavy metals arsenic and cadmium and disinfectant benzalkonium chloride was tested. Isolates from cantaloupes displayed heavy metal and antibiotic resistance (9%) but not the isolates from cucumber fermentation. However, co-resistance was not detected in any of the LAB isolates. Three isolates of *Leuconostoc pseudomesenteroides* from fresh cantaloupe and three (one *L. plantarum* and two *L. pentosus*) isolates from cucumber fermentation were studied for conjugative transfer of *erm*(B) and *tet*(M) genes by plate mating from *Enterococcus faecalis* and *Listeria monocytogenes*, respectively. Only two *L. pseudomesenteroides* isolates participated in conjugative transfer of the above genes at frequency of $\leq 10^{-7}$ per recipient. None of the isolates from cucumber fermentation yielded transconjugants suggesting that their physiology and source of origin may impact the conjugative transfer of the genes tested. In conclusion, the autochthonous *L. pentosus* and *L. plantarum*

isolates in this study have a potential to be starter cultures for industrial pickle manufacturers to achieve a consistent, safe and quality fermentation and final product. The tests in this study could serve as guidelines for evaluating starter culture properties for cucumber fermentation and possibly other similar vegetable fermentations.

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Characterization of *L. plantarum/pentosus* for Starter Cultures in Cucumber Fermentation and
Conjugative Transferability of Antibiotic Resistance in Lactic Acid Bacteria

by
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DEDICATION

To my beloved family and friends.

BIOGRAPHY

Kartheek Anekella was born and raised in India where he obtained Bachelors in Biotechnology from Vellore Institute of Technology University. Kartheek earned his Master's degree in Bioresource Engineering at McGill University, Canada. Kartheek did an internship at Riddet Institute (Massey University), New Zealand before starting his PhD at NC State University in Food Science. He received the prestigious IFT Feeding Tomorrow Scholarship as part of which he did an internship with General Mills in the summer of 2015. Kartheek wishes to join the food industry in the area of R&D and product development upon graduation.

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CHAPTER 1

Literature Review

INTRODUCTION

COMMERCIAL CUCUMBER FERMENTATIONS

Cucumber fermentation begins with a washing step of cucumbers, followed by cover brine (rich in NaCl) addition and acidification by acids in brine and natural fermentation by bacteria naturally present on the cucumbers. In the US, cucumber fermentations are performed in 8-10,000 gallons open-top glass fiber tanks (Breidt and others 2013). Fermentation is completed in 7-12 days at 25-30 °C (75-85 °F) ambient temperatures depending on the location. Air purging is intermittently applied during the cucumber fermentation to release the carbon dioxide produced by microbial activity and fruit respiration to prevent bloater (hollow cavity) formation (Etchells and others 1973; Perez-Diaz and others 2013). Fermentation can be variable depending on cucumber cultivar type, microbial load, ambient weather conditions and quality of the water used for cover brine and final product may vary from tank to tank and year to year (Etchells and others 1973). The initial pH of brined cucumber systems is usually between 5 and 6.5 and final pH of the pickles after fermentation is between 3.2 and 3.6 (Perez-Diaz and others 2013). Depending on the final form of the pickle, acetic acid and lactic acids are present at a concentration of 0.6 to 2.5% and 0.02 to 0.35%, respectively (Perez-Diaz and others 2013).

Traditionally 5-8% salt is used during fermentation and 8-16% during bulk storage post fermentation (Chavasit and others 1991). Salt inhibits undesired Gram-negative pathogens giving a selective advantage for the Lactic Acid Bacteria (LAB) to predominate (Daeschel and Fleming 1984; Perez-Diaz and others 2013; Wouters and others 2013). Salt tolerant bacteria can survive up to 4-6 weeks between 25 and 30 °C (Etchells and others 1975). Salt concentration and temperature predominantly govern the wild fermentations by bacteria. Acetic acid is usually

added at the beginning of fermentation for buffering and flavoring purposes (Perez-Díaz and others 2013). Lower pH at the initiation of fermentation also helps in the release of excess CO₂ formed during fermentation and inhibits acid-sensitive spoilage and pathogenic microorganisms from proliferating (Breidt and others 2013; Perez-Díaz and others 2013). A low level of calcium chloride (0.1-0.4% and equilibrated to 20-40 mM) is added along with NaCl for firmness retention of the final product (McFeeters and Pérez-Díaz 2010). The fermented products are desalted to 2% NaCl by a washing step and then processed into various forms such as chips, spears, mixed vegetables and relishes before packing. Preservatives such as potassium sorbate (0.04%) may be added along with acetic acid (0.16%) to the final product and pasteurized (Breidt and others 2013). Pasteurization of the final product may not be necessary if other preservatives such as sugar or acids are added and stored at cold temperatures (Perez-Díaz and others 2013).

Microbial and Metabolic Changes during Fermentation:

During the initiation of cucumber fermentation both Gram-positive and Gram-negative bacteria are present as a part of natural microbiota from cucumbers. The diversity and number of bacteria in microbiota varies from season to season, environmental factors and pesticide usage (Perez-Díaz and others 2013). A variety of spoilage and pathogenic microorganisms are also naturally present on cucumbers depending on the harvesting and storage conditions (Breidt Jr and others 2006). Aerobic bacteria (~5 log CFU/g), LAB (~3.8 log CFU/g) and Enterobacteriaceae (~4.5 log CFU/g) are present during the initiation phase and may survive through the fermentation depending on the fermentation and environmental conditions (Perez-Díaz and others 2013). Lactic acid bacteria eventually predominate due to higher acid tolerance compared to all other bacteria (Breidt and others 2013). Within LAB, *Lactobacillus plantarum* is

usually found as the predominant species in most vegetable fermentation due to its greater acid tolerance (Breidt and others 2013). Other LAB that are found during cucumber fermentation include *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides* and *Weissella* spp. (Perez-Diaz and others 2013). Interestingly, heterofermentors *Weissella cibaria* and *Weissella paramesenteroides* were the predominant species found in cucumber fermentation of Taiwanese style cucumber pickles. These pickles are usually fermented at a lower temperature for a shorter time and thus probably due to shortage of time and appropriate conditions homofermenting *L. plantarum* or *L. pentosus* are not found as the predominant species (Chen and others 2012). Fermentative yeast strains belonging to the genera *Candida* spp., *Pichia* spp. and *Saccharomyces* spp. are also found that are acid-resistant, that can utilize fermentable sugars and contribute to undesirable flavors and bloating (Perez-Diaz and others 2013). Acid tolerant yeasts may occur towards the end of fermentation and can be active in the tank for several months (Etchells and others 1975; Franco and Perez-Diaz 2012 and 2013). If there is residual sugar present after primary fermentation, sub-surface yeasts such as *Torulopsis* spp., *Sacharomyces* spp. and *Brettanomyces* spp. utilize these sugars and also lactic acid and thus increase in numbers causing spoilage of pickles during storage (Etchells and others 1975; Franco and Perez-Diaz 2012).

A variety of acids including lactic, acetic, propionic, succinic and butyric acids are produced by the bacteria during fermentation (Breidt Jr and others 2006). However, lactic acid is the predominant acid that is produced and gives the characteristic sour flavor expected in a fermented pickle. Along with these acids other inhibitory compounds such as peroxides, thiols and bacteriocins are produced that inhibit undesirable pathogens and spoilage microorganisms thus acting as preservatives (Çon and Karasu 2009; Bourdichon and others 2012; Breidt and

others 2013). Cucumber pickles occasionally have product defect “bloaters” which are hollow spaces inside the pickles formed due to CO₂ production by natural respiration of cucumbers, malic acid decarboxylation, heterofermenting bacteria and yeasts (Fleming and Pharr 1980; Etchells and others 1975). Along with acid production, fermentation by bacteria also improves the bioavailability and digestibility of proteins, micronutrients, minerals and trace elements and may also degrade anti-nutritional factors (Károvičová and Kohajdová 2003 ; Bourdichon and others 2012). Ideally, all the sugars must be fermented during primary fermentation yielding organic acids and a final pH between 3 and 3.5. Protonated organic acids diffuse through the Gram- negative bacterial cell and acidify the cytoplasm after dissociation of the acid internally. Internal accumulation of protons and dissociated anions lead to death of these cells (Breidt Jr and others 2006). After the fermentation is complete, the pickles can be stored up to a year without refrigeration (Breidt Jr and others 2006). However, acid resistant pathogens were discovered in the final products recently that caused foodborne illnesses and thus there is a great interest to understand the effect of organic acids and their ion counterparts on the survival and persistence of pathogens (Breidt Jr and others 2006).

Role of Salt in Fermentation:

In cucumber fermentation, NaCl creates a favorable environment for the desirable homofermentative LAB while inhibiting the undesirable spoilage and pathogenic bacteria (Fleming and others 1985; McFeeters and Pérez-Díaz 2010). Salt also reduces the water activity, oxygen solubility and changes the internal osmolarity of cell and thus inhibits spoilage and pathogenic bacteria (Panagou and others 2011). Gram-negative cells in general lose the turgor pressure during osmotic shock from NaCl (Glaasker and others 1998). Plasmolysis happens when the cytoplasmic membrane retracts from the cell wall but it is less likely to happen in

Gram-positive cells because of the close association of peptidoglycan layer to cytoplasmic membrane (Glaasker and others 1998). Salt also brings the sugars out of the fruit for fermentation by bacteria.

Impact of Salt on Human Health and Environment:

On an average, Americans consume about 3400 mg/day of sodium while the recommended sodium intake per day is 2300 mg and even lower (1500 mg/day) for compromised population with diabetes and hypertension (Bautista-Gallego and others 2013). Even though vegetable-based fermented products like pickles are consumed in limited quantities the risk of high sodium intake is significant because pickles, for example, contain salt up to 1200 mg/100 g of product (Bautista-Gallego and others 2013). Currently, U.S. Environmental Protection Agency (EPA) recommends chloride levels in fresh water to be reduced from 230 ppm to 170 ppm which demands a heavy reduction in salt content of the brine (Perez-Diaz and others 2013). The brine waste is also high in organic matter from fermentation which increases the biological oxygen demand for lakes into which this brine is ultimately disposed (Perez-Diaz and others 2013, 2015). Brine recycling is practiced by some industries but treatments such as adsorption to eliminate softening enzymes and microbial removal is necessary prior to reuse which increases the capital input (Perez-Diaz and others 2013, 2015).

Alternatives to Salt:

Reduction in salt supports the growth of spoilage microorganisms which would lead to spoilage of the final pickle product (Chavasit and others 1991; Guillou and others 1992). The amount of salt required for fermentation was reduced by adding CaCl_2 (0.2-0.44%) and potassium sorbate (0.2%) to obtain a good quality product but the study was conducted in a

controlled anaerobic conditions which is not currently practiced in the industry (Guillou and others 1992). Sulfites may be added in the absence of salt but the controversies around sulfites is discouraging pickle manufacturers to adopt its usage (Perez-Díaz and others 2013). Partial replacement of NaCl by salts such as KCl, MgCl₂ and CaCl₂ and/or their mixtures for various vegetable fermentations is currently investigated (McFeeters and Pérez-Díaz 2010; Panagou and others 2011; Bautista-Gallego and others 2013). The cations of these salts are also essential micronutrients for the human body (Bautista-Gallego and others 2008). However, a single replacement for NaCl by any of these salts is impractical and the individual effects of each type of these salt alternatives are described elsewhere (Bautista-Gallego and others 2013). The selection of salt alternative depends on the ion composition, type of food matrix it is being used in and its role in that food- such as taste, preservation and/or texture (Bautista-Gallego and others 2013). Since the osmotic effects of various salts depend on molar concentration as opposed to percentages, a solid scientific evaluation is necessary before low or no-sodium fermentations are practiced by the industry (Bautista-Gallego and others 2013). Vegetable fermentations such as olives, sauerkraut and cucumbers were attempted with low and/or no salt fermentation with or without alternative salts (Johanningsmeier and others 2007; McFeeters and Pérez-Díaz 2010; Bautista-Gallego and others 2013; Wolkers-Rooijackers and others 2013). A combination of various chloride salts gave similar quality and safety of the final product in sauerkraut with or without the use of starter cultures (Johanningsmeier and others 2007; Wolkers-Rooijackers and others 2013).

STARTER CULTURES

Despite the abundant knowledge on commercial cucumber fermentation process, starter culture bacteria that are autochthonous to cucumber fermentation are still unavailable commercially. Thus, it is important to develop robust starter cultures satisfying certain ideal starter culture properties suitable for commercial cucumber fermentation which is the primary focus of this dissertation. Sterilization/pasteurization of the raw product (cucumbers here) to eliminate native microbiota on fruit has been attempted but not well adopted by industries due to the higher costs. Hence, addition of starter cultures that are capable of rapid fermentation and inhibit undesirable microorganisms was proposed as an alternative (Lee and others 2015).

The desirable technical characteristics of starter cultures depend on the type of raw material and expected qualities of the final product (Holzapfel 2002; Ammor and Mayo 2007; Chang and Chang 2010; Heperkan 2013; Xiong and others 2013; Lee and others 2015). Studies on efforts to develop and implement starter cultures that accelerate cucumber fermentation were started in 1960s (Etchells and others 1964). *L. plantarum* and *Pediococcus cerevisiae* were proposed as the acid resistant starter cultures for cucumber fermentation. When a higher salt is desired, initial addition of *P. cerevisiae* was suggested due to its higher salt tolerance followed by *L. plantarum* for complete glucose utilization (Etchells and others 1973). The inoculum levels and culture addition timing are discussed later. Mixed cultures consisting of LAB and yeasts were proposed to ferment cucumbers mainly to avoid secondary fermentation spoilage (Daeschel and others 1988). Yeasts were added along with LAB to achieve complete sugar utilization without the use of acid buffers. However, ethanol and glycerol production and increase in pH from yeast metabolism, inapplicability and cross contamination from yeast culture addition at an industrial scale rendered this technique impractical.

Role of Starter Cultures in Low/No Salt Fermentation:

Starter cultures were proven efficient for vegetable fermentations to rapidly reduce the pH to inhibit undesirable microorganisms and thus alleviating the negative outcomes of adding low or no salt (Chavasit and others 1991; McFeeters and Pérez-Díaz 2010; Pérez-Díaz and McFeeters 2011). Sauerkraut had a firmer texture and no off-flavors when *Leuconostoc* spp. was used as a starter culture in salt free fermentation (Johanningsmeier and others 2007). McFeeters and Pérez-Díaz reported salt free cucumber fermentation for the first time using only CaCl₂ as the salt source and an added starter culture inoculum (McFeeters and Pérez-Díaz 2010). To understand the effect of salt on LAB, growth of *L. pentosus* was tested at various salt concentrations and it was found that it can withstand up to 5.2% salt (chloride levels) in MRS medium (Bautista-Gallego and others 2008). From a metabolic perspective, it was demonstrated in *L. plantarum* that the cells were more tolerant to NaCl than KCl at the same concentration due to higher intracellular concentration of K⁺ already present in unstressed cells. This indicates that KCl may be inhibitory to cells and osmotic imbalance may not be the only mechanism of cell death (Glaasker and others 1998). A similar range of growth inhibitory concentrations was found for NaCl, KCl and CaCl₂ (Bautista-Gallego and others 2008). However, a change in flavor quality and texture may be noticed when the salt content is altered and thus pilot and commercial scale studies are necessary before a low salt fermentation is scaled up, to save the economic losses. In a wild fermentation, calcium salt causes diffusion of sugars out of the fruit slower than NaCl and thus fermentation was delayed due to slower production of lactic acid. Thus, the addition of starter cultures will circumvent this issue by rapid fermentation (McFeeters and Pérez-Díaz 2010).

Selection of Starter Cultures:

Starter cultures for various vegetable fermentations were selected from their native fermentation source based on their rapid fermentation ability (Karovičová and others 1999; Di Cagno and others 2008; Rao and others 2013). However, their genetic identification was not well classified. Hence, there might be a mixed load of bacteria in the starter culture mixture and there is a possible risk of losing some strains due to propagations over time (Rattanachaikunsopon and Phumkhachorn 2010). In addition, plasmid loss may be seen and functionality of starter cultures may be lost if the desired characteristics are encoded on a plasmid. Thus, it is of great importance to fully characterize the potential starter culture candidates before they are commercialized (Rattanachaikunsopon and Phumkhachorn 2010). Scientific discovery of microorganisms that are intended to be used as starter cultures or probiotics must be well studied according to regulatory authorities such as FDA or EFSA (Bourdichon and others 2012). Polyphasic approaches involving both phenotypic and genotypic characterization are recommended that represents clear variabilities and similarities between existing type strains (Bourdichon and others 2012; Heperkan 2013).

Utilizing autochthonous starter cultures that are native to fermentation is beneficial since they are metabolically evolved to grow and survive in that specific environment (Leroy and De Vuyst 2004; Di Cagno and others 2008, 2009; Rattanachaikunsopon and Phumkhachorn 2010). Allochthonous starter cultures are bacteria that are of different origin than the intended food matrix. They may not exhibit a comparable functionality as autochthonous starter cultures because it was demonstrated that food fermentations with autochthonous starter cultures are more efficient and produced higher quality products compared to allochthonous or wild fermentation (Di Cagno and others 2008, 2009). Currently, most commercial starter cultures

available in the US are allochthonous to cucumber fermentations. Significant differences were seen in the vitamin content, color and sensory profile of the final products between autochthonous and allochthonous fermentations. Selected secondary metabolites such as bacteriocins, exopolysaccharides and vitamins specifically produced by autochthonous starter cultures in their native food matrix, are often considered added-value for the final product (Di Cagno and others 2009).

CHARACTERISTICS OF STARTER CULTURES

Along with rapid fermentation, starter cultures should not produce undesirable compounds such as D-lactate and biogenic amines (Holzapfel 2002; Leroy and De Vuyst 2004). The metabolism of lactic acid bacteria normally slows down at lower temperatures (< 20°C) and since the ambient weather is colder in the northern regions of the US, the ideal starter culture(s) must be able to function at various temperatures (Etchells and others 1975). Starter cultures must readily adapt to the environment which depends on factors such as pH, salt and temperature (Di Cagno and others 2008; Karasu and others 2010). Starter cultures should also inhibit yeasts and heterofermentative bacteria which are commonly responsible for production of off flavors and softening enzymes that compromise the quality of the final product (Geisen and Holzapfel 1996; Heperkan 2013). Starter cultures also act as preservatives by producing acids, peroxides and antimicrobial peptides like bacteriocins and thus their production is desirable in some products (De Vuyst 2000; Chang and Chang 2010). Partial inhibition of endogenous pectinolytic enzymes and lipoxygenases that cause texture defects and off-flavors was observed when starter cultures are used in various vegetable fermentations and thus a better sensory profile and texture is expected (Di Cagno and others 2008). Safety assessment for production of any undesirable compounds that may negatively impact the safety and quality must be performed prior to their

use (Buckenhüskes 1993; Geisen and Holzapfel 1996; Holzapfel 2002; Di Cagno and others 2008).

Dominance of a particular species/strain is possible through the production of bacteriocins that can kill closely related bacteria (Rao and others 2013). Bacteriocinogenic starter are of special interest due to their ability to kill other undesirable bacteria in the fermentation and thus it avoids the necessity to add preservatives during storage (Holzapfel 2002; Chang and Chang 2010; Di Cagno and others 2013; Rao and others 2013). Film forming surface yeasts cause spoilage by utilizing residual sugars and lactic acid to increase the pH to a level where coliforms and other molds start growing (Etchells and others 1975). Thus, the ideal starter culture should completely utilize glucose and also inhibit these spoilage associated bacteria and yeasts. Antibiotic resistance gene transfer from starter cultures to opportunistic and pathogenic bacteria in humans is of significant concern currently (Ammor and Mayo 2007). The attributes that are relevant to cucumber fermentation were studied throughout this work and discussed in the following sections.

CARBOHYDRATE UTILIZATION

Cucumber fermentation is a dynamic process with variables such as size of cucumbers, amount of sugars available for fermentation, composition of natural microbiota and their nature of metabolism (Breidt and others 2013). There are approximately 2% fermentable sugars in cucumbers including xylose, arabinose, mannose, galactose with glucose and fructose as the two predominating sugars (Etchells and others 1975; Miller 1989). During maturation of cucumbers, stachyose is converted to sucrose in the peduncle and enters the fruit breaking down into glucose and fructose (Handley and others 1983). Glucose and fructose are the primary free sugars available for fermentation along with traces of sucrose (Handley and others 1983). Malate and

citrate are also formed during cucumber maturation. Mechanical stress can induce changes in the sugar components due to enzyme activity. For example, chilling can affect glucose and galactose contents in cucumbers during storage due to temperature dependent enzyme activity (Miller 1989).

Sugar Transport in LAB:

The phosphoenol pyruvate-phosphotransferase System (PEP-PTS) was supposedly limited only to homofermentative LAB that utilize glucose through Embden-Meyerhoff-Parnas (EMP) pathway (Romano and others 1979). However, this theory has changed because the pathway is used based on the growth conditions such as aerobic vs. anaerobic conditions. For example, heterofermenting *L. brevis* can transport and metabolize fructose through PTS usually only under anaerobic conditions (Saier and others 1996; Axelsson 2004). Organisms with PEP-PTS systems are involved in transport and metabolism of specific sugars that are catabolized or anabolized depending on the nutrient and growth conditions (Romano and others 1979; Axelsson 2004). The metabolism of glucose and fructose is similar in most LAB except at the initial transport step via which they enter the cell to undergo glycolysis. Activation of sugar through phosphorylation is usually the first step before they enter any metabolic pathway (Axelsson 2004). Glucose is transported via PEP-PTS transporters EII^{glc} and EII^{man} while fructose is transported through EII^{fru} and EII^{man} . However, EII^{man} has a greater affinity for transport of glucose compared to fructose when both the sugars are present together and thus varying sugar utilization rate is observed (Lu and others 2001). Disaccharides can also enter the cell either in active phosphorylated form or native form and then broken down into individual sugars. Sucrose can also enter the cell through a permease and is broken down into glucose and fructose by sucrose hydrolase. If sucrose is transported via PEP-PTS, it is hydrolyzed to glucose-6-phosphate

and fructose. Sucrose may also act as precursor for EPS production in some LAB (Axelsson 2004). Most sugars enter the major pathways as glucose-6-phosphate or fructose-6-phosphate except galactose that uses tagatose-6-phosphate pathway (Axelsson 2004). Pentoses are phosphorylated and converted to ribulose-5-phosphate or xylulose-5-phosphate and enters the phosphoketolase (PK) pathway. This pathway is not just restricted to heterofermentors and in fact seen in facultative heterofermenting LAB such as *L. pentosus* and *L. plantarum*.

Fate of Sugars and End Products:

Substrate level phosphorylation is the main energy generating pathway in LAB since they have low O₂ tolerance and do not use electron transport chain (Axelsson 2004). The microorganism's natural habitat, presence of various carbohydrates and stress factors determine the metabolic pathways LAB adopt. In fact, *L. plantarum* and *L. mesenteroides* possess a higher number of pyruvate metabolizing genes compared to other plant LAB such as *L. brevis* or *P. cerevisiae* common prevalence of *L. plantarum* and *L. mesenteroides* in high acid vegetable fermentation (Breidt and others 2013). Two molecules of pyruvate are yielded from glucose by EMP homofermentative pathway whereas heterofermentative pathways such as anaerobic Entner-Doudoroff or PK pathway yields only one pyruvate per glucose molecule. Thus, it is metabolically disadvantageous for heterofermentative bacteria to use its only PEP molecule for transport purpose and not for energy production (Romano and others 1979). Fructose-bisphosphate (FBP) drives lactate dehydrogenase (Ldh) to form lactate but if there is not enough FBP the intermediate pyruvate is driven to form metabolic end products of heterofermentation. Changes in pH can induce LAB to switch from homo to heterofermentation. It was found that *Lactobacillus bulgaricus* synthesized less Ldh at higher pH value, whereas the opposite was observed with *L. plantarum* at which higher pH drives more Ldh activity. Lactobacilli can

produce both D- and L- forms of lactic acid. In *L. plantarum*, racemases convert L- to D- lactate when there is an accumulation of L-lactate (Tseng and Montville 1993). Under excess oxygen, enzymes such as pyruvate oxidase, acetate kinase and NADH oxidase are increased and hence heterofermentative product acetate is produced along with lactate. Under aerobic conditions, oxygen sensitive pyruvate formate lyase is inactive and thus pyruvate oxidase and NADH oxidase are activated. Pyruvate oxidase and acetate kinase pathway leads to the formation of acetate under aerobic conditions and NADH oxidase regenerates NAD^+ (Bobillo and Marshall 1992). Production of metabolites other than lactate, such as acetate, helps the cell maintain its pH homeostasis and thus could be an advantage however quality challenges due to production of carbon dioxide is undesirable if the cell follows a heterofermentative pathway (Bobillo and Marshall 1992).

Carrier-mediated influx of protons and efflux of catabolic end products occurs in lactobacilli. There is an active transport of protons from the exterior and catabolic products from the interior of the cell. A positive membrane potential is generated for every two protons pumped inside and one monovalent anion pumped out (Tseng and Montville 1993). Thus, a continuous proton motive force is achieved which contributes towards metabolic ATP energy. At least one additional ATP can be generated by this carrier mediated lactate-proton efflux pump. Proton motive force is also critical for metabolic processes such as energy production and amino acid transport (Ferain and others 1994). On the other hand, ATPase effluxes protons out of the cell while consuming ATP to maintain pH homeostasis. Under alkaline and aerobic conditions *L. plantarum* generates acetate as well as lactate. Acetate efflux yields more protons than lactate and thus creates a higher membrane potential and higher ATP generation (Tseng and Montville 1993). Under glucose depriving and aerobic conditions, *L. plantarum* produces acetate through

pyruvate oxidase and acetate kinase. This step along with NADH oxidase can catalyze the production of hydrogen peroxide which are toxic to the cell (Quatravaux and others 2006). NADH peroxidase and Mn^{+2} dependent pseudocatalases are involved in detoxifying the toxic effects of peroxides (Quatravaux and others 2006).

Metabolism of D-xylose:

Metabolism of pentose sugars such as D-xylose yields pyruvate and acetyl phosphate which are converted to lactate and acetate, respectively. The final products lactic acid, CO_2 and acetic acid/ethanol ratios can vary depending upon the presence of oxygen and electron acceptors (Bustos and others 2005). Lactic acid was consumed by *L. pentosus* after 72 h when there was no other primary carbon source was present in deMan, Rogosa and Sharpe (MRS) medium. There was production of acetic acid from other complex nutrients like yeast extract and animal peptone. Xylose (20 g/L) was completely depleted in bioreactor was after 48 hours (Xavier 2011). Xylose transportation comprises of internalization of D-xylose by transport proteins XylP and XylQ, isomerization to D-xylulose by D-xylose isomerase (XylA) which is then phosphorylated to D-xylulose-5-phosphate (X5P) by phosphorylase enzyme (XylB) followed by its catabolism to acetylphosphate and glyceraldehyde-3-phosphate (G3P) via pentose-phosphate/phosphoketolase pathway (Lokman and others 1991; Posthuma and others 2002). EII^{Man} transport system is involved in catalyzing the transport of D-xylose across the membrane (Chaillou and others 1999; Posthuma and others 2002). D-xylose transport proteins are required for the uptake of D-xylose from the environment and its mutants showed no growth. In fact, transport of D-xylose was the rate limiting step for the strains with plasmid encoded for xylose catabolic genes (Chaillou and others 1999).

L. pentosus produces one mole each of lactic and acetic acid per one mole of xylose consumed (Lampen and others 1951). The 5 carbon sugar is split into C₃ and C₂ fragments by aldolase type enzyme to 2-keto pentose. Lactic acid is formed from the C₃ fragment following reduction of pyruvate and C₂ is a versatile precursor that can form acetic acid or carbon dioxide. Acetic acid is produced from the aldehyde component of the xylose (Lampen and others 1951; Nutting and Carson 1952). However, at low pH C₂ fragment was found to be in lactate because there was more than 1 mole of lactate per 1 mole of D-xylose. It was hypothesized that condensation of C₂ and C₁ (CO₂) produced the 3 carbon lactate (Nutting and Carson 1952). Phosphoketolase pathway is used in facultative heterofermentative *L. pentosus* to produce lactic acid from pentose sugars using xylulose-5-phosphate phosphoketolase (XpkA) as the key enzyme (Posthuma and others 2002). Acetyl-P is formed from breakdown of X5P which is metabolized to acetate or ethanol through an intermediate acetyl-coA. Glyceraldehyde-3-phosphate on the other hand is converted to pyruvate and then to lactate. Production of ATP is involved in both reactions (acetate and lactate formation) (Lokman and others 1997; Chaillou and others 1999; Posthuma and others 2002; Bustos and others 2005).

The *xyl* operon includes genes that encode xylose transport proteins (*xylP* and *xylQ*), D-xylose isomerase (*xylA*), D-xylulose kinase (*xylB*) and a regulatory repressor (*xylR*). Uptake of xylose by membrane bound transport proteins is positively correlated to growth rate of *L. pentosus* (Lokman and others 1997). XylR acts as a repressor protein of *xylA* and *xylB* and serves as a negative control factor (Lokman and others 1997). *xylR* is expressed lesser during inducing conditions (presence of D-xylose) to propagate the co-transcription of *xylA* and *xylB* (Lokman and others 1997). In the presence of sugars other than D-xylose, XylR is produced at levels enough to repress transcription of *xylA* and *xylB*. *Lactobacillus* spp. that are unable to utilize D-

xylose are deficient in one or more *xyl* genes (Lokman and others 1991). Two genes *xylP* and *xylQ* are found upstream of *xylR* whose deduced amino acid sequences are significantly similar to membrane bound proteins of other microorganisms. So, it was hypothesized that these proteins have a transport of D-xylose into the cell (Lokman and others 1994). Regulation of *xyl* operon occurs at the transcriptional level following induction by D-xylose (Lokman and others 1994; Lokman and others 1997). Global catabolite control protein (CcpA) also regulates the phosphoketolase pathway through catabolite regulation elements (cre) near the catabolite genes in the *xyl* operon in *L. pentosus* (Posthuma and others 2002; Mozzi and others 2010). In the presence of glucose or other sugars in environment, CcpA or cre represses the expression of *xyl* genes (Lokman and others 1994; Lokman and others 1997; Posthuma and others 2002). While glucose metabolism genes are constitutive, pentose metabolism genes are usually inducible and are repressed under high glucose conditions (Kandler 1983; Lokman and others 1997). Upon induction, pentose metabolism follows heterofermentative pathway producing lactate and acetate and thus some homofermentative bacteria such as *L. pentosus* and *L. plantarum* were classified as facultative heterofermentative (Kandler 1983). *L. pentosus ccpA* mutant had a significantly longer lag phase in D-xylose growth medium indicating its role in D-xylose metabolism. Repression of *xylA* in the presence of glucose was not observed in this mutant confirming its regulatory role in transcription of *xyl*. When *xyl* genes were introduced into xylose non-fermenting strain of *Lactobacillus casei* through plasmid transformation they were able to ferment D-xylose. The growth however was still lower when compared to glucose as the carbon source (Posno and others 1991).

In certain bacteria such as *L. brevis*, xylose and glucose can be metabolized simultaneously. Simultaneous utilization of carbohydrates is beneficial for a rapid fermentation

especially in cucumbers since there is glucose and fructose present (Kim and others 2009). From the recent whole genome sequencing of *L. pentosus* KCA1 it was revealed that there are ~457 putative carbohydrate metabolism associated genes similar to other heterofermentative bacteria. Sugar transport by PTS systems for various sugars was identified including three mannose specific systems (Anukam and others 2013). “Sugar island” contains various gene cassettes responsible for various sugar metabolism in *L. pentosus*. As discussed earlier, D-xylose is transported via EII^{Man} PTS proteins that are constitutively expressed at high levels in *L. pentosus* and thus is expected to metabolize various pentose sugars (Anukam and others 2013).

Effect of Buffers on Sugar Utilization:

Excess amounts of lactic acid and low pH were the main causes for incomplete glucose utilization and hence buffers were added to the system (Lu and others 2001). Acetate was added to buffer the internal and external environment of the cell but it was not successful in achieving a complete utilization of glucose and lead to secondary fermentation by yeasts (Lu and others 2001). It was shown that high concentration of anions (> 60 mM) had an inhibitory effect on starter cultures for cucumber fermentation. Fructose utilization was higher than glucose at a higher inorganic anion concentration (10mM) (Lu and others 2002a). However, different organic acid anions had different effects on glucose utilization during fermentation (Lu and others 2002a). For example, citrate anion increased glucose utilization and lactate ions decreased its utilization while acetate anion had no effect. Fructose in general was more readily metabolized than glucose in the presence of these ions (Lu and others 2002a). Inorganic acid anions had no effect on buffering the final pH of the fermentation. Nitrates however decreased the activity of the starter cultures (Lu and others 2002a).

Among cations, Mn^{+2} plays a major role as a cofactor for various enzymes in glycolysis, as an activator for PEP-PTS sugar transport and also defense against superoxides by scavenging the radicals (Lu and others 2002a). It is also involved in ribosomal and cell wall stability. Spices that are high in Mn^{+2} such as cinnamon, ginger and celery stimulated acid production (Lu and others 2002a). Increasing Mn^{+2} in cucumber juice medium increased fructose utilization, but, beyond 120 mM both glucose and fructose utilization was lowered indicating an inhibitory effect (Lu and others 2002a). Other cations had no effect at lower concentrations and a negative effect at a high concentration due to osmotic stress. Divalent cations had more inhibitory effect than monovalent due to higher ionic strength at a given concentration. Brine composition is thus crucial in sugar utilization by the LAB during fermentation. Perhaps adding Mn^{+2} and acetate buffer could enhance sugar utilization and thus achieve rapid decrease in pH (Lu and others 2002a). Bigger cucumbers have higher amount of sugars and thus higher residual glucose is remained by the end of fermentation compared to the smaller cucumbers (Lu and others 2002b). Smaller fruits however have a better natural buffering capacity than bigger fruits since acid production is relatively lower. Thus, external buffers (such as calcium acetate) are added during fermentation of larger fruits, however, this may increase pH which promotes the growth of spoilage bacteria and yeasts (Lu and others 2002b). It was also found that blanching had no effect in promoting sugar utilization and acid production (Lu and others 2002b).

D- AND L- LACTIC ACID PRODUCTION

The ratio of lactic acid isomers' production is dependent on the presence of the enzymes L-lactate dehydrogenase (LdhL) for L-lactic acid, D-lactate dehydrogenase (LdhD) for D-lactic acid and lactate racemase for interconversion between D- and L- lactic acid forms or vice-versa (Ferain and others 1994; Hofvendahl and Hahn-Hägerdal 2000; Goffin and others 2005). The

ratio of racemate mixture changes according to conditions of growth and bacterial metabolism (Ferain and others 1994). In fact, the lactate racemate mixture formed by *L. plantarum* can also vary with aeration and salt concentration (Bobillo and Marshall 1992). During low pH conditions, acetate and L-lactic acid concentrations decreased whereas D-lactate increased in medium by *L. plantarum* (Bobillo and Marshall 1992). Fructose-bis-phosphate (FBP) acts as an activator of NAD dependent Ldh enzymes and some organisms need Mn^{+2} as a cofactor to activate Ldh enzymes. There are also NAD independent Ldh that are involved in lactate oxidation rather than their production (Kandler 1983; Plumed-Ferrer and others 2008).

It was observed that *L. plantarum* mutants overexpressing *ldhL* produced more L-lactic acid than the wild type but the ratio of D- and L- lactate still remained constant indicating that lactate racemase activity was activated by L- lactate (Ferain and others 1994). Ferrain and others concluded that conversion of pyruvate to L- and D- forms of lactate in *L. plantarum* is an equilibrium reaction and absence of LdhL did not affect the final ratio of these isomers (Ferain and others 1994). *ldhD* mutant strain showed poor growth due to dysfunctional peptidoglycan biosynthesis (Goffin and others 2005). It also showed a reduced expression of genes in *lar* (lactate racemase operon) when D-lactate was externally added to the medium suggesting that the ratio of L-/D- lactate regulates *ldhD*'s expression. The *lar* operon was activated in *ldhL* mutant proving that L-lactic acid is the inducer of gene for racemase enzyme in *L. plantarum* (Goffin and others 2005). The *lar* operon in *L. plantarum* is induced by L-lactate at transcription level and there may be genes outside of this operon which may or may not be dependent on the presence of L-lactate (Goffin and others 2005).

The ratio of $NADH/NAD^+$ also affects the production and activity of Ldh enzymes and under glucose limiting conditions this ratio was found to play a key role in switching from homo

to heterofermentation (Hofvendahl and Hahn-Hägerdal 2000). Under aerobic and glucose depriving conditions, *L. plantarum* produced acetate along with lactate. Fructose-bis-phosphate and levels of Ldh enzymes are the regulators in switching from homo to heterofermentation in *L. plantarum*. Interestingly, heterofermentative byproduct acetate was less prevalently seen when the cultures grown in presence of salt even at a low pH. When the same culture was grown at pH 4.5 without salt it produced acetate while produced no acetate in the presence of salt (Bobillo and Marshall 1991). This phenomenon may partly be responsible for bloater formation in no salt fermentation by heterofermentative production of acetate and CO₂. Factors that influence antimicrobial activity of weak organic acids such as lactic acid include: internal buffering capacity of the cell, effects of acids/anions on the enzyme and membranes, proton efflux and facilitated transport of acids out of the cell (Breidt Jr and others 2006). Lactic acid disrupts the lipopolysaccharide membrane in various Gram-negative pathogens and thus sensitizing outer membrane to other antimicrobial agents. Other mechanisms of lactate's antimicrobial activity include lowering the water activity, chelating iron and inhibition of metabolic enzymes such as lactate dehydrogenase (Breidt Jr and others 2006).

In humans, L-lactate is formed from pyruvic acid through anaerobic carbohydrate metabolism whereas D-lactate is formed from intestinal microbiota that possess D-lactate hydrogenase or racemases that convert L- to D-lactate (Coronado and others 1995). L-lactic acid is completely metabolized in the human body whereas D-lactic acid is poorly metabolized and only 40% of D-lactic acid is excreted. This excess amount in the stomach can lead to lactic acidosis seen in infants with necrotizing enterocolitis (Henry and others 2012). An elevated D-lactic acid excretion is seen in patients with diabetes, ischemia (restriction in blood supply to tissues, causing a shortage of oxygen and glucose needed for cellular metabolism), patients with

post-operation complications and patients that use antibiotics following short bowel syndrome (SBS) or jejunioileal surgery (Coronado and others 1995; Henry and others 2012). D- lactic acid induced acidosis was also seen in patients with short bowel syndrome that went through a bypass surgery for obesity treatment. Coma, dizziness, change in mental state, blurred speech, disorientation and hyperventilation (in kids) are some of the symptoms of D- lactic acidosis (Connolly and Lönnerdal 2004). Species including *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* were dominant in patients with SBS and produced high amounts of D- lactic acid that was not metabolized (Connolly and Lönnerdal 2004). In infants, excess D-lactate was not metabolized and excreted and also caused weight loss. However, orally induced D- lactic acid through yogurt did not cause any adverse effects in human adults. *Lactobacillus reuteri* that produces D- and L- lactic acid has been shown to be safe as a probiotic supplement that confers multiple health benefits. There was no bacterial overgrowth or imbalanced gut microbiota seen in the studies that used *L. reuteri* as a probiotic supplement (Connolly and Lönnerdal 2004). Lactate is consumed in the gut by other commensal bacteria and also D-/L- forms are interchanged by D-/L- lactate racemase enzyme when L-lactate alone is produced in high quantity. Starter cultures were isolated from Sauerkraut produced exclusively only L-lactic acid and not D- lactic acid for the above reasons (Fleming and others 1985).

BLOATING OF PICKLES

Bloating occurs due to physical and chemical reactions that lead to diffusion of CO₂ into the cucumbers at super saturated levels causing a structural deformity (Fleming and Pharr 1980). The liquid layer surrounding the cucumber mesocarp acts as a differentially permeable barrier through which CO₂ is diffused and entrapped. The gas displaces carpel that has seeds and flesh and pushes them towards the outer surface of the cucumbers. Tissues rupture due to

accumulation of CO₂ causes hollow empty spaces creating honey comb or partial balloon structure (Fleming and Pharr 1980). A supersaturated level of CO₂ is required to cause bloating which is dependent on bacterial population, temperature and salt conditions. However, bloating was observed even at subsaturated levels of CO₂ indicating that the initial internal gas composition also had an effect on the extent of bloating (Fleming and Pharr 1980). This was proved by adding sodium bicarbonate in the brine, causing supersaturation of carbon dioxide which diffused into the cucumbers and caused bloating similarly to regular fermentation (Fleming and Pharr 1980).

Heterofermenting LAB such as *L. brevis*, *L. buchneri* and fermentative yeasts produce CO₂ and thus contribute to bloating especially at salt concentration between 3 and 4.5% (Etchells and others 1968; Etchells and others 1975; Perez-Diaz and others 2013). *L. brevis* cannot withstand salt levels above 10% but it can predominate in a mixed microbiota at 8% salt levels and cause bloating during fermentation (Etchells and others 1968). Homofermenting LAB that are capable of malic acid decarboxylation also contribute to bloating together with the cucumber tissue respiration and release CO₂ (Perez-Diaz and others 2013). Small cucumbers with flowers and plant debris contain pectinolytic molds that contribute to softening (Perez-Diaz and others 2013). Purging N₂ gas during fermentation into the tanks removes the CO₂ from the cover brine, thus minimizing bloater development (Fleming and others 1973). Nitrogen purging sweeps the CO₂ along with it to the surface and then into atmosphere. Nitrogen is inert and thus does not interfere with flavor or taste of the final products (Etchells and others 1973). Even though air purging is effective and currently practiced by most pickle industries, it may promote softening and off-flavor formation from mold growth if it is not implemented carefully. It also includes a higher capital investment to purge the tanks during fermentation. Adding antimicrobial agents

such as potassium sorbate (0.04%) and acetic acid (~0.16%) reduces heterofermentative bacterial and yeast population and thus minimize bloating to an extent (Perez-Diaz and others 2013).

Malic Acid Decarboxylation:

Malic acid decarboxylation is one of the main causes for bloating as discussed previously. Phosphorylation is one of the principle source of ATP generation in bacteria which includes oxidative, substrate level and photophosphorylation. Since LAB are fermentative, oxidative phosphorylation is not found in them and substrate level phosphorylation is one of the main ATP driver (Nanatani and Abe 2011). Decarboxylation dependent phosphorylation was discovered in several LAB that derive ATP using organic acids and/or amino acids as the precursors. Some common precursor/byproducts that produce ATP through decarboxylation include malate/lactate, histidine/histamine, tyrosine/tyramine, lysine/cadaverine and citrate/lactate. After the precursors are internalized, they are decarboxylated to their respective byproduct yielding a proton gradient. The entry of precursor is usually through gradient based membrane bound antiporter mechanism where the substrate enters cell while the decarboxylated product leaves cell yielding a negative membrane potential. Thus, a chemiosmotic based proton motive force (PMF) is generated which is used to derive ATP through F_0 -ATPase system (Nanatani and Abe 2011). The cell interior becomes less acidic during decarboxylation reaction due to loss of protons and hence this reaction also aids in maintaining acid homeostasis and tolerance (Nanatani and Abe 2011). In fact, decarboxylation reaction based ATP synthesis via cell membrane is a major pathway of ATP synthesis in some LAB (Nanatani and Abe 2011). Once the decarboxylation byproduct accumulates at high levels, it inhibits the PMF gradient and also ATP synthesis by F_0 -ATPases (Nanatani and Abe 2011). The charge of the imported substrate and exported product is critical in determining the energy generation and chemiosmotic

gradient because protons are necessary to create membrane potential. At a lower pH where the substrate is usually in the protonated form, little pH gradient is generated. However, when the external pH is high there is an active PMF generated due to the presence of ionic forms of substrate and thus generation of ATP. A negative membrane potential ($\Delta\Psi$) is created during the precursor: product exchange. Internally, protons replace the liberated carboxyl group (as CO_2) and thus they are removed from the cell making the internal environment more neutral (ΔpH) and this internal pH rise also favors MDC enzyme since its optimum activity is at pH 6 (Olsen and others 1991). This combination of $\Delta\Psi$ and ΔpH drives ATP synthesis by F_0 -ATPases. This reaction is irreversible and thermodynamically stable due to the liberation of carbon dioxide (Nanatani and Abe 2011).

However, in *L. plantarum* malate/lactate are not transported through antiport mechanism and instead at low malate concentrations, L-malate is transported into cell with malate- H^+ symport mechanism and lactate is effluxed out separately or with protons (Olsen and others 1991). A lactate efflux is created and this external lactate decreases growth rate. At high malate concentration, the gradient serves as passive diffusion force to drive the malate in. Along with pH, the decarboxylation reactions are dependent on the co-factor pyridoxal-5'-phosphate. L-histidine: histamine electrogenic efflux is well studied in *L. buchneri* which creates a negative membrane potential and an increased internal pH. Thus, both metabolic energy production and pH regulation are achieved through decarboxylation reactions (Nanatani and Abe 2011). Although several methods exist to detect malic acid decarboxylation, most of them are indirect which measures the substrate or byproduct. The direct methods are long and expensive and do not allow a rapid detection when the culture collection is vast. The external acidifying property

can be visually seen on agar by adding bromocresol green which turns yellow when malic acid is not converted to lactate and a decrease in pH (Breidt and Fleming 1992).

PRODUCTION OF BACTERIOCINS

Due to the growing interest in “all natural” and “preservative free” foods, fermentation industry is driving towards providing more preservative free and natural products (Cleveland and others 2001; Chen and Hoover 2003). Bacteriocins are small peptides that are naturally produced by bacteria and have been consumed by humans for a long time without any known side effects (Cleveland and others 2001). Both Gram-positive and Gram-negative bacteria produce bacteriocins but Gram-positive bacteriocins are more diverse and well explored. Bacteriocins are cationic, amphiphilic and ribosomally synthesized that are usually 30-60 amino acids long. They can have either bactericidal or bacteriostatic effect on host microorganisms (Gillor and others 2008; Settanni and Corsetti 2008). *L. plantarum* produces ‘plantaricin’ - a bacteriocin that can kill other lactic acid bacteria and closely related Gram-positive bacteria. Similarly, pentocin is produced by *L. pentosus* and pediocin by *P. pentosaceus* with similar capabilities.

Bacteriocins can be added to food as a natural purified concentrate or as a layer on the packaging material or as a bacteriocinogenic starter or co-culture bacteria during fermentation (Gálvez and others 2007). Bacteriocinogenic starter cultures for vegetable fermentation are advantageous as they are biopreservatives that control pathogens and spoilage bacteria as well as providing health benefits associated with live cultures (Chen and Hoover 2003). Bacteriocinogenic *L. plantarum* has been successfully used as a starter culture for olive fermentation. Bacteriocin producing ability of this strain was hypothesized to be the reason for its dominance over other existing natural microbiota on olives (Leal-Sánchez and others 2003;

Chang and Chang 2010). Bacteriocins aid bacteria to invade an existing niche to establish itself and dominate the niche. They can inhibit an invasive foreign species entry into its occupied environment and mediate quorum sensing signals to interact with other bacteria (Gillor and others 2008). Clinical studies have proven that bacteriocins are also effective against treating gingivitis, dental plaque and preventing tooth decay. Bacteriocin producing strains were shown to be effective in protecting mice against *Listeria monocytogenes*, *Staphylococcus aureus* and *Helicobacter pylori* infections indicating that they could be of probiotic health benefit to the host (Gillor and others 2008). Bacteriocins have also been widely explored as oral hygiene supplements to prevent dental caries. While the bacteriocins are degraded in the gut when ingested orally, the bacteriocinogenic strains however have an advantage of competing with the native microbiota to establish themselves in the gut (Cotter and others 2005). This antimicrobial nature is especially useful in eliminating any pathogens that may have invaded human gut. Bacteriocins are considered safe since they are easily degraded by the proteases in the human host and thus do not affect the intestinal microbiota and do not induce cross resistance to antibiotics (Gálvez and others 2007).

Bacteriocin like Inhibitory Substances (BLIS) commonly refer to compounds whose amino acid sequence is not determined yet but which show a potential bacteriocin like characteristics. The positively charged bacteriocins interact with negatively charged phosphoric acid on the cell wall and hydrophobic patches of bacteriocins interact with hydrophobic part of the cell membrane (Cleveland and others 2001; Chen and others 2014). Some bacteriocins also bind to negatively charged groups such as teichoic, lipoteichoic and teichuronic acids on the cell wall and activate autolytic enzymes that cause lysis of the cell wall (Klaenhammer 1993; Abee and others 1995). Bacteriocins degrade the murein layer and depolarize cell membrane of Gram-

positive bacteria (Settanni and Corsetti 2008). The mode of action, biochemistry and genetic aspects of production of bacteriocins by lactic acid bacteria is well characterized in some strains (De Vuyst and Vandamme 1994; Gillor and others 2008; Settanni and Corsetti 2008). They produce immunity proteins that are integrated at cytoplasmic membrane for self-protection against their own bacteriocins discussed later in this section. Bacteriocin activity on host can be receptor mediated or receptor independent. Bacteriocins are usually produced during a switch from log to stationary phase (Kawai and Saito 2011).

Classes of Bacteriocins:

There are four classes of bacteriocins; however, class I and class II are the widely explored bacteriocins in LAB (Klaenhammer 1993; Cleveland and others 2001; Chen and Hoover 2003; Cotter and others 2005; Gillor and others 2008). Class I are post translationally modified bacteriocins called lantibiotics and class II are heat stable and minimally modified. Class I bacteriocins are slightly broad spectrum in their activity where it was shown to inhibit even distantly related Gram-positive organisms such as *Listeria* spp., *Staphylococcus* spp., and *Clostridium* spp. along with closely related *Lactobacillus* spp., *Lactococcus* spp. and *Leuconostoc* spp. (Chen and Hoover 2003). Type I lantibiotics are further divided into three sub classes: Type A, that comprises Nisin, the best studied bacteriocin which is screw shaped and positively charged. It creates pores through cell membranes, perturbs energy (ATP) producing PMF and dissipates the intracellular components (Dykes 1995). Type B interferes with cellular metabolism such as cell wall synthesis. The unusual amino acid lanthionine that is formed during post translational modification supposedly contributes to an increased stability, resistance to oxidation, pH, temperature and proteolysis (De Vuyst and Vandamme 1994; Cintas and others 2001). Presence of cations reduces the efficiency of pore formation of nisin because they

neutralize and condense the anionic phospholipids increasing the rigidity of cell wall (Abee and others 1995; Cintas and others 2001). The legal safe limits of nisin addition to foods varies from country to country, however, FAO recommends no more than a daily intake of 60 mg pure nisin for a 70 Kg person (Chen and Hoover 2003). Type II bacteriocins are non-lanthionine containing heat stable peptide chains that kill the hosts by forming pores through permeabilization of membranes and leakage of intracellular components. They are amphiphilic, relatively narrow in spectrum and can inhibit only closely related bacteria such as *Lactobacillus* spp., *Lactococcus* spp. and *Enterococcus* spp. (Chen and Hoover 2003). Class III bacteriocins are large and heat sensitive which include helveticin J or lactacin B. Little is known about Class IV bacteriocins (Klaenhammer 1993; Cintas and others 2001; Gillor and others 2008).

Effect of Food Matrix on Bacteriocin Activity:

Food matrix can affect the efficacy of bacteriocins or bacteriocinogenic strains due to the surrounding complex ecosystem (Gálvez and others 2007). Factors including pH, presence of oils, fats and proteins can affect the binding and efficiency of the bacteriocins (Cleveland and others 2001). The preservative benefits of bacteriocins can be used as a part of hurdle technology along with other preservatives such as acids, thermal and non-thermal treatments (Abee and others 1995; Settanni and Corsetti 2008). For example, nisin was effective in hurdle technology that combined one or more antimicrobial treatments such as EDTA, acids, pulsed electric field processing, high pressure processing and modified atmosphere packaging to eliminate Gram-positive pathogens and even structurally compromised Gram-negative pathogens (Cleveland and others 2001; Cotter and others 2005; Gálvez and others 2007; Settanni and Corsetti 2008). Some pathogens may survive in food due to sub lethal exposure to bacteriocins and thus having multiple antimicrobial treatments will ensure a complete elimination of pathogen load. The

choice of hurdle treatment with bacteriocins depends on the type of target pathogen for example: acid treatment works well for acid sensitive pathogens whereas high heat treatment is required for spore formers (Cotter and others 2005; Gálvez and others 2007).

Some of the common problems encountered while using bacteriocins in food are storage conditions, presence of acids, enzymes, sugars, redox potential, adsorption of bacteriocins to the food components, instability of bacteriocins and presence of interfering microbial load (Gálvez and others 2007). Organic acids, nitrites, ethanol, essential oils (antioxidant components) and lysozyme were shown to work synergistically with nisin in inhibiting the pathogens. EDTA dissipates Ca^{+2} and Mg^{+2} ions and thus destabilize the lipopolysaccharide layer of Gram-negative bacteria rendering them susceptible to bacteriocins (Gálvez and others 2007). Combination of bacteriocins can also be effective in eliminating certain pathogens. Sterile food model system is routinely used to imitate an actual food matrix to evaluate the efficiency of bacteriocins (Settanni and Corsetti 2008). Bacteriocins were able reduce the pathogenic and spoilage spore forming vegetative bacteria in food systems such as canned vegetables, juices and vegetable purees (Chen and Hoover 2003; Settanni and Corsetti 2008). Combined effects of preservatives and bacteriocins were effective in reducing *L. monocytogenes* and *Clostridium* spp. in dairy products, meat, fresh cut fruits and vegetables (Settanni and Corsetti 2008; Cleveland and others 2001; Chen and Hoover 2003). Recently, bacteriocin based packaging was developed that slowly release bacteriocins during short term storage. Various packaging materials were tested which incorporated EDTA and other agents to enhance the activity of bacteriocins. These packages have successfully eliminated pathogens such as *Listeria* spp. and *Staphylococcus* spp. (Chen and Hoover 2003).

Role of Bacteriocins in Fermentation:

From the perspective of selecting an ideal starter culture, bacteriocinogenic strains have an advantage because a rapid predominance of desired starter strain(s) during the initial stage of fermentation allows for a more consistent and stable fermentation throughout the process. In fact, inhibition of other competing bacteria through action of bacteriocin is a common amensalism strategy used by LAB to predominate in a complex system such as cucumber fermentation (Di Cagno and others 2013; Ivey and others 2013). So, this study aimed at characterizing starter cultures that have an ability to inhibit common spoilage and undesirable heterofermenting and spoilage bacteria associated with fermented cucumbers. These starter cultures may also be used as co-cultures in other fermentations such as wine, meat and Kimchi if they possess a broad bacteriocinogenic potential (Cotter and others 2005; Di Cagno and others 2013). However, when the bacteriocinogenic strains are used as an adjunct co-cultures, the intended starter culture strain must be resistant to bacteriocins and should not interfere with normal fermentation (Gálvez and others 2007). Daeschel and others reported bacteriocin production by *L. plantarum* from cucumber fermentation that was bacteriocidal, proteinaceous and heat stable with an optimal pH between 4.0 and 6.5 (Daeschel and Fleming 1984). This bacteriocin was able to inhibit other competing lactobacilli during fermentation and outcompeted natural microbiota (Klaenhammer 1988). It would be advantageous during storage and in cases of temperature abuse if the bacteriocinogenic strain may also act as spoilage agent indicating quality or safety concern (Gálvez and others 2007; Di Cagno and others 2013). Salt, which is a major component in most vegetable fermentations, was shown to have a positive effect on the activity of bacteriocins by the starter strains. NaCl interacts with ions that might otherwise interfere with activity and efficiency of bacteriocins (Gálvez and others 2007). Since bacteriocins work synergistically in

hurdle technology, severe heat treatments can be mitigated which may otherwise compromise the quality of final product (Gálvez and others 2007).

Factors Affecting the Activity of Bacteriocins:

Some impeding factors that limit the efficacy of the bacteriocinogenic strains are the loss of ability to produce bacteriocins, competition with other bacteria in the food, insufficient cell numbers to produce enough bacteriocin quantities and composition of food matrix (Gálvez and others 2007). Ability to produce bacteriocins may be lost from plasmid instability if the genes are encoded on plasmid or if there are mutations on bacteriocin associated genes. The ability can also be lost if the production is too little with a high dilution of nutrients in environment. It was shown that there were no bacteriocins produced when the cell concentration in fresh culture was less than 4 log units. Bacteriocin mutant strains can be inducible to produce bacteriocins when induction factors were supplemented in the medium (Cintas and others 2001). Resistance to bacteriocins is not well studied although some Gram-positive organisms were shown to produce nisinase that degrade nisin (Abee and others 1995; Cleveland and others 2001). It is also possible to develop resistance by continuous exposure where the host bacteria modify fatty acid chain composition affecting the fluidity of cell membrane (Cleveland and others 2001; Cotter and others 2005). When the cell membrane becomes more rigid it is harder for the bacteriocins to exert their action (Abee and others 1995). Organisms that are resistant to antibiotics are usually not cross-resistant to bacteriocins since bacteriocin resistance mechanism must involve modifying their basic cell wall composition (Cleveland and others 2001). It was discovered in nisin resistant strain of *L. lactis* that this resistance has a complex phenotype with mechanisms involving prevention of insertion into cell wall and transporting the inserted nisin out of cell wall (Cotter and others 2005; Kramer and others 2006; Gálvez and others 2007).

Plantaricin locus (*pln*):

Plantaricins (Pln) are class IIb bacteriocins encoded in the *pln* locus in strains of *L. plantarum*. The locus consists of 22-25 genes in 5-6 operons in chromosome or plasmid depending on the strain (Diep and others 2009; Kawai and Saito 2011). They are two peptide bacteriocins whose optimal activity is dependent on the presence of both peptide components (Diep and others 2009). The *pln* operon has a conserved region and a variable region within several *L. plantarum* strains and includes genes encoding for class IIb bacteriocins, specific ABC transporter and quorum sensing peptides (Diep and others 2009; Di Cagno and others 2010; Kawai and Saito 2011). *L. plantarum* C11 (vegetable origin) was widely studied for its *pln* synthesis and was shown to produce different plantaricins including PlnA (48 amino acids), PlnE (33 aa), PlnF (34 aa), PlnJ (25 aa), PlnK (32 aa) and PlnN (Kawai and Saito 2011). PlnA works as a quorum sensing pheromone for inducing bacteriocin production and also as an antimicrobial peptide (Diep and Nes 1995; Diep and others 2009). PlnE and PlnF (encoded by *plnE/F*) and PlnJ and PlnK (by *plnJ/K*) are hypothesized to be cationic complementary bacteriocins whose maximum activity is detected when both of them are present together (Hauge and others 1999; Kawai and Saito 2011). Regulatory genes *PlnB* and *PlnCD* encode histidine kinase and putative two component regulatory system respectively for bacteriocin production (Kawai and Saito 2011). The pheromonal signal transduction peptide PlnA induces expression of *plnABCD* operon and also *plnEFI* and *plnJKLR* that encode PlnE/F and Pln J/K two component bacteriocins (Diep and others 2003; Kawai and Saito 2011). PlnC and PlnD regulate the transcription of bacteriocin encoding genes by activation and repression, respectively (Kawai and Saito 2011). *PlnMP*, *plnI* and *plnL* code for putative bacteriocin immunity proteins that confer immunity to the producing cell against its own bacteriocins (Kawai and Saito 2011). *plnGHSTUV* encode ABC transporter

PlnG and accessory protein PlnH for processing and transport of the plantaricins to exterior of the cell. Loci *plnEFI* and *plnGHSTUVW* are conserved while the other regulatory loci are less conserved (Diep and others 2009). *plnMNOP* is hypothesized to be involved in PlnN synthesis that is part of N-terminal double glycine leader.

PlnA interacts with cell membrane of the sensitive target cells and display antibacterial capacity. PlnA contains cationic amphiphilic core that permeabilizes through negatively charged membrane to create pores and dissipate the membrane potential and transmembrane pH gradient and also serves the purpose of pheromone (Diep and Nes 1995; Kawai and Saito 2011). PlnA senses exterior of the cell and stimulates histidine protein kinase (HPK-PlnB) and homologous cytoplasmic regulators (PlnC/D) that regulate and activate the promoters of bacteriocin operon (Diep and Nes 1995). In fact, PlnA by itself may be considered as a class IIc bacteriocin (no post-translational modification) with a narrower spectrum than PlnEF or PlnJK. PlnA does not have a corresponding immunity protein and it can be considered as an evolutionary protein between the induction factor and antimicrobial peptide (Diep and Nes 1995; Diep and others 2009). In a liquid culture where PlnA could be easily diluted before reaching a critical concentration, the bacteriocin synthesis may be inhibited due to lack of pheromonal signals (Diep and others 2009).

The interaction between pheromone and HPK is specific and thus triggers the phosphorylation of regulatory response proteins in a specific manner only when it is required (Diep and Nes 1995; Diep and others 2009). During the initial phase, PlnC binds strongly to *pln* locus causing an outburst of plantaricin production and after a critical limit, PlnD replaces the bound PlnC ceasing any further expression of *pln* associated genes (Diep and others 2009). Another possible mechanism by which PlnC is regulated is through production of truncated

version of PlnC with alternative start codons which autorepresses its own regulon (Diep and others 2009). Strains C11 and WCFS1 share a high homology in *pln* loci, however, WCFS1 contain *plnXY* gene following *plnGHSTUW* that is involved in plasmid maintenance. Its role in Pln synthesis however is not yet determined (Diep and others 2009).

The genes *plnE* and *plnF* are located next to each other on *plnEFI* operon which is highly conserved among various *L. plantarum* strains studied. They may possess bacteriocin activity even as individual peptide molecule (except PlnE) in *L. plantarum* C11. They form α -helical cells with polar residues on one side and non-polar on the other exhibiting a bactericidal mode of action by permeabilizing through the membrane and creating pores (Hauge and others 1999; Diep and others 2009; Kawai and Saito 2011). PlnEF creates pores through interaction with small monovalent cations whereas PlnJK interacts with specific anions indicating that the charge of lipid membrane is critical for their activity (Kawai and Saito 2011). PlnI and PlnLR serve as immunity proteins however their specific mechanism is yet to be determined. They could either serve in the sugar uptake system or prevent its own bacteriocin from binding to it or it could enzymatically modify the structure of the bacteriocin making the producer cells insusceptible to its own plantaricins (Diep and others 2009).

Pln NC8 is produced by the *L. plantarum* NC8 (isolated from grass silage) that is also a class IIb bacteriocin with two peptides. Strain J23 (isolated from grape must) is also similar to NC8 with operon containing genes encoding for N-terminal leader sequence, immunity protein, 3-component regulatory system with an induction factor, histidine kinase and only one response regulator (unlike C11 with two response regulators PlnC/D) (Kawai and Saito 2011). PlnNC8 and J23 production was induced by the presence of both live and dead cells of various Gram-positive organisms indicating that their expression is dependent on surrounding environment and

they are produced only during stress or competition (Kawai and Saito 2011; Diep and others 2009). Plantaricin C produced by *L. plantarum* LL441 (isolated from homemade cheese whey) also creates pores in a voltage independent manner but does not require a specific receptor to kill the target cell membrane. It is also different from other plantaricins in that it is a lanthionine containing class I bacteriocin. Plantaricin W is a lantibiotic from the strain LMG2379 (isolated from wine) has α and β peptide chains that act in synergy similar to PlnEF or PlnJK (Kawai and Saito 2011). Plantaricin S was characterized from the strain LPCO10 (from Spanish olive fermentation) that is similar to other plantaricins and includes an N-terminal leader, histidine kinase and response regulators (Kawai and Saito 2011). The optimal conditions for plantaricin S were found to be 22-27 °C temperature, 2.3-2.5% NaCl and an inoculum level of at least 7.3 log CFU/mL (Leal-Sánchez and others 2003; Kawai and Saito 2011). A recent study on *L. plantarum* V90 (from fermented meat) revealed a novel plantaricin that is similar to C11. However, V90 encoded transposon element next to *plnQ* which was not identified in other *L. plantarum* strains. In fact, strain V90's plantaricin production is constitutive unlike C11 which needs a pheromonal trigger by PlnA. Presence of this transposon element downstream of the regulatory operon *plnABCD* is hypothesized to be the reason for this new phenotype observation (Diep and others 2009). Strains that possessed *pln* genes were one of the predominating during cucumber and various dairy fermentations (Singh and Ramesh 2008; Diep and others 2009). However, genes encoding plantaricin S and W encoded strains originally isolated from olive and wine fermentation respectively are rarely found (Diep and others 2009).

Prior to using bacteriocins or bacteriocinogenic strains in foods their safety features, spectrum of inhibition, biochemical characteristics and optimal production conditions must be evaluated (Yang and Ray 1994). Agar spot assays and well diffusion assays are commonly used

to assess the antimicrobial capacity of the bacteriocins or BLIS. The cell free supernatant of bacteriocinogenic culture is treated with alkali and catalase to eliminate the inhibitory effect of acid and peroxides, respectively (Klaenhammer 1988). Bacteriocins are extracted by centrifuging the cell culture and precipitating the supernatant protein by ammonium sulfate dialysis. The activity is tested against competitor strains in fermentation that are commonly the spoilage and pathogenic microorganisms. They are later analyzed by chromatographic techniques or/and MALDI TOF (Cintas and others 2001). Some of the factors that affect the bacteriocin activity *in vitro* are the purity of the sample preparation, nutrient composition of the growth medium and sensitivity of the test strains (Corsetti and others 2004).

PRODUCTION OF EXOPOLYSACCHARIDES

Exopolysaccharides (EPS) have a wide variety of applications in industries including food, material science, pharmaceutical and biotechnology (Freitas and others 2011). Food industry is of special significance as they are used as texture modifiers in a wide range of dairy and baked products and they are considered GRAS (Vuyst and Degeest 1999; De Vuyst and others 2001; Duboc and Mollet 2001). Some EPS that are widely used in the food industry are xanthan gum, gellan gum, alginate and glucan. Exopolysaccharides impart desired rheological aspects like thickening, gelling, emulsifying, stabilizer, viscosifying agent, adding mouth feel to various dairy products (Vuyst and Degeest 1999; Badel and others 2011). They are broadly divided based on the type of individual sugars that make up the polysaccharide chain-homoexopolysaccharides (HoPS- 10^4 - 10^6 Da in size) such as dextran and levan; heteroexopolysaccharides (HePS- 10^4 - 10^7 Da in size) such as xanthan gum and gellan (Vuyst and Degeest 1999; Badel and others 2011; Nwodo and others 2012). Non-carbohydrate moieties such as phosphate, acetyl, uronic acid and glycerol can also be present as a part of EPS

macromolecule (Nwodo and others 2012). The sugar monomers that commonly make up EPS from *L. plantarum* and *L. pentosus* are glucose, fructose, galactose, rhamnose and mannose in varying ratios (Vuyst and Degeest 1999; Tallon and others 2003; Rodríguez-Carvajal and others 2008; Ismail and Nampoothiri 2010). A given strain can produce EPS with different monomer composition depending on the carbon source supplied in the medium and growth conditions (Vuyst and Degeest 1999). As the EPS chain length increases the complexity in entanglement and inter/intra molecular interactions change and thus the physicochemical behavior (Nichols and others 2005a; Kim and Yim 2007). The size, shape and structural rearrangements of EPS are critical in determining their intermolecular interactions and thus the functionality and applications (Vuyst and Degeest 1999).

Role of EPS in Fermentation:

Exopolysaccharides mask the cell surface, protect cell barrier properties and thus prevent cells from adversities (Patel and Prajapati 2013). Exopolysaccharides aid bacteria in adapting to stressful environments and are not likely to be used as an energy source (Cerning and others 1994; Donot and others 2012; Harutoshi 2013). Ecologically, EPS form defensive microstructures around bacteria to help them survive against extreme conditions (Kim and Yim 2007). Exopolysaccharides can sequester/degrade metal ions due to their anionic nature and also allow a controlled diffusion of stress factors such as metal intoxication, antibiotics or surfactants and thus protecting the cell (Badel and others 2011; Donot and others 2012; Patel and others 2012). They also aid in survival against stress during processing (Patel and Prajapati 2013). Even though the energy expenditure and carbon investment for EPS production is high, microbial cells produce EPS for defensive and protective purposes during fermentation (Kim and Yim 2007; Poli and others 2010).

Cryoprotective Role of EPS:

Apart from the role of niche establishment through EPS production, some marine Arctic and Antarctic polar bacteria produce EPS as a survival strategy in extreme habitats for cryoprotection, in high temperature (deep sea thermal vents), osmolarity (hyper salinity) and pressure (Nichols and others 2005a; Kim and Yim 2007; Poli and others 2010). Exopolysaccharides were found to be a major component in the particulate content of Arctic and Antarctic ice microbial communities enabling them to survive against extreme cold conditions (Nichols and others 2005b; Kim and Yim 2007). It was found that EPS synthesis can be stimulated by low temperature in Antarctic bacteria (Nichols and others 2005b). In fact, Junge and others found that Arctic bacteria produce EPS only during winter time concluding that EPS are involved in a strong cryoprotective role (Junge and others 2004). Glucose, galactose and mannose were the major sugars abundant in these Antarctic derived EPS. When EPS producing wild type and mutant strains of *Pseudomonas* spp. were frozen at -20°C and -80°C and thawed, the wild type had a significantly higher viability at both the freezing temperatures (Carrión and others 2015). Studies also compared the cryoprotective role of Antarctic EPS to commercial cryoprotectants such as glycerol and found similar functionality (Kim and Yim 2007; Carrión and others 2015). However, the precise cryoprotective mechanism is yet to be established. The structure of EPS is less ordered at high temperatures and in absence of ions they tend to be in solid state and thus they could also an effect of the texture of final product (Poli and others 2010). We anticipate that the EPS producers in this study have a similar strategy of survival seen in high salt and low temperature storage conditions which is a common scenario in the US Midwestern pickling industries. They may also aid in preservation and protection against spoilage during long storage months in winter if they produce bacteriocins to inhibit spoilage

bacteria and also by maintaining a low pH. During the winter months the finished products are stored in 12-15% of salt until packed. Thus, NaCl levels may be reduced during storage and subsequent washing and disposal steps if EPS offer cryo-effective benefit for long term winter storage of fermented products.

Biosynthesis of EPS:

Most EPS are produced intracellularly and transported actively or passively to exterior. Exopolysaccharide synthesis is active during exponential and stationary phase and it is dependent on carbon substrate along with minerals, nitrogen, temperature and pH (Cerning 1990; Cerning and others 1992; Rodríguez-Carvajal and others 2008; Ismail and Nampoothiri 2010; Poli and others 2010). Presence of different sugar transport systems for entry of mono and disaccharides also has an effect on EPS production (Ruas-Madiedo and de los Reyes-Gavilán 2005). The genes involved in EPS production can be present on plasmid or chromosome (Donot and others 2012). Exopolysaccharides specific gene cluster is well documented in dairy starter cultures *S. thermophilus*, *L. delbrueckii* and *L. lactis* (De Vuyst and others 2001). In *L. lactis*, they were encoded on plasmids and conserved in most strains (De Vuyst and others 2001). Phosphoglucomutase (Pgm) that converts glucose-6-phosphate to glucose-1-phosphate has a critical role in regulating pathways to synthesize backbone sugars that constitute EPS (Welman and Maddox 2003). Exopolysaccharide production is an energy expensive process involving ATP expenditure throughout the process (De Vuyst and others 2001). Sucrose in general is the most preferred sugar for EPS biosynthesis (Vuyst and Degeest 1999). From a production point of view, inexpensive waste products may be fed as substrate to the EPS producing microorganisms but undesirable byproducts may accumulate. Process and strain improvement are the two most

common strategies to increase the yield and quality of EPS production (Vuyst and Degeest 1999).

Homo-exopolysaccharides can be synthesized intra or extracellularly using a single substrate whereas HePS are synthesized using various precursors intracellularly with polymerization occurring externally after translocation through the membrane. However, it was recently discovered that HoPS such as pullulan and curdlan are synthesized only intracellularly and exported outside the cell. Flippase protein is hypothesized to translocate the monomer chains through the membrane and assemble the final EPS outside the cell (Donot and others 2012). Numerous housekeeping genes and EPS biosynthesis specific genes are involved in the process of EPS production (De Vuyst and others 2001; Ruas-Madiedo and others 2002). Specifically, HoPS production is less energy intense since glycosyltransferase is the only enzyme that is required for sugar addition to the growing EPS chain. The sugars in homo-EPS are linked to one another by glycosyltransferases (Gtf) or fructosyltransferases (Ftf) at exterior of the cell. There is no dedicated transport process or use of phosphorylated sugars in HoPS and thus the energy requirement is low (Lebeer and others 2008). The genes involved in EPS production are not necessarily confined and are also involved in other cellular functions (Vuyst and Degeest 1999). However, they are present as a cluster in many LAB in one direction and transcribed together as a single polycistronic mRNA (Welman and Maddox 2003; Bindhumol 2012). Unlike HoPS, a series of steps are involved in HePS production including- translocation of monomer sugars into cytosol, sequential addition of activated monomer sugars by Gtf/Ftf, transport of these sugars to a lipid carrier at cell membrane, release of the repeating units to exterior through cell membrane and a final assembly of thousands of repeating units by polymerase (De Vuyst and others 2001; Welman and Maddox 2003; Donot and others 2012; Patel and others 2012; Harutoshi 2013).

The Gtf involved in attaching sugar moieties to increasing chain are the rate limiting enzymes that affect EPS production in most LAB (Vuyst and Degeest 1999).

Genes encoding for EPS can be found either on plasmids or chromosome and sometimes plasmids containing EPS gene cluster may be lost (Duboc and Mollet 2001; Welman and Maddox 2003). It was shown that EPS production can be increased if carbon metabolism is diverted from production of lactic acid by knocking out D-/L- lactic dehydrogenase genes. The Gtf genes can be manipulated to produce different types of EPS which are involved in polymerization and export of final product (Welman and Maddox 2003). Omission of certain vitamins, minerals and amino acids also led to a reduced yield of EPS indicating that non carbon components in the media have an effect on EPS production (Vuyst and Degeest 1999). Prolonged incubation may degrade the EPS due to glycohydrolase activity from cells and also unfavorable culture conditions (Vuyst and Degeest 1999; De Vuyst and others 2001). Higher growth temperatures, loss of plasmids encoding EPS can lead to loss of EPS production capability (De Vuyst and others 2001; Harutoshi 2013). Temperatures lower than optimal growth temperature favored EPS production in some organisms since less carbon is utilized towards energy production due to slower metabolism (Carrión and others 2015). In addition, cells grow slower at lower temperature and thus cell wall synthesis is slower, making carbon sources and their byproducts more available for EPS biosynthesis (Cerning and others 1992). However, contradicting results were observed on the effect of temperature where higher growth temperature was suggested as ideal conditions for higher EPS production and thus the effect of temperature varies from strain to strain along with other factors like pH and oxygen tension (Kimmel and others 1998; Vaningelgem and others 2004; Bennama and others 2011). In some *L. lactis* and *L. delbrueckii* strains it was found that EPS production was lower when fructose is

used as carbon source compared to glucose. The precursor sugar nucleotides are produced in lower quantities when fructose is used as the main carbon source. When fructose was the main sugar, EPS production took place in stationary phase when there is no requirement for sugar any more (De Vuyst and others 2001). Glucose was found to be preferred sugar for HePS biosynthesis due to a higher production of precursor sugar nucleotides (Welman and Maddox 2003). Designer polysaccharides may be developed depending on the application if the genetics, production can be controlled (Vuyst and Degeest 1999).

Probiotic Role of EPS:

Exopolysaccharides can also serve as nutraceuticals with prebiotic effects, anti-tumor, anti-ulcer, anti-mutagenic (against cyclic amines), immune-stimulatory roles, form physical intestinal barrier and cholesterol lowering ability (Ruas-Madiedo and others 2002; Ruas-Madiedo and others 2006b; Aswathy and others 2008; Wu and others 2010; Lebeer and others 2011; López and others 2012; Harutoshi 2013; Patel and Prajapati 2013; Hidalgo-Cantabrana and others 2014). Adherence to cell lines was higher in EPS producing *Bifidobacterium* spp. and in murine cells by *Lactobacillus rhamnosus* GG (Lebeer and others 2011; López and others 2012). Exopolysaccharides also improve adherence properties of probiotics during gut transit thus increasing the colonization and transit time (Welman and Maddox 2003; Ruas-Madiedo and others 2006b; Badel and others 2011; Hidalgo-Cantabrana and others 2014). More studies on relationship between physiological function and structure of EPS are needed to propose a definite mechanism of adherence (De Vuyst and others 2001; Jolly and others 2002; Ruas-Madiedo and others 2006a; Patel and others 2012). Exopolysaccharides can form a protective barrier directly on the mucus and competitively inhibit the binding or surround the producer cell which decreases the binding efficiency of pathogens (Ruas-Madiedo and others 2006b).

Exopolysaccharides stimulated splenocytes from mice and produced lesser proinflammatory cytokines when they were incubated together indicating a potential immunostimulatory activity (Hidalgo-Cantabrana and others 2014). Exopolysaccharides from *Bifidobacterium longum* also showed an antimicrobial activity against various foodborne pathogenic bacteria such as *Bacillus* spp., *Salmonella* spp., *Escherichia coli* and *Staphylococcus* spp. (Wu and others 2010; Patel and others 2014). Exopolysaccharides can potentially also act as virulence factors due to the above reasons. Exopolysaccharides act as prebiotics which are fermented by the gut bacteria and reduce pH to inhibit undesirable bacteria and enhance absorption of minerals (Patel and others 2012). They serve as precursors to short chain fatty acids (SCFA) such as γ -amino butyric acid (GABA) that are anti-carcinogenic in nature (Duboc and Mollet 2001; Jolly and others 2002). Some EPS are a long polymerized chain (>25) subunits and may not qualify as prebiotic. Such polysaccharides must be enzymatically broken down to use them as prebiotics (Badel and others 2011).

Characterization of EPS:

It is essential to understand the chemical nature, molecular mass and structure of EPS since the physiological properties and textural effects are dependent on the individual sugar moieties that make up the backbone of EPS (Ruas-Madiedo and others 2002; Kodali and others 2009). Exopolysaccharides are extracted by centrifugation or solvent based precipitation such as isopropanol, methanol followed by drying the final product (Freitas and others 2011). Exopolysaccharides production can be anywhere between 50-700 mg/L but can go up to 2700 in some LAB (Harutoshi 2013). Biochemical characterization EPS usually starts with visual classification as either “ropy” or “slimy”. A sharp pointed tooth pick is used to test the ropiness or sliminess on the plate. The culture medium also turns viscous and it is obvious during

pipetting of the culture that falls off the tip (Ruas-Madiedo and de los Reyes-Gavilán 2005). Ruthenium red has been used to identify EPS and non-EPS producing *Streptococcus thermophilus* strains where the former produces white color colonies and the latter makes pink colonies due to uptake of the dye (Ruas-Madiedo and de los Reyes-Gavilán 2005). This method is particularly efficient if the EPS are ropy in nature.

Exopolysaccharide screening medium with a high concentration of glucose was developed to screen for the EPS producing strains from various fermentation origins. Traditional MRS with excess sugar concentration (up to 100 g/L) of various sugars was also used to screen the EPS producing strains. Sucrose-rich MRS medium in general was able to support the EPS production the most although it varies from strain to strain (Ruas-Madiedo and de los Reyes-Gavilán 2005). Crude EPS is obtained after protein precipitation using trichloroacetic acid or pronase digestion and centrifugation followed by concentration with ethanol or acetone (Ruas-Madiedo and de los Reyes-Gavilán 2005). This is followed by purification and concentration steps such as dialysis, microfiltration and/or ultrafiltration at refrigeration temperature depending on the size and then subjected to acid hydrolysis to determine the monomer sugar composition by HPLC (Smitinont and others 1999; Mishra and Jha 2013). Quantification of carbohydrates in EPS determined by assays such as phenol-sulfuric acid method (total carbohydrate content), Anthrone test or dinitrosalicylic acid test for reduced sugars. The chemical composition and structure of the EPS is studied using GC-MS, ion exchange/size exclusion chromatography, MALDI-TOF and NMR spectroscopy (Rodríguez-Carvajal and others 2008; Donot and others 2012). Chemical structure of EPS can be tailored to specific use, increasing the versatility and adaptability of its application in various industries.

PRODUCTION OF BIOGENIC AMINES

Biogenic amines are low molecular weight nitrogen based compounds formed from the decarboxylation of amino acids in fermented foods. They are formed from precursors such as tyrosine, histidine, arginine, lysine and ornithine. Biogenic amines can be present as monoamines such as histamine, tyramine and tryptamine or diamines including putrescine, spermidine and cadaverine (Špička and others 2002). Diamines are frequently associated with meat and wine spoilage emitting off odors that can be carcinogenic. Putrescine can also be formed from deimination of agmatine (EFSA 2011). They occur in fermented foods such as wine, cheese, beer and sausages. They are involved in various physiological roles in many microorganisms, plants and animals. The metabolic significance of biogenic amine formation in LAB is similar to that of malic acid decarboxylation discussed in previous sections. In humans they are involved in brain regulatory functions like maintaining homeostasis in stomach, immune and allergic response, blood pressure control, and gastric juice secretion (Ladero and others 2010; EFSA 2011). However, excessive consumption of BA is undesirable due to many side effects. Histamine intake > 100 mg/day can cause moderate poisoning and the toxicity levels for a healthy individual are 50-100 mg and 100-800 mg/day for tyramine (toxic at >1080 mg) (Nout 1994; Önal 2007). Humans can ingest up to 180 mg of pure histamine orally without any side effects but intravenous ingestion of as low as 0.007 mg can cause increase in heart rate and vasodilation (Weiss and others 1932). Due to these discrepant toxic limits and variable detoxification system of individuals it is difficult to estimate an exact toxic dosage for a range of population.

Decarboxylating enzymes histidine decarboxylase (Hdc) decarboxylates histidine to histamine, tyrosine decarboxylase (Tdc) decarboxylates tyrosine to tyramine, ornithine decarboxylase (Odc) decarboxylates ornithine to putrescine and lysine decarboxylase (Ldc)

decarboxylates lysine to cadaverine (Marcobal and others 2005). The genes *odc* and *hdc* are frequently detected on plasmids and found only in certain bacteria which are acquired possibly through horizontal gene transfer (Coton and others 2010). In fact, *L. plantarum* is frequently associated as biogenic amine forming bacteria in fermented sausages (Straub and others 1995; Komprda and others 2010). Hdc activity in *Lactobacillus* spp. and most Gram-positive organisms for that matter is pyrovoyl dependent whereas Tdc enzymes are pyridoxal phosphate cofactor dependent (Coton and Coton 2005). Addition of starter cultures during vegetable fermentation reduced the concentration of putrescine compared to the wild fermented samples indicating that rapid acid production will hinder biogenic amine producing bacteria (Karovičová and others 1999). Agar based color change methods and HPLC quantification methods are routinely employed to detect BA. There have been discrepancies and false positives that were detected by agar based pH detection due to alkaline compound formation (Marcobal and others 2005). A combination of phenotypic, molecular methods such as detection of decarboxylating genes by PCR and quantitative HPLC methods are suggested as the most reliable methods (Bover-Cid and Holzapfel 1999; Önal 2007; Buňková and others 2009; Spano and others 2010).

PRESENCE OF ANTIBIOTIC RESISTANCE

Lactic acid bacteria from fermented foods are one of the predominant carriers of transferable antibiotic resistance genes in food chain (Toomey and others 2009). Even though LAB were classified as GRAS by FDA due to their safe historic usage in various fermented foods, recent discoveries on antibiotic resistance gene transfer subjected them to a thorough scrutiny before they can be used as starter cultures (Klare and others 2007). Commensal gut bacteria harbor antibiotic resistant genes that are transferable to other neighboring gut bacteria and sometimes to pathogens through promiscuous horizontal gene flow. The prevalence of

multidrug resistant organisms in farm animals is high which can potentially come in contact with vegetable farms through animal waste disposal. Starter cultures and commercial probiotics were also found to be resistant towards several antibiotics (Danielsen and Wind 2003; Kastner and others 2006). When antibiotic resistant microbes are ingested into human gut, a transient residence is observed during which gene transfer can occur between these resistant bacteria and native gut microbiota (Teuber and others 1999; Danielsen and Wind 2003). It is not the bacteria itself that is risky but the ability to disseminate antibiotic resistant genes to pathogens is the concern. Thus, live cells being delivered through food chain must not code for these transferable antibiotic genes as they can be transient residents in gut (Danielsen and Wind 2003; Clementi and Aquilanti 2011; Devirgiliis and others 2013).

Intrinsic and Acquired Antibiotic Resistance:

Intrinsic antibiotic resistance is naturally present in bacteria whereas acquired resistance is transferred through conjugation and/or transformation of transposons or plasmids encoding antibiotic resistance genes and mutation of intrinsic genes (EFSA 2008; EFSA 2012). Intrinsic resistance mechanisms could be due to absence or modification of targets for the drug to act upon, low affinity for the drug uptake or permeability, non-specific antibiotic drug efflux and drug concentration below the inhibition levels. Intrinsic resistance in general is not horizontally transferable between bacteria because the enzymes are also involved other physiological and metabolic functions of the cell (Nawaz and others 2011). Bacteria with intrinsic resistance to antibiotics are naturally resistant towards some antibiotics. For example, even though phenotypic presence of resistance towards tetracycline, ciprofloxacin and vancomycin was determined in *L. brevis* KB290, no evidence of genes or gene transfer was detected between *E. faecalis* indicating that *L. brevis* is naturally resistant to those antibiotics (Fukao and others 2009).

Acquired or non-intrinsic resistance occurs from uptake of genes that code for enzymes that specifically inactivate the drug or genes that reduce the susceptibility of microorganism to the drugs by target modification or drug efflux (EFSA 2008). Thus, acquired resistance has a greater potential of transfer to other bacteria than intrinsic resistance. The gene uptake occurs through mobilizable and conjugative elements including plasmids, transposons, lytic phages, non-conjugative plasmids and transposons without replication machinery that can move from plasmid to plasmid or plasmid to chromosome or vice-versa (Clementi and Aquilanti 2011; Devirgiliis and others 2013). Horizontal transfer of integrons that code for several antibiotic resistance gene cassettes was also detected (EFSA 2008). Conjugational plasmids and lytic phages are one of the common natural carriers of antibiotic resistance transfer in LAB (Teuber and others 1999). The probability of antibiotic resistance being transferable is studied based on the presence of the acquired resistance gene as opposed to intrinsic or natural presence of the antibiotic resistance genes on the chromosome. Distinction between intrinsic and acquired antibiotic resistance may be difficult since most of these organisms were studied for these properties during the pre-antibiotic era. Studies have shown that identical genes responsible of antibiotic resistance can be transferred between various genera or species. For example, streptomycin and erythromycin resistance genes were identical in various enterococci, lactobacilli and streptococci (Teuber and others 1999). Bacteria acquire antibiotic resistance genes from donors as a mechanism of survival when they are exposed to antibiotics repeatedly. Chromosomal conjugation of antibiotic resistance genes is also observed in some enterococci and lactococci by conjugative transposons which involve excision and incorporation of these genes into the host genome (Clementi and Aquilanti 2011). In fact, chromosomal resistance through mutations may augment the chances of resistance through subsequent horizontal gene

transfer (Teuber and others 1999). The distinction between intrinsic and acquired resistance must be clearly made before a starter culture or a probiotic is being used (EFSA 2008; EFSA 2012). European Food Safety Authority (EFSA) publishes the break points of various microorganisms against various antibiotics every few years to track the incidences of acquired antibiotic resistance. When a strain displays higher breakpoint value than the rest of the strains, it may be implied that there was an acquired antibiotic resistance and thus it has a potential to transfer to other bacteria (EFSA 2012).

Susceptibility of LAB to Various Types of Antibiotics:

When microorganisms are exposed to a particular antibiotic, it can induce resistance towards similar antibiotics of that same class or with same mode of action. Lactic acid bacteria are usually sensitive to protein synthesis inhibiting antibiotics such as macrolides, tetracycline, erythromycin and chloramphenicol (Klare and others 2007). Most lactobacilli seem to have natural resistance towards nucleic acid synthesis inhibitors such as sulphamethoxazole, trimethoprim, ceftiofur, vancomycin and metronidazole (Ammor and others 2007; Lahtinen and others 2009; Clementi and Aquilanti 2011). *L. plantarum* is naturally resistant towards vancomycin due to the presence of the enzyme D-alanine: D-alanine ligase (Ddl). Vancomycin acts on the D-ala:D-ala peptidoglycan which is interfered by Ddl (Kuzin and others 2000). It was also found that the terminal peptidoglycan molecule of *L. plantarum* is D-ala:D-lactate instead of D-ala:D-ala and thus they are naturally resistant towards vancomycin due to absence of target site (Goffin and others 2005).

Some of the antibiotics that are regularly tested for resistance include ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol (EFSA 2012). They are classified as resistant or sensitive based on minimum

inhibitory concentration (MIC) that is above or below the breakpoint value. The guidelines on MIC are updated frequently by organizations such as European Food Safety Authority (EFSA) under Qualified Presumption of Safety (QPS), FDA, National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) and The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). The growth media, dilution method and incubation parameters play a role in determining the MICs (EFSA 2008; EFSA 2012). Antibiotics to which the bacterial strains developed a resistance must not be used as feed additive according to the EFSA guidelines. According to EFSA if breakpoint is higher for an antibiotic in a species than previously reported, genetic basis of the antibiotic resistance must also be studied (Clementi and Aquilanti 2011). Identifying and sequencing the genes that code for antibiotic resistance determines the transferability of those genes to other bacteria and their similarities with in closely related species or genera. Risk assessment procedures must also be performed when such a new resistance to any antibiotics is noticed (Lahtinen and others 2009).

Transfer of Antibiotic Resistance:

Lactic acid bacteria participate in horizontal exchange of genetic material and thus receive/donate the genes associated with antibiotic resistance (Devergiliis and other 2013). Lactobacilli are able to transfer macrolide antibiotic resistance genes to enterococci (Jacobsen and others 2007; Ouoba and others 2008). Factors such as type of selection pressure, route of transmission, complexity of food vehicle, persistence and expression of those genes play an important role in understanding the antibiotic gene resistance *in vitro* or *in vivo* (Ouoba and others 2008). Analyzing these patterns in food based matrix is more insightful since gene expression is dependent on the environment the bacteria is present. There is a significant concern when LAB exchanges antibiotic resistance genes between pathogens such as *Listeria* which can

be subsequently transferred to other gut bacteria and pathogens. *L. monocytogenes* is a pathogen of special interest as it is widely found in foods such as milk, chilled foods, fermented dairy foods such as cheeses and also in animal gut and natural environments (Charpentier and Courvalin 1999). Repeated exposure to stress conditions such as disinfectants and mild-intense processing can also induce antibiotic resistance in *Listeria* spp. (Allen and others 2016). Plasmids encoding antibiotic resistance genes were widely transferred between various species including *Listeria* transconjugates. Intrinsic resistance mechanisms such as multidrug cell wall efflux pumps and two-component signal transduction systems are common ways for *L. monocytogenes* to counteract antimicrobials (Allen and others 2016). However, Bertsch and others demonstrated the conjugative transfer of trimethoprim resistance encoding transposon between *E. faecalis* and *L. monocytogenes* demonstrating the horizontal gene transfer capability (Bertsch and others 2014).

Tetracycline Resistance:

Tetracycline resistance is widely used for treatment of various animal and human infections. The genes encoding for tetracycline resistance are commonly transferred between bacteria through mobile genetic elements including plasmids and/or transposons. There are also bacteria that acquired tetracycline resistance through spontaneous mutation (Roberts and Schwarz 2009). There are three main mechanisms of tetracycline resistance: 1) production of Ribosomal Protection Proteins (RPP) that protect ribosomes from the action of tetracycline, 2) energy dependent efflux of tetracycline from the cells to outwards and 3) tetracycline inactivating enzymes (Aminov and others 2004; Roberts 2005). There are over 35 genes discovered in various bacteria that are involved in tetracycline resistance along with some genes of unknown functions (Roberts and Schwarz 2009). More than 11 genes were found that encode

RPP such as *tet(M)*, *tet(O)*, *tet(Q)*, *tet(S)*, *tet(T)* and *tet(W)* (Aminov and others 2004; Roberts 2005; Hedayatianfard and others 2014). The amino acid sequence of RPP is similar to elongation factor proteins involved in peptide synthesis (Roberts 2005; Roberts and Schwarz 2009). When tetracycline binds to the ribosome initially, the allosteric conformation of the binding site changes and thus tetracycline is released from the ribosomal attachment. Tetracycline is unable to bind to the new conformation and thus protein synthesis continues normally (Roberts and Schwarz 2009). The genes encoding for RPP are more widely disseminated among various genera as it is usually encoded on conjugative transposons that are promiscuous.

The energy dependent transport proteins involved in effluxing tetracycline out of cell have a structure similar to major facilitator super family (MFS) transmembrane segments (Aminov and others 2004; Roberts and Schwarz 2009). They are membrane bound and often involved in multidrug resistance, quaternary ammonium and heavy metal resistances (Roberts and Schwarz 2009). More than 23 genes were identified that code for transport membranes including *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)* and *tet(G)* among many others (Aminov and others 2004; Roberts 2005). However, *tet(K)*, *tet(L)*, *tet(M)*, *tet(O)* and *tet(S)* are commonly found in Gram-positive organisms (Hedayatianfard and others 2014). *tet(S)* was found in a recent study in Tn6000 which is hypothesized to possess anti restriction proteins and methyltransferases that protect the transposons from host endonucleases before integration and they also possess restriction enzymes that blocks further integration within the transposon once it is successfully integrated into the host chromosome (Roberts and Mullany 2011). The prevalence and diversity of tetracycline resistance genes is currently increasing including genes coding for tetracycline inactivating enzymes such as *tet(X)*, *tet(34)* and *tet(37)* (Roberts 2005). *tet(X)* codes for

NADPH dependent oxidoreductase that inactivates tetracycline but it is not fully understood yet (Roberts 2005; Roberts and Schwarz 2009).

tet(M) is the most studied and widely found genes in both Gram-negative and positive organisms of different origins that is involved in production of ribosomal protection proteins. *tet(M)* via conjugative transposons are present on either chromosome or plasmid (Zonenschain and others 2009; Devirgiliis and others 2010; Frazzon and others 2010; Gevers and others 2003b; Gevers and others 2003a; Roberts and Schwarz 2009). In LAB, *tet(M)* is commonly found on transposon Tn916 or Tn916 like transposons that share a close sequence homology (Flórez and others 2008). *tet(M)* can be transferred via mobile genetic elements by homologous recombination such as Tn916 like elements that were exchanged between *E. faecalis*, *S. aureus*, *L. lactis* and *Lactobacillus* spp. demonstrating the promiscuity of this transposon and broad recipient transfer spectrum including pathogenic bacteria (Flórez and others 2008; Gevers and others 2003b; Roberts 2005). Conjugative transposon Tn916 encoding *tet(M)* was transferable between *Enterococcus* spp. and *Listeria* spp. and also within the *Listeria* strains (Charpentier and Courvalin 1999; Jahan and Holley 2016). Tn916 like transposons can also pick up other accessory genes beyond antibiotic resistance such as heavy metal resistance and other potentially functional genes which are yet to be understood.

tet(M) is commonly found on transposon Tn916 and Tn916 like transposons Tn918, Tn925, Tn3702 and Tn6084 detected in bacteria isolated from humans, processed meat, swine and dogs (Roberts and Mullany 2011). In *L. lactis* it was found to be present on a transposon located in a plasmid sharing a close homology with the conjugative transposon Tn916-Tn1545 family of other LAB (Roberts 2005; Flórez and others 2008). Tn916 was first discovered in *E. faecalis* (called *Streptococcus faecalis* at the time of discovery) while Tn1545 was discovered in

Streptococcus pneumoniae (Roberts and Mullany 2011). This family of transposons also encodes genes of resistance towards tetracycline, macrolides, lincosamides, streptogramins and kanamycin (Roberts and Mullany 2011). It is transferred through mobile genetic elements by homologous recombination indicating a broad range of hosts and a conjugative transfer between a variety of bacteria (Hedayatianfard and others 2014; Devirgiliis and others 2010; Frazzon and others 2010; Gevers and others 2003a). They are transferred between various bacteria through conjugation, where they are excised from donor cells followed by formation of a circular structure and integrated in to recipient DNA by recombinases (Roberts and Mullany 2011). They usually enter the host cell and integrate into genome by the action of integrase and site specific recombinases (Roberts and Mullany 2011). The Tn916-Tn1545 family is found in various Gram-positive and negative organisms that are also able to integrate new transposons within their existing transposon. Thus, new transposons are evolving constantly that are similar to the original Tn916 like genetic framework (Roberts 2005; Roberts and Mullany 2011). This transposon family contains ORF with functional genes encoding for conjugation, excision, insertion, transcription regulation and accessory function such as antibiotic resistance. Tn5397 is an another transposon with *tet(M)* that is site specific in binding and integration into the host genome of *Clostridium difficile* (Roberts and Mullany 2011). In a study conducted by Devergiliis and others on Gram-positive dairy bacteria, it was found that *erm(B)* and *tet(S)* were genetically linked in double resistant strains while *tet(M)* was found when bacteria were only resistant to tetracycline (Devergiliis and others 2010). Mobilization of non-conjugative plasmids can also occur through the Tn916 like transposons but the genetic variations that these transposons cause in a host are beyond just making the host resistant to antibiotics (Devergiliis and others 2013).

Erythromycin Resistance:

Acquired erythromycin resistance is commonly conferred by methylases that act on 23S rRNA during post translational modification and thus protecting the bacterium from erythromycin. *erm(B)* is the most common erythromycin resistance gene found in most LAB that codes for methylase (Zonenschain and others 2009; Devirgiliis and others 2010). *erm(B)* confers a higher degree of erythromycin resistance than other erythromycin resistance genes and thus it is widely studied (Varaldo and others 2009). Plasmid pAM β 1 is a 17 MDa sized highly promiscuous plasmid that commonly encodes for *erm(B)* with a wide host range of transmission including *Listeria* spp., *Bacillus* spp., *Leuconostoc* spp., *Streptococcus* spp., *Lactobacillus* spp. and *Lactococcus* spp. (Shrago and others 1986; Vescovo and others 1983; Tannock 1987; Gibson and others 1979; Flamm and others 1984; Pucci and others 1988). The plasmid carrying erythromycin resistance pAM β 1 was first discovered in *E. faecalis* (*Streptococcus faecalis* at the time of discovery) (Leblanc and Lee 1984). Conjugative plasmid pAM β 1 shared a high homology between *L. monocytogenes*, enterococci and streptococci strains indicating a common ancestry (Toomey and others 2009b). In fact, *erm(B)/tet(M)* were present together and/or separate in transposons including Tn6002, Tn6003, Tn1545 and Tn2010 that are 'Tn916 like' in their genetic makeup (Varaldo and others 2009).

Additionally, these methylases also confer a broader resistance to Macrolide, Lincosamide and StreptoGramin (MLS) resistance phenotype along with erythromycin. The genes involved in MLS resistance can be expressed constitutively or inducibly in the presence of erythromycin (Varaldo and others 2009). Genes such as *erm(A)* and *erm(TR)* are recently discovered whose resistance mechanism depends on the type of drug efflux pump and it is usually a low level resistance (Varaldo and others 2009). Efflux associated protein coding genes

such as *mef(A)* and *mef(E)* first discovered in *Streptococcus* spp. also confer a low level erythromycin resistance. Under sublethal concentrations of erythromycin, the resistance conferring genes can be transferred to transmissible plasmids which can be subsequently transferred to various recipients (Varaldo and others 2009). This was observed with *erm(B)* resistance on Tn917 gene in *E. faecalis* which is linked to Tn916 that encodes *tet(M)*.

Environmental Factors Affecting Antibiotic Resistance Spread:

The ability of antibiotic resistance genes to disseminate to broader genera depends on whether these genes are associated with promiscuous conjugative transposons or plasmids (such as the Tn916-Tn1545 family and pAM β 1) or non-conjugative elements (Roberts 2005). Studies on antibiotic resistance gene transfer has been conducted *in vitro* for several decades; however, in recent years studies are focusing more on *in vivo* food matrices to imitate and assess the practical implication of such transfers. Factors such as effect of growth matrix, surrounding environment, presence of other microbiota can be better understood when native matrices (such as food, water or biofilms) are used in these conjugation studies (Kruse and Sørum 1994; Kajiura and others 2006; Toomey and others 2009b). Production of biofilms and subsequent close association with neighboring cells creates an ideal environment for persistence and horizontal gene transfer (Angles and others 1993; Rachid and others 2000; Harrison and others 2005; Kajiura and others 2006; Madsen and others 2012). The interconnection between biofilm formation and horizontal gene transfer is well documented (Madsen and others 2012; Luo and others 2005). In fact, *L. lactis* produced transconjugants with pAM β 1 at a frequency of 10,000 times higher than its mutant strain with no biofilm forming capability (Luo and others 2005). Horizontal gene transfer between cells in a biofilm type matrix was mathematically modelled and the factors involved were demonstrated (Merkey and others 2011). Lactic acid bacteria were

able exchange antibiotic resistance genes between *Salmonella* spp., *E.coli* and *Staphylococcus* but not with *Listeria* spp. *in vitro* and in milk demonstrating the effect of growth/food matrix and surrounding environment (Allen and others 2016). Stress adaptation in bacteria such as *E. coli* and LAB showed an increased rate of conjugative plasmid transfer during prolonged exposure to sub lethal stresses (Allen and others 2016).

In a meat fermentation study, before processing and fermentation, both *tet(M)* and *tet(S)* genes were found in the raw samples, however, only *tet(M)* genes were found in the final product after fermentation. This study highlighted the loss of genetic diversity due to processing while demonstrating the persistence of *tet(M)* even after harsh treatments (Gevers and others 2003c). Loss of microbial diversity was attributed as the main cause for the loss of tetracycline resistance gene diversity. In a different study, antibiotic gene recipients displayed 3 times the lower resistance to tetracycline compared to donor even though the plasmid encoding for tetracycline resistance was successfully transferred during the mating. Along with tetracycline resistance encoding plasmid, erythromycin resistance was also co-transferred to the recipient from the same plasmid (Gevers and others 2003b). Regardless of the ecological origin, transfer of antibiotic resistance genes can take place even between distantly or unrelated genera and of various origins such as human, animal or fish. Kruse and Sorum demonstrated that day to day commodities such as a towel dipped in bovine milk with mastitis resistant bacteria and cutting board that was used to chop pork meat and fish can also serve as matrices for antibiotic gene transfer (Kruse and Sørnum 1994).

Studies also highlight that the recipient/donor ratio is critical for conjugation and it can vary depending on the food matrix (Kajiura and others 2006; Lampkowska and others 2008; Kruse and Sørnum 1994). Sasaki and others demonstrated that 10:1 ratio yielded higher *erm(B)*

transfer frequency (a high as 10^{-6}) of *L. plantarum* with *E. faecalis* containing pAM β 1, Shrago and others proposed 1:10 with transfer frequency of 10^{-7} while West and others proposed 1:1 with frequencies $> 10^{-7}$ (West and others 1985; Shrago and others 1986; Sasaki and others 1988). Spontaneous mutants of donor or recipients were not reported in these studies. However, the double selective medium for transconjugant detection varied in different studies. Agents such as lactose, rifampicin, melezitose, streptomycin were used along with erythromycin (West and others 1985; Shrago and others 1986; Sasaki and others 1988). pH could also have an effect on *in vitro* mating, however, there are no consistent reports because some studies indicate low pH stress promotes conjugal mating while other reports indicate the opposite or no effect of pH (Toomey and others 2009c; Haack and others 1996; Mc Mahon and others 2007). In most studies, it is not clear if it is the donor's ability to donate or recipient's ability to accept the genetic material that is affected under varying physiological conditions. The optimal pH for conjugation depends on the cells involved in mating, type of plasmid and growth conditions (Toomey and others 2009c).

Co-selection of Heavy Metal and Antibiotic Resistance:

Co-selection phenomenon is observed when genes encoding resistance towards different stresses such as heavy metals, sanitizers, disinfectants and antibiotics are present on the same plasmid (Allen and others 2016). Common resistance mechanisms between heavy metals and antibiotics such as membrane impermeability, target modification and/or sequestration and efflux are well documented (Baker-Austin and others 2006; Chapman 2003). For example, efflux pumps encoding for both disinfectants and antibiotics are present on the same plasmid, exposure to one of them concomitantly leads to increased resistance towards other stresses (Allen and others 2016). Sub lethal stress exposure to any one of these stresses can also induce resistance

towards other stresses because the genes involved are cotranscribed on the same plasmid (Allen and others 2016). Recent studies also show that exposure to heavy metals such as arsenic, copper, mercury and cadmium or sanitizing agents such as quaternary ammonium compounds can co-select antibiotic resistance especially if their associated genes are physically present closely and transcribed together on the same genetic element (Chapman 2003; Ug and Ceylan 2003; Baker-Austin and others 2006; Katharios-Lanwermeier and others 2012). Co-selection of heavy metals (mercury, silver, cobalt and copper) and antibiotic (erythromycin, streptomycin and vancomycin) resistance is well documented in various gut and oral bacteria in humans, swine and Enterobacteriaceae (Baker-Austin and others 2006; Summers and others 1993; Ghosh and others 2000; Davis and others 2005).

Co-regulation at a transcriptional level can also lead to co-selection of multiple resistances demonstrated in *Pseudomonas aeruginosa* where a lethal exposure to zinc increased resistance towards cadmium, cobalt and also carbapenem class antibiotics via over production of efflux pumps (Perron and others 2004). Comparison between heavy metal resistant and sensitive bacteria revealed that the former were resistant to antibiotics along with heavy metals (Berg and others 2005). However, their direct correlation is yet to be established. This co-resistance phenomenon of heavy metals along with antibiotics is demonstrated in various ecosystems and food matrices such as agricultural soil, river water, drinking water, primate dental fillings, cattle, swine and poultry (Calomiris and others 1984; Summers and others 1993; Hasman and Aarestrup 2002; Berg and others 2005; Baker-Austin and others 2006). Understanding the transfer mechanisms and range of recipients for antibiotic resistance genes can give further insights into hyper virulence, drug persistence evolution and epidemic control of various pathogenic bacteria (Roberts and Mullany 2011).

OTHER DESIRABLE STARTER CULTURE TRAITS

Flavor compounds are sometimes produced by non-starter bacteria during the course of fermentation (Aurand and others 1965; McFeeters 2004; Heperkan 2013). So, it is important that the flavor of the final product is not compromised by allowing only specific strain(s) to predominate during whole fermentation process (Leroy and De Vuyst 2004; Rattanachaikunsopon and Phumkhachorn 2010; Heperkan 2013). Functional starter cultures that can deliver additional health benefits through secondary metabolite production and probiotic health effects along with the primary rapid fermentation is gaining momentum and thus desirable (Hansen 2002; Ammor and Mayo 2007; Xiong and others 2013). Factors such as degradation of anti-nutritional compounds (trypsin inhibitors), detoxification of toxic compounds if any, improved bioavailability of nutrients, vitamins and amino acids are also desirable (Holzapfel 2002; Çon and Karasu 2009). Stachyose, raffinose and other oligosaccharides present in cucumbers are not easily digested by humans and thus ideal starter cultures that can hydrolyze these carbohydrates to avoid outcomes such as gastric discomfort and flatulence would be ideal (Holzapfel 2002).

Single strain starter cultures offer a better control and predictability of process and the final product. However, they are susceptible to bacteriophage infections (Holzapfel 2002). This problem can be circumvented by the use of multi strain/species starter inoculum which are comparatively more adaptive to any fluctuations during the process (Holzapfel 2002). However, when a mixed culture of starter cultures is proposed, it is also important to determine the compatibility between strains (Houle and others 1989; Gardner and others 2001). During the post-processing of fermented pickles, it would be ideal if bacterial cell mass deposit at the

bottom at the end of fermentation which eases the white cell mass removal by filtration (Daeschel and Fleming 1984).

Technological Approach for Starter Culture Selection:

The foremost step to develop starter cultures for any type of fermentation is to understand the predominant bacteria of the fermentation ecosystem. With the advent of novel and affordable genetic and omics tools, it is possible to determine these bacteria and their metabolites (Hansen 2002). Genotypic techniques such as 16S rRNA sequencing, housekeeping gene sequencing, Multi Locus Sequence Typing (MLST), Random Amplified Polymorphic DNA (RAPD), Pulsed Field Gel Electrophoresis (PFGE) and Denatured Gradient Gel Electrophoresis (DGGE) are widely used to identify and characterize bacteria of interest (Heperkan 2013). Understanding the metabolomics of these bacteria is also crucial to determine the nutritional requirements, metabolic byproducts (primary and secondary), antimicrobial/bio-protective compounds and desirable/off-flavor compounds (Hansen 2002). Phage defense mechanisms and antibacterial activities are well understood now using molecular genetics approach. A key factor in technical understanding of starter culture development is the rapid acidification and type of fermentation i.e., homo vs. heterofermentation (Holzapfel 2002). Vegetable juice medium is commonly used to test the potential starter cultures to imitate the native fermentation medium and conditions as close as possible (Di Cagno and others 2008). Factors such as temperature, salt, and pH must be tested at various levels are critical during lab scale selection to ensure confidence in predicting their potential on industrial scale fermentation (Hansen 2002; Di Cagno and others 2013; Lee and others 2015). Proper inoculation conditions ensure rapid and controlled acidification, microbiological evolution and dominance during the initial phase of fermentation (Leal-Sánchez and others 2003).

Frozen or freeze dried culture offers the most flexible and safe option of inoculating starter cultures into fermentation tanks (Hansen 2002). However, higher costs involved with refrigeration and freeze drying limits their use in large scale cucumber fermentation. Perez-Díaz and McFeeters described an efficient way of growing starter culture inoculum preadapted to cucumber fermentation. This method involves propagating the culture in commercial fresh pack pickle jars aseptically and adding them to fermentation tanks just before initiation (Pérez-Díaz and McFeeters 2011). Initial adjustment of pH to 5.2 (from 4) of fresh pack jars and an incubation temperature between 20-30 °C resulted in high counts of the starter culture as well as final lactic acid greater than 100 mM compared to pH unadjusted jars (Pérez-Díaz and McFeeters 2011). An initial inoculum of 10^5 CFU/mL was used as starter cultures to drive the commercial cucumber fermentation (Pérez-Díaz and McFeeters 2011). The starter cultures can be grown in commercial fresh pack pickle jars processed in plant and thus allowing the bacteria adapt to the high acid environment and also use a medium of non-animal origin for kosher purpose. The major drawback is the large volumes of starter cultures preparation in jars depending on the size of the plant. Bulk preparation of starter culture that meets Kosher guidelines is possible now which does not require freeze drying. This method is also economical as the pickle industries do not need to set up a separate microbiological unit to maintain these starter cultures (Pérez-Díaz and McFeeters 2011). It is possible to use these starter cultures for not just vegetable fermentations but also other fermentations wherever they are deemed and also as health promoting probiotics (Hansen 2002).

The two main hindrances to commercial production and adaptation of starter cultures for cucumber fermentation include 1) lack of clear picture of the predominant bacteria, metabolite production and safety and 2) lack of proper guidelines for selection of appropriate starter

cultures. Thus, the current work aims at addressing both of these issues using a polyphasic approach. In addition, prevalence of antibiotic resistance, co-resistance of antibiotics with heavy metals and conjugative transfer of antibiotic resistance genes is also studied.

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CHAPTER 2

Screening and Selection of *Lactobacillus plantarum* and *Lactobacillus pentosus* Isolates from Commercial Cucumber Fermentation as Candidates for Starter Cultures

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the journal guidelines)

ABSTRACT

Aims: To identify the robust fermenting bacteria from commercial cucumber fermentation for potential starter cultures. Knowing the predominant bacteria responsible for commercial cucumber fermentation will open new ventures for starter culture development and value added fermented cucumbers.

Methods and Results: A group of 243 tentative *Lactobacillus plantarum/pentosus* isolated from commercial cucumber fermentations on various days, was evaluated for the ability to rapidly decrease the pH in cucumber juice medium. Treatment combinations of salt content (0 or 6%), initial pH (4.0 or 5.2) and incubation temperatures (15 or 30 °C) were developed using a fractional factorial screening design. Eighty-three isolates were selected based on rapid acidification under these treatment combinations and antimicrobial assay was performed to select isolates that inhibited *Lactobacillus buchneri* but not *Lactobacillus brevis*. Seven isolates were selected based on the above criteria and genotypically characterized using Multi Locus Sequence Typing and housekeeping genes including *recA*, *dnaK*, *pheS* and *rpoA*. All the isolates were identified as *L. pentosus* and only one as *L. plantarum*. Random Amplified Polymorphic DNA genotyping identified 2 unique strain patterns among the final 7 *L. pentosus* isolates. *recA* based differentiation was performed on all the tentative *L. plantarum* isolates (n=252) from commercial cucumber fermentation tanks from two geographically distinct locations in the US (NC and MN). More than 90% of the tentative *L. plantarum* isolates from NC and 70% from MN were identified as *L. pentosus*.

Conclusions: The robust bacteria are selected mostly from days 1 and 3 of fermentation. *L. pentosus* is the predominant and robust species found in commercial cucumber fermentation as opposed to the traditionally associated *L. plantarum*.

Significance and impact of study: This is the first study to isolate bacteria from commercial cucumber fermentation from two geographically distinct locations. The isolates from this study have a potential as starter cultures for pickle industries for rapid and consistent fermentation. A diligent elucidation of their technological and safety properties in future would allow pickle manufacturers to utilize these isolates as functional starter cultures.

Keywords: Antimicrobial Activity, Cucumber Fermentation, Lactic Acid Bacteria, Starter Culture

INTRODUCTION

Development of starter cultures for commercial cucumber fermentation becomes imperative with the inception of technologies for low-salt pickle processing in the U.S (Fleming *et al.* 2002; McFeeters and Pérez-Díaz 2010). While starter cultures are widely used by the dairy industry worldwide, this practice is less common in the commercial production of fermented vegetables. Starter cultures improve the fermentation process by providing a predictability, control and consistency of the process and final safety and quality of the products along with improving the shelf life (Hansen 2002; Holzapfel 2002; Ammor and Mayo 2007; Çon and Karasu 2009; Chang and Chang 2010; Heperkan 2013; Lee *et al.* 2015). The initiation phase of fermentation can be considerably reduced with the use of starter cultures (Holzapfel 2002). In addition to food manufacturers, consumers are also moving towards “all natural”, “clean label” and “home fermented” food and life style choices which demands the knowledge of predominant fermenting bacteria to develop starter cultures (Holzapfel 2002).

The majority of pickle processors worldwide currently rely on the indigenous microbiota and application of cover brine with at least 5.6% sodium chloride (NaCl) to achieve high quality, stable and safe fermentation and final products (discussed more in the literature review). Hypertonic cover brines aid in the inhibition of unwanted pathogenic and spoilage associated microbes naturally present in the fruits at the initiation of a cucumber fermentation, thus creating a competitive advantage for the desired Lactic Acid Bacteria (LAB). The use of starter cultures in low salt cucumber fermentations would induce rapid lactic acid production resulting in a quick significant reduction of pH and thus an early exclusion of undesired microbes. However, starter cultures specifically selected for cucumber fermentations and low salt processing are currently

not produced. In addition, the sources for alternative commercial LAB starter cultures that are of vegetable fermentation origin are limited.

A process to prepare lab scale starter cultures for cucumber fermentations was developed by Pérez-Díaz and McFeeters using *L. plantarum* LA 0445 (McDonald *et al.* 1993; Pérez-Díaz and McFeeters 2011). In the translation of the technology to ferment cucumbers with CaCl₂ instead of NaCl to commercial operations, it was observed in the laboratory scale studies that the addition of a starter culture (*L. plantarum* LA 0445) aided in achieving a higher production of lactic acid (75 mM) as compared to a fermentation producing 55 mM lactic acid with just indigenous microbiota (McFeeters and Pérez-Díaz 2010). *L. plantarum* LA 0445 was originally isolated from a commercial pilot scale anaerobic cucumber fermentation tank as the dominant microorganism found at the end of the process that was inoculated with a *L. plantarum* to 10⁵ CFU.mL⁻¹ (Fleming *et al.* 1988). So, it was presumed that *L. plantarum* LA 0445 is resistant to pH as low as 3.3. This particular bacterium is a naturally occurring from the cucumbers and streptomycin resistant which was the preferred starter culture for experimental cucumber fermentations at the time of isolation (McDonald *et al.* 1993; Fleming *et al.* 1988). Although *L. plantarum* LA 0445 has been used successfully in a number of cucumber fermentation research studies, a systematic search of suitable candidates for robust starter cultures has not been conducted.

Homofermentative metabolism prevents the production of CO₂ and consequently reduces the incidence of the formation of hollow cavities inside the fermented fruits, a processing defect known as bloating (discussed more in the literature review) (Daeschel *et al.* 1984). Thus, quick production of lactic acid is desirable because it is effective against pathogens of public health significance assuring safety of the fermented goods (Holzapfel 2002; Lu *et al.* 2011). On the

other hand, low concentrations of acetic acid (0.65-0.7%) from heterofermenting bacteria help to maintain the texture of the final product (Etchells *et al.* 1972). *L. brevis* is one such abundant heterofermentative species during fermentation that produces acetic acid. Thus, *L. brevis* is desirable as adjunct bacteria for their ability to form small amounts of acetic acid while co-fermenting with *L. plantarum/pentosus*. Spoilage of fermented cucumbers by LAB such as *Lactobacillus buchneri* and *Lactobacillus parafarraginis* in concert with *Lactobacillus rafi* leads to the formation of acetic and propionic acids at the expense of lactic acid, resulting in a rise in pH especially in low salt fermentations (Franco *et al.* 2012; Franco and Pérez-Díaz 2012; Johanningsmeier *et al.* 2012; Lu *et al.* 2012; Breidt *et al.* 2013; Franco and Pérez-Díaz 2013). Addition of starter cultures can prevent the proliferation of spoilage microbes and thus aid in maintaining stable products through time. Microbiological stability is of significant concern in the application of new technologies lacking the preservation hurdle derived from high salt concentrations.

Microbial succession is commonly seen in a complex fermentation ecosystem such as cucumbers and the predominating bacteria during fermentation is dependent on its ability to rapidly proliferate, produce acids, bacteriocins, biofilms and/or other quorum sensing strategies to have a competitive advantage over other bacteria (Singh and Ramesh 2008). Factors including spatial and temporal heterogeneity arise during a non-starter commercial fermentation which may lead to uneven quality of the final product (Giraffa 2004). Cell-cell and cell-food matrix interactions also play a vital role in determining the predominant bacteria during fermentation (Giraffa 2004). So, it is advantageous to isolate samples on various days and at different parts of the tank to achieve a better understanding of the ecosystem. Furthermore, microbial nomenclature of closely related bacteria is constantly evolving especially based on

metabolic diversities. For example, *L. pentosus* was once regarded as same species as *L. plantarum* and later it was reinstated as a separate species due to some confounding metabolic differences (Zanoni *et al.* 1987). Thus, it is essential that the bacteria intended for commercialization be well characterized.

Knowing the robust and predominant microorganisms during a commercial fermentation allows manufacturer to eventually select starter cultures for an efficient fermentation. In fact, microbial diversity during fermentation and their taxonomic identification is one of the first steps towards developing starter cultures (Giraffa 2004). The tools used to study such complex ecosystems have tremendously improved in the past few years and it is now possible to identify and differentiate the bacteria up to strain level quick and inexpensive. Inter- and intraspecific differentiation of microorganisms would also allow for a wide selection of bacteria with varying technological, sensorial aspects and potential health benefits of live cultures before commercialization (de las Rivas *et al.* 2006). Next generation sequencing tools such as 16S rRNA based identification is well established and has been successfully used as the basic approach to identify the population in various fermentation systems such as cucumbers, sourdough, maize and other fermented foods (ben Omar and Ampe 2000; Randazzo *et al.* 2002; Miambi *et al.* 2003; Giraffa 2004; Bokulich 2012). Amplification and sequencing of ubiquitous and conserved protein coding genes using Multi Locus Sequence Typing (MLST) and other housekeeping genes is commonly used to confirm the identity of microbial isolates (de Las Rivas *et al.* 2006; Anukam *et al.* 2013). The sequences that are obtained are matched against reference database and thus the choice of an accurate and updated database is also critical (Bokulich 2012). Housekeeping gene sequence based approaches were successfully used in understanding the qualitative and quantitative detection of the predominant bacteria during small scale vegetable

and meat fermentation (Giraffa 2004; Singh and Ramesh 2008). Singh and Ramesh utilized 16S *rRNA* and bacteriocin gene primers sequentially to understand the dynamics of microbial succession and prevalence of bacteriocins that aid in dominance during cucumber fermentation (Singh and Ramesh 2008). Randomly Amplified polymorphic DNA (RAPD) is a rapid non-sequence based approach routinely used for inter and intra species identification (Singh *et al.* 2009). Prior knowledge of the sequence is not necessary and thus varying sizes of DNA bands are produced on the gel that is unique to species and/or strain. Some species are in fact more definitively identified by RAPD than any other genetic methods (Huang and Lee 2009; Singh *et al.* 2009). RAPD based differentiation of successive population and persistence was successfully used in understanding the dynamics of fermented foods such as Spanish table olives, meats and sourdough breads (Giraffa 2004; Rodríguez-Gómez *et al.* 2013).

Recent studies on the microbiota of commercial cucumber fermentations suggest that LAB such as *L. plantarum*, *L. pentosus*, *Pediococcus ethanolidurans* and *L. brevis* co-dominate in commercial cucumber fermentations brined with 6% NaCl (unpublished). However, the heterofermentor *L. brevis* seems to be at a disadvantage in this competition, due to bacteriophage attacks (Lu *et al.* 2012). Moreover, *L. plantarum* and *L. pentosus* reach maximum cell counts faster than *L. brevis* in commercial cucumber fermentations and remain dominant until the end of the process with lactic acid as the main catabolite (unpublished). Thus, a commercially viable starter culture for cucumber fermentations must be able to proliferate in a variety of temperatures, initial pH, salt concentrations and convert glucose and fructose to lactic acid and co-ferment with *L. brevis* while inhibiting spoilage bacteria such as *L. buchneri* (Etchells *et al.* 1973).

The objective of this study was to screen a collection of tentative *L. plantarum/pentosus* isolates from commercial fermentation tanks for the ability to rapidly initiate fermentation in a cucumber juice medium. These isolates were obtained from two geographically distant commercial fermentations that were selected based ability to ferment in a cucumber juice medium (CJM) with variable initial pH and salt concentration and growth temperatures. Robust isolates that were able to ferment in at least three or more conditions were further tested for their ability to inhibit spoilage associated bacteria (*L. buchneri*) and co-ferment with *L. brevis*. The final selected isolates were differentiated at species level using MLST, various housekeeping genes and further discriminated at the strain level using Randomly Amplified Polymorphic DNA (RAPD). The collection of *L. plantarum* isolates from both the tanks from two locations was further identified based on the PCR product size of *recA*.

MATERIALS AND METHODS

The sample collection, isolation of bacteria, fermentation screening design, dendrograms based on hierarchical cluster analysis of the isolates from commercial cucumber fermentation tank in North Carolina were developed and implemented by Drs. Ilenys Pérez-Díaz, Suzanne Johanningsmeier, Consuelo Arellano and Ms. Janet Hayes. The agar spot assay was modified and developed by the current author Kartheek Anekella that was used for the final selection of isolates (Anas *et al.* 2008). The same approach and methods were used for identifying and screening bacterial isolates from the tank in Minnesota during which the current author was involved in the project. After selection of the tentative *L. plantarum* isolates, all the subsequent experiments were performed by the current author.

Microbial Culture Collection and Media:

Microbial isolates originated from the cover brine solutions of two commercial cucumber fermentation tanks in NC and MN were selected for this study. This group of bacteria was found to predominate during the peak of commercial fermentation and these isolates were tentatively identified as *L. plantarum/pentosus* (referred to as isolates throughout the paper) using the partial sequence of their 16S rRNA (Table 2.1, Amann et al. 1995). The isolates were streaked for purification on deMan, Rogosa and Sharpe (MRS; Cat No. 288130, Difco™ Laboratories, MI, USA) agar prior to the preparation of frozen stocks in MRS broth containing 15% glycerol (Cat No. G5516, Sigma, MO, USA).

Fresh pickling cucumbers procured locally and their juice was prepared using commercial juice maker (Warring Commercial, CT, USA). The juice was strained through cheesecloth to remove large particulates prior to addition of NaCl (Morton Pickling Salt, IL, USA) and dilution with distilled water. The Cucumber Juice Medium (CJM) was composed of 60% (v/v) cucumber juice and 40% (v/v) distilled water. The unadjusted pH of the CJM with 2% salt was 5.6-5.8. The CJM was spun at 3220xg for 60 min at ambient temperature using a bucket rotor (Eppendorf Centrifuge Model 5810, Hamburg, Germany) and filter-sterilized using 0.22 micron filtration units (Nalgene®-Rapid Flow™, Thermo Scientific, CA, USA). Pure cultures were transferred from frozen stocks to CJM containing 2% NaCl (Morton Salt) and incubated aerobically for 4 d at 30 °C prior to the inoculation of the experimental media. During this transfer each isolate was concomitantly streaked onto MRS agar to verify purity. Aliquots of 1.2 mL of the filter-sterilized CJM (with 2% salt) were aseptically transferred to sterile microcentrifuge tubes to serve as the medium for proliferation of the individual cultures. Cultures were calibrated upon inoculation into the experimental CJM by measuring optical

density at 600 nm using a spectrophotometer (OD₆₀₀, Thermo Spectronic Genesys20 Model 4001/4, MA, USA). An initial OD₆₀₀ of 0.05 was targeted during inoculation. Cultures were diluted 10X prior to measuring OD₆₀₀, unless turbidity corresponded to an OD₆₀₀ of less than 0.60.

Fermentation Screening Design and Experimental Media Preparation:

The group of 243 pure *L. plantarum* isolates was tested for their ability to ferment cucumbers under various pH, NaCl, and temperature using CJM as a model system. A fractional factorial screening design (JMP 10.0, SAS, NC, USA) was used to generate various treatment combinations as follows: Treatment 1 (T1)- (pH 5.4; 0% NaCl; 15 °C incubation), Treatment 2 (T2)- (pH 4.0; 6% NaCl; 15 °C incubation), Treatment 3 (T3) (pH 5.4; 6% NaCl; 30 °C incubation) and Treatment 4 (T4) (pH 4.0; 0% NaCl; 30 °C incubation). The pH of the experimental CJM was adjusted (Acumet[®] Research 25 pH meter, Fisher Scientific, CA, USA) using minimal volumes of 3N HCl (Spectrum Chemicals, NJ, USA). The unadjusted pH of the experimental CJM without salt was 5.4 ± 0.1. Lowering the pH to 4.0 in treatments 2 and 4 formed precipitates which were eliminated subsequently in the pellet after the centrifugation step. The CJM was spun at 3220xg for 30 min at ambient temperature (Eppendorf Centrifuge Model 5810). The CJM was filter-sterilized using 0.22 micron filtration units (Nalgene[®]-Rapid Flow[™], Thermo Scientific). Aliquots of 1 mL of the experimental CJM were aseptically dispensed into sterile microcentrifuge tubes and incubated at the corresponding treatment temperatures for 48 h prior to inoculation. The experimental CJM was inoculated with the pre-calibrated pure cultures and incubated for 48 h at either 15 or 30 °C under static aerobic conditions.

The ability of each isolate to rapidly initiate fermentation under each of the four treatment conditions was assessed by measuring the fermentation pH (Denver Instruments, model UB-5, NY, USA) after 48 h incubation time. pH meters were combined with Gel-Filled Pencil-Thin pH Combination Electrodes (Acumet Fisher Scientific, PA, USA) so that pH measurements could be made directly from the CJM cultures in microcentrifuge tubes. The tubes were allowed to reach ambient temperature prior to measuring pH. This experiment was performed in duplicate using different lots of cucumbers for the experimental and growth media and independent culturing of each isolate. Hierarchical two-way Cluster Analysis (HCA) of *L. plantarum/pentosus* isolates was performed based on their average final pH after 48 h in each of the four treatments (JMP 10.0, SAS, NC, USA) to generate a dendrogram.

Antimicrobial Assay:

Isolates that were able to reach the cut-off pH values in at least 3 out of the 4 treatments in the fermentation screening, were considered for further characterization using an antimicrobial assay by agar spot method (Anas *et al.* 2008). The selected isolates were grown in MRS broth and incubated at 30 °C for 24 h. The MRS agar plates (Difco Laboratories, Detroit, MI, USA) were spotted with 2 µL of 4 to 6 isolates per plate. *L. brevis* LA 0036 (ATCC 14869), *L. buchneri* LA 1147 and LA 1149 were used as indicator organisms (USDA-ARS Food Science Research Unit culture collection, Raleigh, NC, USA). *L. brevis* LA 0036 and *L. buchneri* LA 1147 and LA 1149 were cultured in MRS broth at 30 °C under anaerobic conditions (Coy Anaerobic Chamber, MI, USA) for 48 h and 96 h, respectively. One hundred µL of the indicator strains were added to 10 mL soft agar (0.7%) overlay to inoculate approximately 10^4 - 10^5 cells.mL⁻¹. The soft agar was allowed to solidify for at least 20 min before inverting the plates and incubated at 30 °C for 48 h under aerobic conditions for plates inoculated with *L. brevis* and

under anaerobic conditions for 72 h for plates inoculated with *L. buchneri* as the indicator. Zone of inhibition (ZOI) was measured as the width of the halo clearance zone around the spot in millimeters. Any ZOI <0.5 mm was recorded as a negative (-) result and >0.5 mm as a positive (+). Isolates that showed an antimicrobial activity against *L. buchneri* but not *L. brevis* were selected for further genotyping.

Genotyping of the Tentative *L. plantarum/pentosus*:

Genomic DNA extraction from pure cultures of the isolates grown overnight in MRS broth was performed using the MasterPure™ DNA purification kit (Cat No.: MCD85201, Epicentre, WI, USA) following the manufacturer's instructions. Extracted DNA was stored at -20 °C until further use. All the PCR reactions were performed in 25 µL volumes with the following ingredients: PCR Master Mix (Cat No: 166-5009EDU; BioRad, CA, USA), 10 µM rehydrated primer stocks (IDT Inc., CA, USA), 1 µL of whole genomic DNA and nuclease free water (Cat No.: AM9937; Ambion®, Life Technologies, NY, USA) to fill the volume to 25 µL. Table 2.1 describes the primers and PCR conditions that were used in this study with the corresponding references. PCR amplicons were confirmed by gel electrophoresis with a 2.0% agar gel supplemented with 0.1% SYBR® safe (Cat No.: S33102; Invitrogen™, Life Technologies) using a 0.5X Tris/Borate/EDTA (TBE) buffer. Amplicon sequences were obtained using Sanger sequencing (Eton Biosciences, NC, USA). Sequence data was formatted and analyzed using BioEdit 7 software (www.mbio.ncsu.edu/bioedit) and subjected to the basic local alignment search tool (BLAST) using the non-redundant nucleotide database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the identity of the strains (Altschul *et al.* 1990; Benson *et al.* 2002; Pruitt *et al.* 2002). All the experiments were performed in duplicate. Results of the isolates' species level identification based on the housekeeping gene sequence

matches using the NCBI-BLAST and corresponding % similarity to those species in the database are presented in Table 2.3.

Multi Locus Sequence Typing (MLST) based on six *L. plantarum* housekeeping genes was performed as described previously (de las Rivas *et al.* 2006). Housekeeping genes including recombinaseA (*recA*), heat shock protein 70 (*dnaK*), phenylalanine t-RNA synthetase (*pheS*) and RNA polymerase α -subunit (*rpoA*) were used to identify the isolates using the primers described in Table 2.1 (Amann *et al.* 1995; Torriani *et al.* 2001; Naser *et al.* 2007; Huang *et al.* 2010). *L. pentosus* ATCC 8041, *L. plantarum* WCSF1 and *L. plantarum* ATCC 14917 were used as standards for identification. Dendrograms and similarity coefficients for the amplicons obtained for *recA*, *pheS*, *rpoS* and *dnaK* from the seven isolates were constructed using the Maximum Likelihood method based on the Tamura-Nei model with a bootstrap value of 1000. All positions containing gaps and missing data were eliminated (Tamura *et al.* 2013).

Strain level differentiation of isolates was performed based on RAPD using primers GTG₅, COC, LP1 and OPL5 with PCR conditions previously described (Versalovic *et al.* 1994; Cocconcelli *et al.* 1995; Torriani *et al.* 1996; Zapparoli *et al.* 2000; Plengvidhya *et al.* 2004). Magnesium chloride was added to the PCR reaction mixes from a 10 mM stock solution (Cat No. 42-303; Apex™, Genesee Scientific, CA, USA) to a concentration of 2.15 μ M for COC, 2.2 μ M for LP1, 2.05 μ M for OPL5 and 2.2 μ M for M14. Amplicons were run in a 1% agarose gel electrophoresis supplemented with 0.1% SYBR[®] safe (Cat No.: S33102; Invitrogen™, Life Technologies) using 0.5X Tris/Borate/EDTA (TBE) buffer. Dendrograms based on percent similarity coefficient by Pearson correlation were constructed by clustering using Unweighted Pair Group Method with the Arithmetic Mean (UPGMA) method (Bionumerics 6.6, Applied Maths, Sint-Martens-Latem, Belgium). Amplifications were performed in duplicate.

Differentiation of *L. plantarum* and *L. pentosus* from the Collection:

The isolates that were originally designated as *L. plantarum* based on 16S rRNA sequencing were revisited to confirm their identity. There were 190 and 62 isolates that were tentatively identified as *L. plantarum* from NC and MN fermentation tanks, respectively. *recA* based PCR was performed on all these tentative *L. plantarum* isolates from both the locations (NC and MN) to differentiate between *L. plantarum* and *L. pentosus* based on the PCR product size.

RESULTS

Fermentation Screening:

The effect of temperature, salt concentration and initial pH on the ability of the tentative *L. plantarum/pentosus* isolates to rapidly initiate fermentation was investigated in the CJM model system. The mean pH reached by the cultures at the end of fermentation in treatments T1, T2, T3 and T4 were 4.80 ± 0.32 , 3.87 ± 0.12 , 3.59 ± 0.46 and 3.54 ± 0.32 , respectively. Based on these averages, the pH cut-off values in for selection of rapid fermenters for T1, T2, T3 and T4 were set at 4.8, 3.9, 3.4 and 3.5 respectively. A manual selection of the 83 isolates that were able to rapidly reduce the pH below these cut-off values for at least three treatments was selected for further studies. A majority of the isolates were eliminated due to their inability to rapidly ferment and reach a pH below the cut-off value (Appendix Figure A.2.1). Treatment 3 conditions (initial pH 5.4, 6% NaCl, and 30 °C incubation) that are more close to current cucumber fermentation practice was able to discriminate most isolates based on the pH reduction. Clusters 1, 2, and 3 represent the robust predominating bacteria that were able to reach the minimum cut-off pH values in three or more treatments.

Antimicrobial Assay

Antimicrobial activity of the selected 83 isolates was tested against *L. brevis* and *L. buchneri*. The majority of the isolates showed zones of inhibition against one or both of the spoilage-associated *L. buchneri* strains tested (Appendix Table A.2.1). Sixty-three out of the 83 isolates were able to prevent growth of the two *L. buchneri* spoilage strains tested. Only 5 isolates did not produce any zone of inhibition, suggesting that most isolates inhibit *L. brevis*. Interestingly, no isolates of *L. plantarum* were detected from MN that were able to co-ferment with *L. brevis* while inhibiting *L. buchneri*. An example of the agar spot assay with zone of inhibition is shown in Appendix Figure A.2.2.

Genotypic Differentiation of *L. plantarum/pentosus*

Identification based on MLST revealed that all the isolates possessed sequences for *mutS* (mismatch repair protein), *purK1* (ATPase subunit of phosphoribosylaminoimidazole carboxylase), *ddl* (D-alanine-D-alanine Ligase) and *gdh* (glutamate dehydrogenase). All the isolates shared 93-99% similarity to those found in previously identified *L. pentosus* (Table 2.2) except isolate 3.2.8 which presented gene similarities to *L. plantarum* (Table 2.2) in the NCBI nucleotide database. However, sequences of *gyrB* (B subunit of DNA gyrase) and *pgm* (phosphoglucomutase) products were amplified only in the control strain WCFS1 and isolate 3.2.8 with >99% sequence similarity. The GenBank accession numbers of the amplified gene products include between KT185461 to KT185491. The MLST analysis indicated that majority of the final seven isolates in this study may be *L. pentosus* except one.

To further confirm this hypothesis other housekeeping genes routinely used for differentiating *L. plantarum* cluster were used. All the isolates except 3.2.8, *L. plantarum* WCFS1 and ATCC 14917 yielded 218 bp fragments for *recA*, indicating these isolates indeed

are *L. pentosus* while isolate 3.2.8 yielded 318 bp PCR product indicating that it is an *L. plantarum* (Figure 2.1). Alignment of the sequences against the NCBI database confirmed their identity as *L. pentosus* with 100% coefficient of similarity (GenBank accession numbers KT027550 to KT027560). Dendrograms constructed based on the *recA* sequences showed that the isolates tentatively identified as *L. pentosus* by MLST associated genes (Table 2.2) clustered with the type strain *L. pentosus* ATCC 8041 with a 100% similarity coefficient (Figure 2.2). Isolate 3.2.8 clustered with previously characterized *L. plantarum* strains including ATCC 14917 and WCFS1 (Figure 2.2). Similarly, sequences of the amplicons obtained from the isolates for *pheS* (GenBank accession numbers KT124117 to KT124127), *rpoA* (GenBank accession numbers KT124128 to KT124138), and *dnaK* (GenBank accession numbers KT124106 to KT124116) presented at least 99% similarity to those previously reported for *L. pentosus* except 3.2.8 which matched with *L. plantarum*. Figure 2.2 shows dendrograms of the four housekeeping gene sequences, based on the maximum likelihood method. Three different clusters were defined from the more polymorphic *pheS* amplicon derived sequences (Figure 2.2). The isolates were further investigated to determine the number of unique strains that were selected through the screening process. Genotypic variability among the seven isolates was further characterized using RAPD. Primers M14 and OPL5 for RAPD did not yield differentiable band patterns among the tested isolates. PCR amplification using primer LP1 produced band patterns that differentiated only a few *L. pentosus* cultures (data not shown). However, PCR amplification using primers GTG₅ and COC yielded defined band patterns differentiating the heterogeneity among the *L. pentosus* and *L. plantarum* isolates (Figures 2.3 and 2.4). Similarity coefficients of 85% and 75% were calculated for the band patterns generated using the GTG₅ primer for RAPD among the *L. pentosus* and *L. plantarum* isolates, respectively.

Differentiation of *L. plantarum* and *L. pentosus* from the Collection:

The collection of commercial cucumber fermentation isolates tentatively identified as *L. plantarum/pentosus* and *Lactobacillus* spp. using 16S rRNA partial sequence, was further scrutinized for the presence of *recA* amplicons of different sizes (Appendix Figure A.2.3 and Table 2.3). Out of 190 isolates from NC fermentation tank, 95% presented *recA* amplicon corresponding to *L. pentosus*, only 4% presented an amplicon size representative of that found in *L. plantarum* and 1% did not yield an amplicon. Similarly, out of 62 isolates from MN fermentation tank 71% belonged to *L. pentosus*, only 22% were identified as *L. plantarum* and 7% did not yield any product. It is likely that the isolates that did not yield an amplicon do not belong to either *L. pentosus* or *L. plantarum* but closely related to *L. plantarum* based on the 16S rRNA sequence.

DISCUSSION

One of the primary objectives of this research was to screen a collection of *L. plantarum/pentosus* isolates from commercial cucumber fermentation for their ability to quickly convert glucose and fructose naturally present in the fruits to lactic acid. A model system composed of CJM, containing water soluble components in the fresh fruits, and distilled water in a 60:40 (v/v %) ratio was used to screen a large number of isolates. Cucumber fermentations worldwide are often performed in open-top tanks exposed to ambient temperatures using either fresh or recycled cover brine solutions. The active fermentation period proceeds during the late spring to early Fall with temperatures ranging from 10 to 32 °C (50 to 90 °F) (McFeeters and Pérez-Díaz 2010). The need to reduce the chloride content in waste waters generated from tank yards, has forced the continuous recycling of cover brine solutions, which mildly acidify the fermentation at its start. Commercially, cucumbers in fresh cover brines would typically

equilibrate to an initial pH of 5.2 ± 0.2 (personal communication with pickle processors) depending on the buffering capacity of the fruits. The use of recycled cover brines may reduce the initial cover brine pH to as low as 3.8 ± 0.1 (personal communication with pickle processors). Therefore, the ideal starter culture for low salt cucumber fermentations should conduct a rapid fermentation under a variety of temperatures and a diversity of initial pH ranging from 5.4 to 3.8. It is one of the long term goals to use these potential starter culture candidates for NaCl-free cucumber fermentations and thus the need to evaluate performance of the isolates in the absence of the salt. Performing the screening under various treatment conditions provided an opportunity to explore the robustness of these isolates to ferment at different pH, salt contents and incubation temperatures. Even though certain isolates performed rapid fermentation in specific treatments here, they did not reduce the pH in other treatments (Appendix Table A.2.1). Thus, only the robust isolates that reduced pH in at least three or more treatments were selected for further studies. The highest number of isolates that presented the desired performance was isolated from the first and third days of the commercial cucumber fermentations (Appendix Table A.2.1), suggesting that microbes dominating early in the process are likely to compete well and produce lactic acid quickly which was unknown in cucumber fermentation as of today.

L. brevis was tested with 83 tentative *L. plantarum* isolates for the possibility of co-existence in cucumber fermentation and potential use as mixed starter cultures. The inhibition against *L. buchneri* by the isolates was seen regardless of the day they were isolated suggesting that fermentation stage does not influence the ability to outcompete *L. buchneri*. However, all the isolates that met these criteria were from NC tank and none of the isolates were from MN tank. This suggests that *L. plantarum* may not be the only predominant fermenting bacteria and instead it co-ferments with other bacteria such as *L. brevis* in the MN tank. This is also corroborated by

detection of lower number of tentative *L. plantarum* isolates from MN tank (n=62) compared to NC tank (n=190) on various days. While the screening of antimicrobial activity performed in this study provides insight into the potential of these isolates to inhibit growth of the indicator strains, it is relevant to highlight that it is a non-quantitative assay that demonstrates a potential for inhibition or co-fermentation. Further studies are needed to determine if the nature of the antimicrobial activity is by acidification or competition for nutrients and/or production of bacteriocins or other inhibitory compounds (Whittenbury 1964; Daeschel *et al.* 1990; De Vuyst and Leroy 2007; Çon and Karasu 2009). Ultimately, the effectiveness of an *L. plantarum/pentosus* culture in preventing spoilage induced by *L. buchneri* is dependent on the mode of inhibition.

Members of the *L. plantarum* group, composed of *L. plantarum*, *L. pentosus* and *Lactobacillus paraplantarum*, are indistinguishable based on their 16S rRNA sequences because they share greater than 99% similarity (Collins *et al.* 1991; Bringel *et al.* 2001; Torriani *et al.* 2001; Singh *et al.* 2009; Ghotbi *et al.* 2011). In line with previous reports, identification of the seven isolates by the partial sequencing of 16S rRNA presented ambiguous identification as either *L. plantarum* or *L. pentosus* (Table 2.2; GenBank accession numbers KT025927 to KT025937) (Bringel *et al.* 2001; Torriani *et al.* 2001; Yu *et al.* 2012). The similarities in some of the genes of MLST scheme suggest that they are highly conserved in both *L. pentosus* and *L. plantarum*. Among the genes whose products were successfully amplified, the nucleotide sequence matches were with *L. pentosus* and not *L. plantarum* except for isolate 3.2.8. Sequence homology comparison (by Clustal Omega) of *gyrB* (KCA1_RS07990 and IG1 LPENT_01765) and *pgm* (KCA1_RS03040 and IG1 LPENT_01109) of two *L. pentosus* strains KCA1 and IG1, respectively showed that they share a low homology even within *L. pentosus* indicating that they

cannot be used for MLST based identification of *L. pentosus*. This was our first indication that majority of the isolates in this study could potentially be *L. pentosus* and not *L. plantarum*. For future studies on differentiation based on MLST, it should be noted that the bacterial origin also has an impact on the success of this technique (Cooper and Feil 2004; de las Rivas *et al.* 2006; Gosiewski *et al.* 2012). For example, Gosiewski *et al.* were able to characterize *L. plantarum* strains of plant origin but not from human origin using the same MLST scheme suggesting that this technique can be highly variable (Gosiewski *et al.* 2012).

To confirm the identity of tentative *L. pentosus* isolates, more housekeeping genes that can differentiate between *L. plantarum* and *L. pentosus* were used. Amplification and sequencing of additional ubiquitous and conserved proteins' housekeeping genes such as *recA*, *pheS*, *rpoA* and *dnaK* confirmed the observations made from the MLST analysis that the identity of the isolates is in fact *L. pentosus* except 3.2.8 which is an *L. plantarum*. The amplification of *recA* is routinely used for the differentiation of *L. plantarum* and *L. pentosus* given that amplicons with 100 bp size difference are produced (Torriani *et al.* 2001; Anukam *et al.* 2013) (Figure 2.1). The enzyme recombinase A is involved in various intracellular functions including homologous pairing and DNA recombination, SOS induction, binding of single and double stranded DNA, ATP hydrolysis and in cleavage of proteins during DNA damage repair (Bianco and Kowalczykowski 1998). *dnaK* codes for heat shock protein 70 that protects various enzymes against heat stress and ensures proper folding and transport of proteins (Huang *et al.* 2010). *pheS* codes for phenylalanine t-RNA synthetase involved in translation of phenylalanine and *rpoA* codes for RNA polymerase α -subunit which is involved in transcription activation (Naser *et al.* 2005; Naser *et al.* 2007). *pheS* and *rpoA* are exceptionally conserved and widely used for evolutionary based identification purposes of various organisms. Moreover, *pheS* and *rpoA* genes

are present only as a single copy with an interspecies gap of only 10% and 5%, respectively, and thus considered as an effective tool for identification and discrimination of closely related lactobacilli such as *L. plantarum* and *L. pentosus* in this study (Naser *et al.* 2007). Similar to previous reports, *pheS* produced a finer differentiation with respect to similarity among various isolates (Figure 2.2) (Naser *et al.* 2007). Since multiple housekeeping genes combine various molecular clocks it gives more insights at an evolutionary level as well as effectively differentiate various species (Naser *et al.* 2005).

Strain level identification through RAPD using GTG₅ and COC primers concluded that there are two unique strain patterns within *L. pentosus* (patterns displayed by isolate *L. pentosus* LA 0445 and the remaining isolates of *L. pentosus*) (Figure 2.3). Isolate 3.2.8 had a unique pattern close to *L. plantarum* control strains WCFS1 and ATCC 14917. Similarity coefficients of 85% and 75% were calculated for the band patterns generated using the GTG₅ primer for RAPD among the *L. pentosus* and *L. plantarum* isolates, respectively; indicating the closeness in the genetic makeup of these isolates obtained from commercial cucumber fermentation (Figure 2.3). Interestingly, the *L. pentosus* isolates selected in this study showed 71% similarity to the strain LA 0445 isolated from cucumber fermentations in the 1980s which was originally identified as *L. plantarum* (Fleming *et al.* 1988). Similar observations were made with the RAPD fingerprints obtained by the primer COC (Figure 2.4). The confounding factor in using RAPD for identification and differentiation at a strain level is the reproducibility of the technique. However, similar band size patterns were considered in both of our replicates and factors such as amount of DNA added, amount of loading volume, % of agarose gel and voltage of the run were optimized in this study. Together with other housekeeping genes and/or phenotypic tests RAPD

is a great tool for identification and differentiation of lactic acid bacteria from various fermentations (Van Reenen and Dicks 1996; Švec *et al.* 2005; Švec *et al.* 2007).

recA based identification confirmed that more than 95% and 71% of the isolates are *L. pentosus* in NC and MN tanks, respectively. Although, *L. pentosus* has been reported in cucumber fermentations in addition to *L. plantarum*, it was never reported as the dominant microorganism (Breidt Jr. *et al.* 2007). *L. plantarum* and *L. pentosus* commonly originate from plant sources and the ability to utilize pentose sugars (D-xylose and glycerol) is the key metabolic differentiation between the both (Zanoni *et al.* 1987). Recent whole genome sequencing studies also reveal that *L. pentosus* KCA1 possessed higher number and copies of putative sugar metabolizing genes than *L. plantarum* WCFS1. Clustered regularly-interspaced short palindromic repeats (CRISPR) associated gene sequences were also discovered in *L. pentosus* KCA1 which were not reported in *L. plantarum* whole genome sequence (Anukam *et al.* 2013).

In conclusion, after considering the results of the fermentation performance study using a fractional factorial screening design and antimicrobial assay, seven isolates were selected as potential candidates as starter cultures from NC. These seven isolates have a potential to co-exist with *L. brevis* and inhibit the undesired spoilage associated *L. buchneri*. Even though tentative *L. plantarum* isolates were detected from MN tank, none of them met the selection criteria of both rapid acidification and co-existence with *L. brevis* while inhibiting *L. buchneri*. Attempts to discriminate the selected isolates at genetic level resulted in the classification of most of the selected isolates as *L. pentosus*, except for 3.2.8 that was identified as *L. plantarum*. Discrimination of these isolates genetically using RAPD with primers GTG₅ and COC resulted in the identification of two distinct *L. pentosus* strain patterns and one *L. plantarum* strain

pattern. To our knowledge this is the first study that showed *L. pentosus* as the predominant microorganism during commercial cucumber fermentation which changes the traditional picture of cucumber fermentation, where *L. plantarum* has always been proposed as the dominant microorganism.

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TABLES

Table 2.1: Primers and PCR conditions used for genotyping tentative *L. plantarum* and *L. pentosus* isolates

Target Genes	Primers Concentrations (µM)	PCR Conditions
16S rRNA (Amann et al., 1995)	0.4	94 °C for 3 min, 25 cycles of 94 °C for 1 min, 57 °C for 2 min and 72 °C for 2 min and a final extension at 72 °C for 5 min.
Housekeeping Genes of Interest		
<i>recA</i> (Torriani et al., 2001)	0.25	94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 56 °C for 10 sec and 72 °C for 30 sec and a final extension at 72°C for 5 min.
<i>dnaK</i> (Huang et al., 2010)	0.25	94 °C for 5 min, 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1.5 min and a final extension at 72 °C for 7 min.
<i>pheS</i> (Naser and others 2007)	0.5	95 °C for 5 min, 30 cycles of 95 °C for 35 sec, 60 °C for 1.15 min and 72 °C for 1.15 min and a final extension at 72 °C for 7 min
<i>rpoA</i> (Naser et al., 2007)	0.5	95 °C for 5 min, 30 cycles of 95 °C for 35 sec, 56 °C for 1.15 min and 72 °C for 1.15 min and a final extension at 72 °C for 7 min

Table 2.2: Identification of cultures based on 16S rRNA sequencing and MLST associated housekeeping genes

Isolated Culture ID	Best Match / Sequence Similarity (%)						
	16S rRNA	<i>mutS</i>	<i>purk1</i>	<i>ddl</i>	<i>gdh</i>	<i>gyrB</i>	<i>pgm</i>
1.2.11	<i>L. plantarum</i> / 100	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 98	<i>L. pentosus</i> / 98	NA*	NA
1.2.13	<i>L. plantarum</i> / 100	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 98	<i>L. pentosus</i> /94	NA	NA
1.8.6	<i>L. plantarum</i> / 100	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 98	<i>L. pentosus</i> / 98	NA	NA
1.8.9	<i>L. plantarum</i> / 100	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 93	<i>L. pentosus</i> /98	NA	NA
3.8.24	<i>L. plantarum</i> / 100	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 98	NA	NA
3.2.37	<i>L. plantarum</i> / 100	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 98	<i>L. pentosus</i> /98	NA	NA
LA0445	<i>L. plantarum</i> / 100	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 99	<i>L. pentosus</i> / 99	<i>L. pentosus</i> /86	NA	NA
<i>L. pentosus</i> ATCC 8041	<i>L. plantarum</i> / 100	NA	NA	NA	NA	NA	NA
3.2.8	<i>L. plantarum</i> / 100	<i>L. plantarum</i> / 99	<i>L. plantarum</i> / 99	<i>L. plantarum</i> / 98	<i>L. plantarum</i> / 99	<i>L. plantarum</i> / 99	<i>L. plantarum</i> / 100
<i>L. plantarum</i> WCFS1	<i>L. plantarum</i> / 100	<i>L. plantarum</i> / 100	<i>L. plantarum</i> / 99	<i>L. plantarum</i> / 100	<i>L. plantarum</i> / 100	<i>L. plantarum</i> / 100	<i>L. plantarum</i> / 100

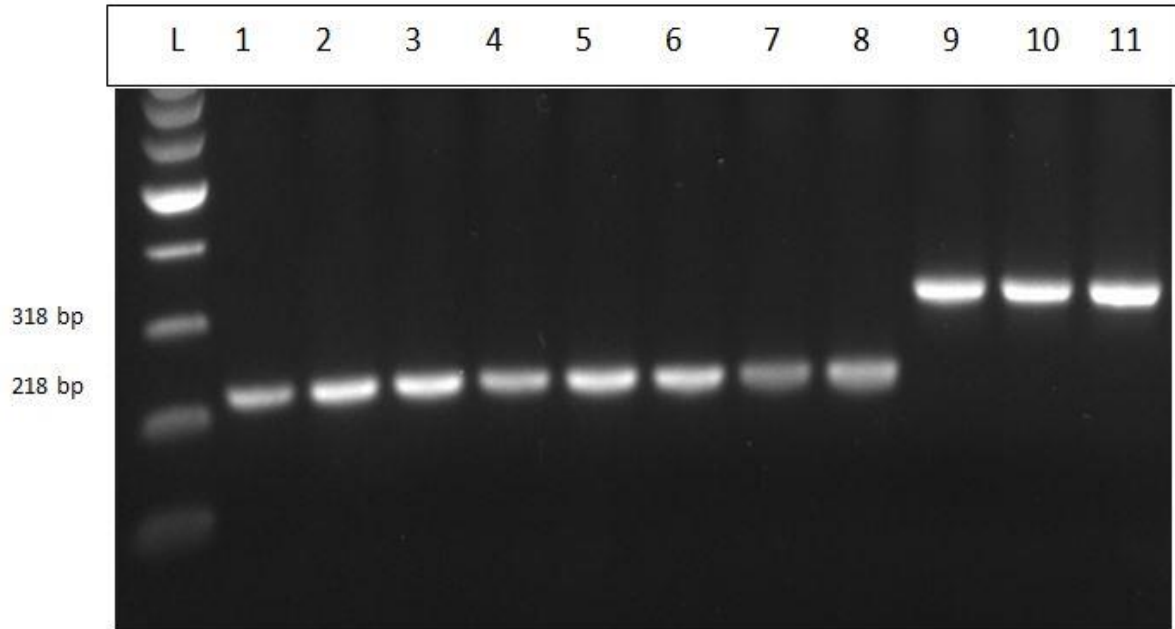
*NA-no amplicon

Table 2.3: Identification and distribution of *L. plantarum* and *L. pentosus* in two commercial cucumber fermentation tanks (NC and MN) using the partial sequences of 16S rRNA and *recA*

	Days	16S rRNA Identification		<i>recA</i> Identification	
		# of <i>L. plantarum</i>	# of <i>L. pentosus</i>	# of <i>L. plantarum</i>	Not Amplified
NC (n=190)	1	39	38	0	1
	3	49	44	5	0
	7	46	42	3	1
	14	56	56	0	0
	Total (%)			180 (95%)	8 (4%)
MN (n=62)	1	0	-	-	-
	3	3	0	1	2
	7	25	11	13	1
	14	34	34	0	0
	Total (%)			45 (71%)	14 (22%)

FIGURES

Figure 2.1: Differentiation of *L. pentosus* (218 bp) and *L. plantarum* (318 bp) based on the different in PCR product on gel electrophoresis



Lanes from left to right: L- 100 bp ladder, 1- 1.2.11, 2- 1.2.13, 3-1.8.6, 4- 1.8.9, 5- 3.8.24, 6- 3.2.37, 7- LA 0445, 8- *L. pentosus* ATCC 8041, 9- 3.2.8, 10- *L. plantarum* WCFS1 and 11- *L. plantarum* ATCC 14917.

Figure 2.2: Dendrogram clusters of the isolates based on the *recA*, *dnaK*, *pheS* and *rpoA* sequences

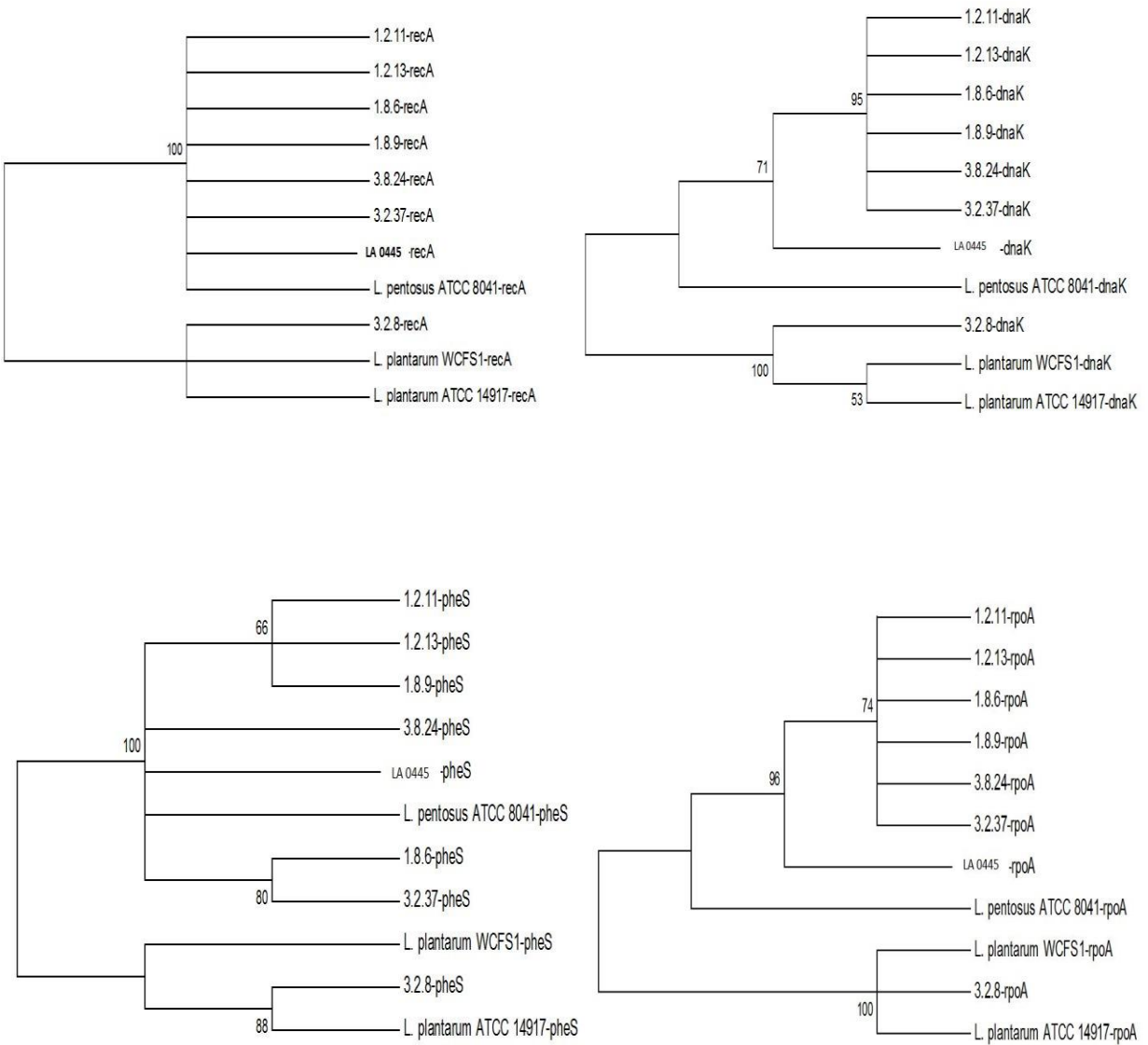


Figure 2.3: Differentiation of isolates based on RAPD using the primer GTG₅. The % similarity between the isolates is shown on the dendrograms

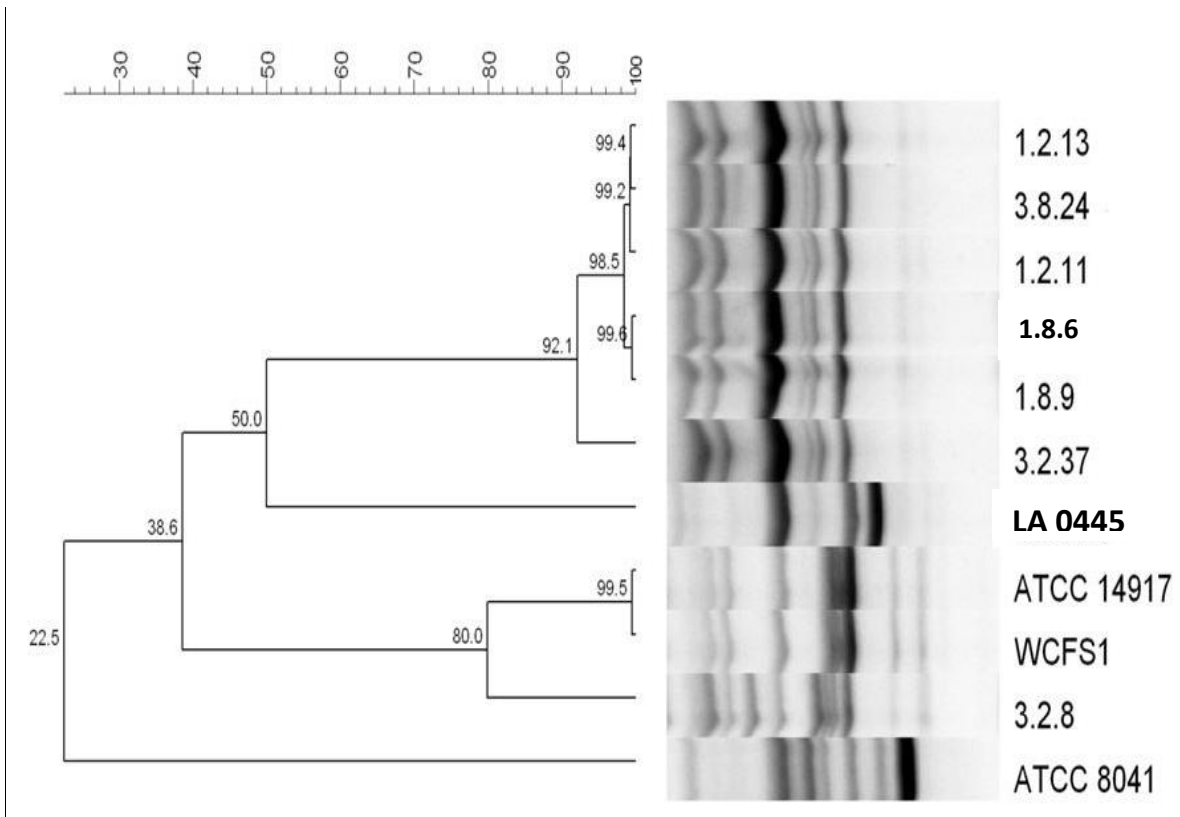
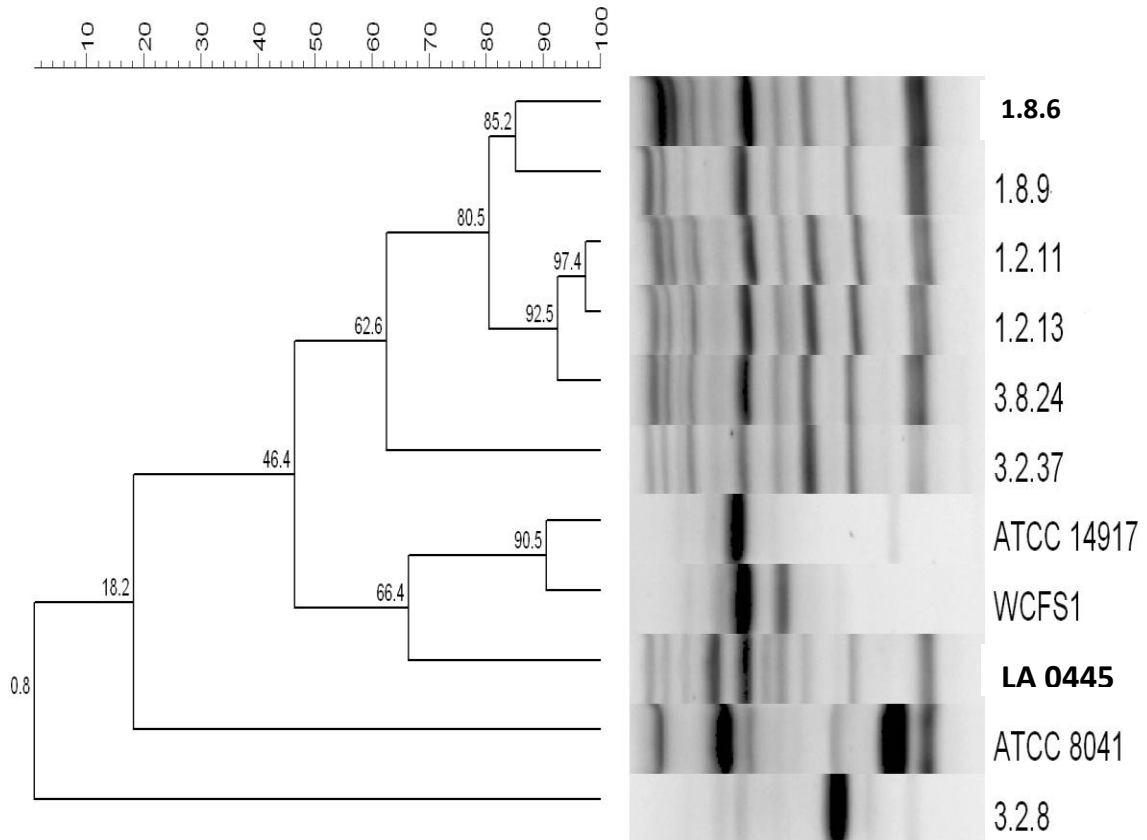


Figure 2.4: Differentiation of the isolates based on RAPD using the primer COC. The % similarity between the isolates is shown on the dendrograms



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CHAPTER 3

Evaluation of *Lactobacillus plantarum* and *Lactobacillus pentosus* isolated from Commercial Cucumber Fermentation as Candidates for Starter Cultures

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the journal guidelines)

ABSTRACT

Aims: *Lactobacillus pentosus* and *Lactobacillus plantarum* isolated from commercial cucumber fermentation were phenotypically and genotypically characterized for utilization as starter cultures for cucumber pickling.

Methods and Results: The isolates presented variable carbohydrate utilization profile as determined by API[®] 50CHL test strips. The *L. pentosus* isolates were not able to utilize D-xylose. Genes associated with biogenic amine (histamine, tyramine, putrescine and cadaverine) production and its phenotypic presence was not detected in any of the isolates. The ratio and amounts of D- and L- lactic acid production was found to be dependent on the growth media. The isolates were able to utilize malic acid which was confirmed on a differential medium rich in malic acid. Exopolysaccharide (EPS) biosynthesis related genes were amplified from all the isolates tested. The isolates produced ropy EPS which was detected using ruthenium red in the medium and their production depended on the type of sugar supplemented. Even though the genes associated with bacteriocin production were detected, the corresponding phenotype was not observed even after co-culturing with competing bacteria found in cucumber fermentation. The antibiotic resistance of the isolates against six antibiotics tested was below the minimum cut-off values.

Conclusions: The *L. pentosus* and *L. plantarum* isolates studied are suitable for use as starter cultures for the commercial production of fermented cucumbers.

Significance and Impact of Study: The spontaneous nature of cucumber fermentations has eliminated the need for the development of starter cultures for commercial process. With the development of low salt cucumber fermentations, it becomes imperative to develop

autochthonous starter cultures to assure the consistency of the metabolic transformations at a commercial scale. The research presented here constitutes efforts towards such goal.

Keywords: starter culture, cucumber fermentation, carbohydrate utilization, exopolysaccharides, malic acid decarboxylation

INTRODUCTION

The perception of food fermentation is moving away from a practical means of preservation to an art of consistent production of characteristic flavors in a microbiologically stable product with potential individualized health benefits (Holzapfel 2002). Additionally, utilization of starter cultures for fermentation enables manufacturers to have some control over the growth of indigenous microbiota, which results in a product with predictable characteristics (Holzapfel 2002). Moreover, addition of starter cultures in vegetable fermentations such as Kimchi, French beans, carrots and marrows has been shown to enhance production of lactic acid and organic volatiles, utilization of fermentable sugars, inhibition of undesirable bacteria such as Enterobacteriaceae and yeasts, increased bioavailability of vitamins, micronutrients and essential amino acids and firmness retention of finished products (Di Cagno *et al.* 2008; Chang and Chang 2010).

Although starter cultures specific to cucumber fermentations are currently commercially unavailable, their utilization for cucumber fermentation has been evaluated (Etchells *et al.* 1964; Etchells *et al.* 1973). *Pediococcus cerevisiae*, *L. plantarum* and *Lactobacillus brevis* have shown to achieve high acidity, cell densities and lower pH in pure cucumber fermentations with 5.4-5.6% sodium chloride (NaCl), conducted with fruits subjected to gamma radiation and blanching (Etchells *et al.* 1964). These Lactic Acid Bacteria (LAB) have also been found to predominate in

modern commercial cucumber fermentations (Pederson and Albury 1956; Tamminen *et al.* 2004). Etchells *et al.* (1973) recommended adding starter cultures at a level of ~4 billion cells per gallon, 18-24 h after the system is fully equilibrated to ~5.6 % NaCl. Additional recommendations include thorough washing of the fresh cucumbers, sanitation of the fermentation vessel, acidification of cover brines to create a buffering system and application of purging to dissipate CO₂ from inside the whole fruits (Etchells *et al.* 1968; Etchells *et al.* 1973). However, as the commercial pickling process changes, in particular to the use of low NaCl concentrations in fermentation cover brines, it becomes imperative to review the recommendations made decades ago for the addition of starter cultures in fermentations and evaluate suitable starter culture characteristics for the current and emerging commercial processes.

Low salt cucumber fermentations represent microbiologically unstable systems that can benefit from the advantages associated with the utilization of starter cultures (Pérez-Díaz and McFeeters 2011). But starter cultures designed for commercial cucumber fermentations that can also meet kosher requirements for vegetable products are unavailable to date (Pérez-Díaz and McFeeters 2011). Ideal starter cultures for cucumber fermentations would outcompete the indigenous fresh cucumber microbiota by rapid acidification of the fruits, adapt to proliferate at temperatures ranging between 15-35 °C (Daeschel and Fleming 1984; Buckenhüskes 1993; Geisen and Holzappel 1996; De Vuyst 2000; Heperkan 2013; Wouters *et al.* 2013), preferably produce L-lactic acid (Coronado *et al.* 1995; Petersen 2005) and co-ferment with other species/strains so that processors can effectively manage possible bacteriophage infections (Károvičová *et al.* 1999; Gardner *et al.* 2001; Holzappel 2002; Di Cagno *et al.* 2013). For cucumber fermentations it is also vital that the selected starter cultures convert most of the sugars

to lactic acid and are unable to decarboxylate malic acid to prevent bloating from the accumulation of CO₂ in the cucumber's mesocarp (discussed more in the literature review) (Etchells *et al.* 1968; Daeschel *et al.* 1984). Homofermentation is a desirable safety feature because the spoilage associated microbes can utilize acetic acid (a byproduct of heterofermentation) as an energy source, and increase the pH (Franco and Pérez-Díaz 2012). Carbon dioxide from heterofermentation also contributes to bloating. In addition, starter cultures of choice for cucumber fermentations should be Generally Recognized As Safe (GRAS), unable to produce biogenic amines and any off-flavors (Di Cagno and others 2008), unable to transmit antibiotic resistance genes (Clementi and Aquilanti 2011) and produce antimicrobial compounds such as acids and bacteriocins against competing spoilage LAB (discussed more in the literature review) (De Vuyst 2000; Rao *et al.* 2013). Bacteriocin producing starter cultures were shown to produce a superior quality product and dominate the microbial ecology during the fermentation and extended shelf life of Kimchi (Chang and Chang 2010). Bacteriocinogenic cultures are also used as co-cultures in fermentations as long as they do not affect the sensory qualities of the finished product (Cotter *et al.* 2005; Gálvez *et al.* 2007). Even though antibiotic resistance started as a clinical problem, it is now widespread in non-clinical situations especially in fermentation and starter culture industries (Kastner *et al.* 2006). Currently, food chain is attributed as one of the predominant routes for antibiotic gene dissemination between pathogens and opportunistic bacteria in humans. It is expected that an understanding of the metabolism and physiology of selected starter cultures for cucumber fermentations, along with their interaction with the food matrix and competing microbiota would allow a more scientifically valid optimization of such cultures and process.

In a preceding study, a group of 243 *L. plantarum/pentosus* isolated from commercial cucumber fermentations were evaluated for their ability to rapidly decrease pH in a cucumber juice model system (Anekella and others, *submitted*). Eighty-three isolates were pre-selected out of the 243 as robust starter culture candidates for cucumber fermentations based on the ability to quickly reduce the pH during fermentation at conditions including 30 or 15 °C, 0 or 6 % NaCl and an initial pH of 4.0 or 5.4 (Anekella and others, *submitted*). Eight robust fermenting *L. plantarum/pentosus* isolates were finally selected based on their ability to co-exist with *L. brevis* and outcompete/inhibit spoilage associated *L. buchneri*. Genotypic characterization of these eight isolates using the sequences of housekeeping genes identified seven cultures as *L. pentosus* and only one as *L. plantarum*. Results of Random Amplified Polymorphic DNA (RAPD) genotyping suggested two unique *L. pentosus* strain patterns and one *L. plantarum* pattern within the group of the eight isolates.

The objective of this research was to characterize these pre-selected seven *L. pentosus* and one *L. plantarum* isolates as candidates for starter cultures for commercial cucumber fermentation. Isolates were phenotypically and genotypically evaluated in light of the desirable attributes discussed above for starter cultures including sugar utilization profile, D-/L-lactic acid production, ability to perform malic acid decarboxylation, production of biogenic amines, exopolysaccharides and bacteriocins and evaluation of antibiotic resistance profile.

MATERIAL AND METHODS

Cultures, Growth Conditions and DNA Extraction: The cultures of LAB used in this study were obtained from the USDA-ARS Food Science Research Unit Culture Collection (NC, USA). Cultures were transferred to deMan, Rogosa and Sharpe plates (MRS; Cat No: 288130, Difco™,

MI, USA) from frozen stocks, and incubated at 30 °C under aerobic conditions, except for *L. buchneri*, *Lactobacillus johnsonii* and *Lactobacillus delbrueckii* which were incubated at 37 °C under anaerobiosis (Coy Anaerobic Chamber, MI, USA). Yeasts were cultured in Yeast-Mold (YM) broth and incubated aerobically at 30 °C for 96 h. Genomic DNA was extracted from pure cultures using the MasterPure™ DNA purification kit (Cat No.: MCD85201, Epicentre, WI, USA) following the manufacturer's instructions. PCR reactions for gene amplification were performed using the Bio-Rad PCR Master Mix (Cat No.: 166-5009EDU, CA, USA) using 10 µM primer stocks (IDT Inc., CA, USA), 1 µL of whole genomic DNA between 40-60 ng.µL⁻¹ and adjusted to 25 µL total volume with nuclease free water (Cat No.: AM9937, Ambion®, Life Technologies, NY, USA). Magnesium chloride was added to the PCR mix as needed from a 10 mM stock (Cat No.: 42-303, Apex™, Genesee Scientific, CA, USA). All the experiments throughout the paper were independently duplicated.

Carbohydrate Utilization Profile: API® 50 CHL carbohydrate utilization kit (API®, BioMérieux, France) was used to evaluate the ability of the selected isolates to metabolize various carbon sources (Figure A.3.1). The isolates were grown overnight in MRS medium at 30 °C in 2 mL centrifuge tubes and spun at 2655 x g for 3 min (Eppendorf Centrifuge Model 5810, Hamburg, Germany). Culture supernatants were decanted and cells were resuspended in phosphate buffer saline (PBS-pH 6.8). The cell suspensions used to inoculate the API® 50CHL strips were adjusted to an OD_{600nm} of 0.1 (Thermo Spectronic Genesys20 Model 4001/4, MA, USA). The strips were incubated aerobically for 48 h at 30 °C and color changes were observed. The results were interpreted as per the manufacturer's guidelines and the sugar utilization pattern was submitted to the reference database APIWeb™ to obtain a matching profile and presumptive identification.

D-xylose Utilization Test and D-xylose Isomerase (*xyIA*) Amplification: The D-xylose utilization assay was performed in MRS without sugar (MRS-N, Cat No.: L1021-06, US biological, MA, USA) either supplemented with 50 mM D-xylose (MRS-X) or 50 mM D-glucose (MRS-G) for the positive control. MRS medium without sugars (MRS-N) was used as negative control for growth. Changes in pH were recorded after 48 h of incubation at 30 °C. *Lactobacillus casei* ATCC 393 was used as negative control for its inability to utilize D-xylose (Posno *et al.* 1991). *L. pentosus* ATCC 8041 was used as the positive control. The cultures were also tested in the M-medium, specifically developed to observe the ability of LAB to utilize D-xylose (Chaillou *et al.* 1999). Pure cultures were transferred to the M-medium supplemented with D-xylose (M-X), D-glucose (M-G) and no sugar (M-N; negative control) separately into 96 well plates and incubated in a plate reader at 37 °C (BioTek Instrument Inc., VT, USA). Growth was monitored for 96 h at OD_{600nm}.

Genotypic detection of the xylose isomerase *xyIA*, was performed with primers *xyIA1-F* (5'-GACTGGCATGAAGGTACTATGG-3') and *xyIA1-R* (5'-GCACCAACA CCACTTTGATAAG-3') with an expected amplicon size of 795 bp. Primers were designed using the PrimerQuest software (<http://www.idtdna.com/primerquest/home/index>) using *L. pentosus* MD353 *xyIA* sequence (Accession ID M57384) as the template. The PCR mix was prepared as described earlier and the following conditions were used: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min and a final extension cycle at 72 °C for 7 min.

Production of D-/L- Lactic Acids: Concentrations of D-/L- lactic acid produced by the isolates in MRS and Cucumber Juice Media (CJM) supernatants were measured using the D-/L- lactic acid rapid assay kit (K/D-LATE, Megazyme, Wicklow, Ireland). Cucumber juice medium (CJM)

was prepared as described by Anekella *et al.*, submitted. Cultures were transferred from frozen stocks to MRS broth prior to inoculation of the experimental media and incubated at 30 °C overnight. The cultures were then transferred from MRS broth to the experimental MRS and CJM to an OD_{600nm} of 0.1 and incubated for 48 and 72 h, respectively, under static aerobic conditions. D-/L- lactic acid concentrations were measured from the culture supernatants following the manufacturer's instructions.

Malic Acid Decarboxylation: The ability of the isolates to decarboxylate malic acid was determined using the Malic acid Decarboxylating (MD) medium as described by Daeschel and others (1984). Cultures were grown overnight in MRS broth at 30 °C, centrifuged in 15 mL tubes at 6300xg (Centrifuge Model 5810, Hamburg, Germany) for 5 min and the pellets were reconstituted in sterile saline solution (0.85% NaCl). Three µL of the pure cultures were spotted on MD agar plates and incubated at 30 °C for 24 h. A color change from blue to yellow was recorded as an indication of no malic acid decarboxylase activity. The mutant strain *L. plantarum* MU0445 with no malic acid decarboxylase (*mdc*⁻) activity was used as a positive control (Daeschel and others, 1984 and 1987).

Production of Biogenic Amines: The detection of putative genes encoding for enzymes involved in the production of biogenic amines was performed using multiplex and uniplex PCR as described previously (Coton and Coton 2005; Marcobal *et al.* 2005; de las Rivas *et al.* 2006). The concentration of MgCl₂ was maintained at 2 mM for all the PCR reactions. Genes encoding for histidine decarboxylase (*hdc*), tyrosine decarboxylase (*tdc*), ornithine decarboxylase (*odc*) and lysine decarboxylase (*ldc*) were considered for this analysis. These enzymes are involved in biosynthesis of histamine, tyramine, putrescine and cadaverine, respectively. *Lactobacillus* 30a

was used as a positive control for the detection of *hdc*, *ldc* and *odc* and *L. brevis* ATCC 8287 for *tdc* detection.

Phenotypic detection of biogenic amine production was performed using biogenic amine media plates containing individual metabolic precursors (Landete *et al.* 2007). Four different sets of plates each with 1% of histidine-HCl, tyrosine, ornithine-HCl or L-lysine (Sigma-Aldrich, MO, USA) were prepared. Cultures were adapted to the biogenic amine media by serially transferring them in MRS supplemented with biogenic amine precursors and pyridoxal-5'-phosphate at least 6 times at 30 °C for 24 h. Cultures were then streaked on their respective biogenic amine agar plates, each containing a different precursor, and incubated for 48 h at 30 °C. A color change from yellow to purple or a clear halo around colonies on tyrosine plates was designated as positive for biogenic amine production.

Exopolysaccharide Production Activity: The ability of the isolates to produce EPS was evaluated using phenotypic and genotypic methods. Genotypic characterization included PCR amplification of genes involved in homo- and heteroexopolysaccharide production as described previously (Low *et al.* 1998; Mozzi *et al.* 2006; Palomba *et al.* 2012). The concentration of MgCl₂ was maintained at 2 mM for all the PCR reactions. Along with the primers proposed in the papers above, two additional primer pairs were designed using the EPS production associated genes in *L. pentosus* IG1 (NCBI locus tags: LPENT_00747 EPS biosynthesis protein (*ebp1*) and LPENT_00748 EPS chain length determinator *Wwz* (*ecl1*) as templates. The following primer pairs were designed in this study using the PrimerQuest software: *ebp1*-F (5'-CTG TTT ACG TGG CAT CTT GAT TT-3') and *ebp1*-R (5'-CAT GAG ATG GTC CCG AGT TAT T-3') and *ecl1*-F (5'-GAT GCG AAC TAT CCC GAA CAT-3') and *ecl1*-R (5'-GTC CTA CTG GTT GAT GCT GAT T-3') with expected amplicon sizes of 411 and 439 bp, respectively. PCR mix

was prepared as described earlier with 2.5 mM MgCl₂ for these two primer pairs using the following conditions : 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min and a final extension cycle at 72 °C for 7 min. The sequences were deposited in NCBI GenBank and accession numbers were obtained.

Phenotypic detection of EPS production was performed on MRS agar plates supplemented with 0.008% ruthenium red (Sigma-Aldrich, CAS No.: R2751, MO, USA) with following sugars individually: 111 mM glucose, 111 mM sucrose, 111 mM fructose or an equimolar ratio of glucose and fructose (55 mM each). The production of ropy EPS, forms a barrier around the colonies on the plate, impeding the absorption of ruthenium red by the cells. Thus, colonies of ropy EPS producing microbes appear white on the pink plates (Stingele *et al.* 1996). Conversely, colonies formed by non-EPS producers appear translucent or pinkish on plates. *Lactobacillus rhamnosus* ATCC 53103 was used as a positive control for EPS production (Lebeer *et al.* 2011).

To mimic the native fermentation matrix, cucumber juice based medium plates were prepared and the isolates were tested for EPS production. Cucumber juice was extracted by blending fresh cucumbers (procured from a local pickle manufacturer) in a commercial juice maker (Warring Commercial, CT, USA) and frozen at -20 °C. The cucumber slurry was thawed right before preparation of the medium and filtered through cheesecloth to remove particulates. The sieved juice was centrifuged at 2060xg (Eppendorf Centrifuge Model 5810, Hamburg, Germany) for 30 min and the supernatant filtered sterilized using a 0.22 micron filtration unit (Rapid Flow[™]-Nalgene[®], Thermo Scientific, CA, USA). Agar solution (45 g.L⁻¹) was autoclaved separately, tempered to approximately 50 °C and added to the cucumber juice at a 3:2 (v/v) ratio of cucumber juice: agar solution. Ruthenium red was sterile filtered and added to the medium to

0.008% just before pouring the plates. Isolates were streaked and incubated for 24 h and checked for their EPS production capability.

Antimicrobial and Bacteriocin Activity Assays: The antimicrobial activity of the *L. plantarum* and *L. pentosus* isolates against heterofermentative LAB *L. brevis* ATCC 14869, *Leuconostoc mesenteroides* ATCC 8293 and *Weissella paramesenteroides* ATCC 33313, fermented cucumber spoilage bacterium *L. buchneri* ATCC 4005 and the yeasts *Candida etchellsii* Y095, *Issatchenkia occidentalis* Y0089, *Pichia manshurica* Y098 and *Zygosaccharomyces globiformis* Y0069 was tested. All the cultures were obtained from the USDA-ARS Food Science Research Unit culture collection.

Agar spot method was performed to determine the antimicrobial activity (Dobson *et al.* 2007; Moraes *et al.* 2010). *L. johnsonii* NCK468 was used as a positive control for bacteriocin production against the sensitive indicator *L. delbrueckii* NCK235 (courtesy of Dr. Todd Klaenhammer, North Carolina State University, NC, USA). Four μL overnight grown test cultures were spotted on MRS agar plates and incubated for 24 h at 30 °C. Ten μL of fully grown indicator bacteria or yeast cultures were added to 10 mL of lactobacilli MRS or YM soft agar (0.7%) respectively. This soft agar was overlaid on MRS agar plates containing the spots of the cultures of interest. The plates were incubated for 48 h at 30 °C to observe the formation of zones of inhibition around the spots. Zone of Inhibition (ZOI) were measured in mm. A ZOI > 1 mm was considered positive. Agar spot assay was subsequently performed by spotting 2 μL of the following enzymes and chemical reagents adjacent to growth spots on MRS agar plates described above: Proteinase K, α -Chymotrypsin, papain, pepsin, trypsin TPCK, catalase and 5M NaOH (Dobson and others 2007; Moraes and others 2010). All the enzymes were reconstituted to 1 mg.mL⁻¹ in phosphate buffer (pH 7). All the reagents were manufactured by Sigma-Aldrich,

MO, USA. The spotted enzymes and reagents were allowed to dry on plates for 20 min prior to overlaying with soft agar. The soft agar was allowed to solidify for 20 min and the plates were incubated at 30 °C for 48 h. A curvature in the ZOI where the proteases, catalase and base were spotted indicates an antimicrobial nature of proteinaceous, peroxidic or acidic, respectively.

Co-culture induction of bacteriocin production by the isolates in the presence of competing heterofermenting and spoilage LAB and yeasts commonly present during commercial cucumber fermentation was performed (Rojo-Bezarez *et al.* 2007). This induction is expected to imitate similar conditions faced in a cucumber fermentation environment. The bacteria and yeasts were grown in 10 mL MRS or YM medium as described previously, centrifuged and the pellet was reconstituted in 1 mL of sterile filtered cucumber juice. A cocktail of the indicator bacteria and yeasts was prepared separately. The test isolates were added to 40 mL of fresh cucumber juice separately at an initial OD₆₀₀ of 0.01 in 50 mL tubes. To each of above test isolates' tubes, bacterial and yeast cocktails were added at an OD₆₀₀ of 0.005 and 0.001, respectively and incubated for 24 h at 30 °C. Supernatants from these tubes were obtained after spinning at 6300xg for 15 min (Eppendorf Centrifuge Model 5810, Hamburg, Germany). The supernatants were filter sterilized using 0.22 µm filters, aliquoted into three equal volumes and treated as follows: 1) SN1- supernatant without any treatment 2) SN2- supernatant neutralized to pH 7 using 5N NaOH (Sigma-Aldrich) to eliminate the effect of acids and 3) SN3- supernatant neutralized to pH 7 and treated with catalase (Sigma-Aldrich) (stock concentration of 5 mg.mL⁻¹) to a concentration of 1 mg.mL⁻¹ and incubated at 30 °C for 1 h to eliminate the effect of both acid and peroxides. Growth kinetics of indicator bacteria in the presence of the 3 supernatants were performed individually in a 96 well plate to determine the antimicrobial potential of these supernatants against the bacterial indicators. A 1:1 volume mixture was prepared in each well

with sterile supernatant and cucumber juice inoculated with each of the bacterial indicators (OD₆₀₀ of 0.05) separately (Patel *et al.* 2014). The 96 well plate was incubated at 30 °C for 24 h (BioTek Instrument Inc., VT, USA). Specific growth rate constant $k = ((\log OD_2 - \log OD_1) / (t_2 - t_1))$ was calculated for all the indicators against each of the supernatants tested.

Genotypic characterization of bacteriocin production was done by PCR based detection of genes associated with plantaricin and pentocin biosynthesis. The primer pair O-48/O-105 was used to detect class IIa bacteriocin coding genes (Yi *et al.* 2010). Class IIb plantaricin associated genes were amplified using PCR primers and conditions developed previously (Diep *et al.* 1996; Rojo-Bezares *et al.* 2007; Cho *et al.* 2010; Doulgeraki *et al.* 2013). Additionally, the primer pair bip1-F (5'- CCT GAC CAA TCG GGG GAT TT-3') and bip1-R (5'- AAG TGC AAC CGG CCC TTA AT-3') with an expected product size of 146 bp was designed in this study targeting the bacteriocin immunity protein gene from *L. pentosus* IG1 using the PrimerQuest software. PCR mix was prepared as described previously with 2.5 mM MgCl₂ and used the following conditions: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 51 °C for 1 min and 72 °C for 30 s and a final extension cycle at 72 °C for 7 min. The sequences were deposited in NCBI Genbank and accession numbers were obtained.

Antibiotic Resistance Profile: Antibiotic resistance profile against six antibiotics and their Minimum Inhibitory Concentration (MIC- µg/mL) was tested against the isolates using Etest[®] strips (Bio-Mérieux, France). The concentration of the antibiotics tested was between 0.016-256 µg/mL and included the following: ampicillin, chloramphenicol, clindamycin, gentamicin, erythromycin and tetracycline. The experiment was performed as per the European Food Safety Authority (EFSA) standards and published methods by Florez and others (2006) on *Lactobacillus*

Susceptibility Medium (LSM) at an inoculum level of $\sim 10^8$ cells/mL (Flórez *et al.* 2006; EFSA 2012). The results were interpreted as per the manufacturer's guide.

RESULTS

Carbohydrate Utilization: Table 3.1 shows that *L. pentosus* cultures were unable to utilize L-rhamnose, D-turanose, amygdaline, arbutine, L-arabinose, gentiobiose, D-lactose, ribose and D-raffinose. The characterization of the *L. pentosus* isolates, including the ATCC type strain was unusual using the miniaturized biochemical strips API[®] 50CHL (Boyd *et al.* 2005; Ghotbi *et al.* 2011).

D-xylose Utilization: While the *L. pentosus* isolates from commercial cucumber fermentations were capable of utilizing glycerol, they did not metabolize D-xylose in the API[®] 50CHL strip test (Table 3.1), in MRS broth supplemented with D-xylose (data not shown), or in the M medium (data not shown) specifically developed to evaluate D-xylose utilization (Chaillou *et al.* 1999). The average OD₆₀₀ in MRS-G inoculated with the *L. pentosus* cultures was 1.2 ± 0.2 ; while no changes in absorbance were observed in the cultures in MRS-X and MRS-N. Similarly no change in growth i.e. increment in OD₆₀₀ was observed in M-X and M-N indicating that these isolates were not able to utilize D-xylose. *xyIA* amplicons were obtained from *L. pentosus* LA 0445 and ATCC 8041 even though their phenotypic activity of D-xylose utilization was not observed (Table A.3.1).

Production of D-/L- Lactic Acids: The D-/L- lactic acid isomers produced by the isolates in CJM were 41.9 ± 3.4 and 33.7 ± 1.3 mM respectively and 129.9 ± 8.9 and 61.2 ± 5.7 mM respectively in lactobacilli MRS broth (Figure 3.1).

Malic acid decarboxylation: No color changes were observed in the MD medium to yellow, suggesting that the *L. plantarum* and *L. pentosus* cultures here possess malic acid decarboxylating activity (Figure 3.2). The positive control *L. plantarum* MU0445, produced a color change to yellow in the MD medium (Breidt and Fleming 1992) (Figure 3.2).

Biogenic Amine Production: A biogenic amine production phenotype was not observed in the corresponding medium even after 6 serial transfers in the inducing medium prior to the test. There were no color changes from yellow to purple or a clear halo around colonies on tyramine plates confirming that there was no detectable biogenic amine production. Primers associated with biogenic amine production, in particular decarboxylases in various lactobacilli, did not produce amplicons from the isolates. The positive controls produced the corresponding changes on the plates and produced expected PCR products (Appendix Figure A.3.2).

Exopolysaccharide Production: All the isolates including the positive control presented a ropy EPS production phenotype in lactobacilli MRS plates supplemented with ruthenium red except *L. pentosus* 1.2.11 and 1.2.13 (Figure 3.3). The ability to produce EPS in lactobacilli MRS agar plates was dependent on the type of sugar (Table 3.2). However, these two cultures (1.2.11 and 1.2.13) showed slightly white colored colonies on cucumber juice plates with ruthenium red dye, suggesting a moderate production of ropy EPS. Amplicons for the primer ecd, associated with EPS chain length determinator, was detected in the seven cultures tested (Appendix Table A.3.1). Amplicons for primer ebp1, coding for the EPS biosynthesis protein, were only obtained from *L. pentosus* 1.2.13, 1.8.6 and LA 0445 (Appendix Table A.3.1).

Production of Bacteriocins: All the cultures inhibited the indicator bacteria in the agar spot assay indicating a potential antimicrobial activity such as acid production, oxidative stress from the production of hydrogen peroxide and/or bacteriocin production (Nieto-Lozano *et al.* 2002).

None of the isolates were able to produce ZOI against the yeast indicators tested (Appendix Figure A.3.3). However, none of the cultures produced a curvature in their ZOI in the presence of proteinases or catalase indicating that this antimicrobial activity may not be proteinaceous or oxidative stress in nature. The positive control produced curvatures in the ZOI, in the vicinity of the enzyme spots (Figure 3.4).

Growth rate constants from SN1 (untreated supernatant with $\text{pH} \leq 3.2$) were significantly lower ($p < 0.05$) from both the neutralized supernatants (SN2 and SN3) with or without catalase indicating that the antimicrobial activity is only due to acid production (Table 3.3). This suggests that the co-culture induction of bacteriocin production by the isolates in the presence of competing LAB and yeasts in CJM did not show any detectable activity of bacteriocins. The growth rate constants of indicator bacteria *L. brevis* ATCC 14869, *L. buchneri* ATCC 4005, *Leuconostoc mesenteroides* ATCC 8293 and *Weissella paramesenteroides* ATCC 33313 in cucumber juice without any supernatants were 0.12 ± 0.002 , 0.095 ± 0.009 , 0.119 ± 0.002 and 0.178 ± 0.006 respectively. No amplicons were produced from primer pair O-48 and O-105, which targets the conserved region of class IIa bacteriocins. However, amplicons were obtained for members of the *pln* operon including *plnD*, *E*, *E/F* and *G* from *L. plantarum* 3.2.8. Products for the primers *plnG* and *bip1* were amplified from all the *L. pentosus* isolates indicating that they could potentially produce class IIb bacteriocins (Appendix Table A.3.1). *L. plantarum* WCFS1 worked as positive control producing amplicons for all the genes tested.

Antibiotic Resistance Profile: All the tested isolates presented an MIC range below the cut-off values proposed by EFSA against ampicillin, chloramphenicol, gentamicin and tetracycline (Appendix Figure A.3.4; Table 3.4). *L. plantarum* ATCC 14917 which was used as the quality control strain displayed MIC values lower than cutoff guidelines for ampicillin, gentamicin and

tetracycline (Table 3.4). Isolates 1.2.13, 1.8.6, 1.8.9, 3.8.24 and ATCC 14917 displayed an intermediate resistance of 2 µg/mL for erythromycin (cut-off value- 1 µg/mL). Isolates 1.8.6, 1.8.9, 3.8.24 and 3.2.37 showed an intermediate resistance against clindamycin (cutoff value > 2 µg/mL).

DISCUSSION

Eight isolates from commercial cucumber fermentation were evaluated for their potential to serve as starter cultures. Carbohydrate utilization is one of the basic strategies to characterize bacterial cultures of interest. While the carbohydrate utilization pattern of *L. pentosus* isolates was found atypical using the miniaturized biochemical strip test, API[®] 50CHL, isolate 3.2.8 confined with the control strain of *L. plantarum* (Table 3.1 and Appendix Figure A.3.1). The difficulties in characterizing *L. pentosus* using API[®] strips and the inability of these cultures to utilize glycerol are in line with previous reports (Bringel *et al.* 1996; Ghotbi *et al.* 2011; Yu *et al.* 2012). *L. pentosus* LA 0445 which was isolated from cucumber fermentation more than 20 years ago for its acid resistance (Fleming *et al.* 1988), metabolized carbohydrates in a pattern different from all the other *L. pentosus* tested here. Even though *L. pentosus* 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.8.24 and 3.2.37 clustered together in a Rapid Amplified Polymorphic DNA (RAPD) analysis, the carbohydrate utilization patterns observed in this study were highly variable (Anekella *et al. submitted*). This observation suggests a high metabolic diversity even among strains of the same origin. Possession of a mosaic of carbohydrate utilization gene cassettes is one of the remarkable features of *L. plantarum* (Tanganurat *et al.* 2009; Siezen *et al.* 2010). *L. plantarum* has been considered as a “natural metabolic engineer” for its ability to adapt to various environments and utilize various sugars (Siezen and van Hylekama Vlieg 2011). A similar trend is expected in *L. pentosus*, a close relative of *L. plantarum*, that possessed numerous sugar utilizing genes in its

“sugar island” (Anukam *et al.* 2013). Discrepancies in carbohydrate metabolism may also be due to the absence of related genes or loss of plasmids encoding unusual carbohydrate metabolism associated genes as a result of evolution and adaptation to specific niches (Siezen and van Hylckama Vlieg 2011).

Reinstating *L. pentosus* as a species divergent from *L. plantarum*, was partially based on the ability of *L. pentosus* to differentially utilize pentoses such as D-xylose and glycerol (Zanoni *et al.* 1987). However, past studies indicate that some *L. pentosus* are unable to utilize D-xylose and some *L. plantarum* can utilize both D-xylose and glycerol (Bringel *et al.* 1996; Shoemaker 2004; Tamminen *et al.* 2004; Ghotbi *et al.* 2011). D-xylose is present in fresh cucumbers in at least 0.4-0.6 mg.g⁻¹ of mesocarp tissue and a higher concentration in the carpel, depending on the size of the cucumbers (McFeeters and Lovdal 1987; Miller 1989). However, it is not the primary fermentation substrate, which may explain the lack of D-xylose utilization ability by the *L. pentosus* isolates tested here. Acidification of fermentation cover brines and cucumbers from the metabolism of the primary sugars, glucose and fructose, may also be preventing the catabolism of D-xylose by *L. pentosus*. Expression of the *xyl* operon that encodes genes involved in D-xylose utilization is induced by xylose and repressed by the presence of glucose and other sugars such as ribose and arabinose (Lokman *et al.* 1997). Lack of xylose utilization by the *L. pentosus* isolated from cucumber fermentations can also be possibly due to loss of plasmids that may be encoding *xyl* operon (Posno *et al.* 1991; Lokman *et al.* 1997).

There was a significant difference in the ratio at which D- and L-lactic acid isomers were produced in the two media tested (Figure 3.1). The physiological advantage associated with formation of either of the lactic acid isomers remained unknown for some time. D-/L- lactic acid production was known to produce metabolic energy by creating an efflux of protons generating

proton motive force and regenerating NAD⁺ (Kandler 1983). However, it is currently understood in that apart from the regular metabolic functions, *L. plantarum* uses D-lactic acid also for peptidoglycan synthesis as a terminal moiety that confers vancomycin resistance (Goffin *et al.* 2005). Thus, during active cell multiplication, D-lactic acid is rapidly produced as a result of the conversion of pyruvic acid by NAD⁺ dependent D-lactate dehydrogenase (LdhD). Even if LdhD is inactivated or not expressed, lactate racemase actively converts L-lactic acid to D-lactic acid (discussed more in the literature review). The *lar* operon which is involved in the expression of lactate racemase coding genes, is induced by L-lactic acid and repressed by excess D-lactic acid in *L. plantarum* (Goffin *et al.* 2005). Thus, the higher D-lactic acid production observed in MRS medium may be associated with active metabolism and fast replication in the presence of excess glucose. Cucumber juice medium contains moderate amounts of glucose and fructose (~60 mM) in solution (McFeeters and Lovdal 1987), which may be translating into a reduced metabolic activity and growth rate as compared to the nutrient rich MRS medium. Lower production of D-lactic acid in CJM also explains lower production of this isomer in cucumber fermentation and thus minimal carry over in finished products.

Malic acid decarboxylation by lactobacilli in cucumber fermentation is one of the main causes of the formation of hollow cavities inside the fruits, better known as the bloating defect (discussed more in the literature review) (Fleming and Pharr 1980). Malic acid is naturally present in cucumbers around 21-29 $\mu\text{moles.g}^{-1}$ of fruit depending on the cultivar (McFeeters *et al.* 1982a; b). It was estimated that 84 mg of CO₂ is produced per 100g of pickled cucumbers from respiration, which contributes to bloating (McFeeters *et al.* 1982a). About 12.5 mM CO₂ is needed to cause cucumber bloating and microbial malic acid decarboxylation can make a difference in reaching such threshold (Daeschel *et al.* 1984). All the isolates in this study were

able to decarboxylate malic acid (Figure 3.2). Thus, utilization of these isolates in cucumber fermentations will require application of air or nitrogen purging at regular intervals to promote dissipation of the CO₂ to the surface of the cover brines. Future research must be directed to identify naturally occurring non-decarboxylating variants of *L. plantarum* or *L. pentosus*.

Cucumbers contain amino acids that can potentially serve as precursors for biogenic amines. Biogenic amines are undesired in foods due to their potential as carcinogenic nitrosamine precursors. They are known to have neurological toxic side effects including headache, fatigue and dizziness including migraine (Landete *et al.* 2007; Spano *et al.* 2010). The European Food Safety Authority (EFSA) set daily intake toxic cut-off levels of histamine and tyramine at 50 and 600 mg, respectively. The minimum toxic levels of putrescine and cadaverine are still unknown (EFSA 2011). None of the isolates in this study presented a biogenic amines production phenotype (Appendix Figure A.3.2). Additionally, the corresponding decarboxylase coding genes were not amplified. These observations suggest that a combination of the *L. plantarum* and *L. pentosus* isolates is unlikely to result in the formation of biogenic amines. The current practice of spontaneous wild fermentation may have higher chances of biogenic amine formation due to the mixed composition of microorganisms present in the environment and the cucumbers (Buckenhüskes 1993). So, addition of these non-biogenic forming starter cultures can potentially outcompete the biogenic amine producers that may constitute natural microbiota.

Formation of biofilms through firm anchoring of EPS to solid surfaces aids bacteria to aggregate, proliferate and thus predominate in fermentations (Patel *et al.* 2012). Exopolysaccharides have also been shown to help in cell survival during processing (Nwodo *et al.* 2012). Hence, EPS production may be influencing the ability of the isolates to thrive in cucumber fermentations (discussed more in the literature review). In fact, production of EPS by

LAB was proposed as a mean for probiotic cultures to exert a health benefit (Lebeer *et al.* 2011; Donot *et al.* 2012; Patel *et al.* 2014). Additionally, production of EPS in CJM but not in MRS, by these isolates highlights the influence of the natural habitat in the induction of such phenotype (Figure 3.3 and Table 3.2). The putative gene encoding for EPS biosynthesis protein was not found in any of the isolates suggesting that such open reading frame is probably not conserved among them or they do not possess this gene (Appendix Table A.3.1). Genes encoding glycosyltransferase for the production of homoexopolysaccharides were not amplified from these isolates, indicating that they may primarily produce heteroexopolysaccharide (Vuyst and Degeest 1999; Palomba *et al.* 2012).

Bacteriocins confer an ecological advantage in an ecosystem because they are often produced under stress and competition as a survival strategy depending on the growth matrix (Ivey *et al.* 2013). For example, bacteriocinogenic *L. plantarum* survived for over 12-weeks as a starter culture in Spanish olive fermentation, whereas a non-bacteriocinogenic starter culture survived for only seven weeks (Ruiz-Barba *et al.* 1994; Dykes 1995). Intentional addition of bacteriocins or the bacteriocinogenic starter cultures to vegetable based products is now emerging along with the fermented products that naturally have such strains (Settanni and Corsetti 2008). Plantaricin, the bacteriocin from *L. plantarum*, production associated genes were considered in this study including- quorum sensing peptide for activation of the *pln* locus (*plnA*), two component regulatory system (*plnD*), ATP binding cassette (ABC) transporter (*plnG*), cationic complementary bacteriocins (*pln E/F* and *pln J/K*) and N-terminal double glycine leader peptide (*plnN*) (discussed more in the literature review) (Diep *et al.* 2009; Kawai and Saito 2011). The bacteriocin immunity protein coding gene from *L. pentosus*, was also targeted here given that it confers immunity to pentocin producing cells. The results suggest that the isolates

possess class IIIb bacteriocins associated with the genes *plnE*, *plnG*, and bacteriocin immunity protein. In addition, the *L. plantarum* cultures possessed *plnD* and *pln E/F* (Appendix Table A.3.1). The genes on the *pln* locus are highly variant among species and the sequences obtained here are different among the isolates tested here (Diep *et al.* 2009).

However, no bacteriocin like activity was detected in the agar spot method or in cucumber juice even after co-culture induction with competing bacteria and yeasts (Table 3.3). Co-culture induction and subsequent growth measurement in the presence of SN3 did not inhibit the indicators. The antimicrobial activity by the supernatants observed against the competing LAB seemed to be only from acid production (Table 3.3). However, indicator *L. buchneri* was inhibited by catalase activity which was observed from the specific growth rates (Table 3.3). The specific growth rates in SN3 are close to cucumber juice with no supernatant added indicating that there was no detectable bacteriocin like activity. The antibacterial effect of bacteriocins is dependent on the strain, growth environment and presence of other agents (Cintas *et al.* 2001). Hence, it can be concluded that the isolates do not produce detectable bacteriocin like inhibitory substances under conditions similar to those present in cucumber fermentations, even though they possessed the genes associated with them. Bacterial ecosystems are often diverse with a heavy competition of spatial and nutrient availability; however, in a laboratory culture where they are grown as monocultures in a sugar rich medium with minimal stress, it is not surprising not to detect bacteriocins (Dykes 1995; Riley and Chavan 2007). Plantaricins from various strains show that they are highly regulated and their activity is dependent on the environment and interspecies cell to cell contact (Riley and Chavan 2007).

Increased popularity in fermented foods containing live cells harbors a risk of being potential donors and/or recipients of antibiotic resistance gene carriers (Ouoba *et al.* 2008;

Schjørring and Krogfelt 2011). Lactobacilli in general are susceptible to protein and cell wall biosynthesis inhibiting antibiotics which were tested here. The MIC values of *L. pentosus* ATCC 8041 were reported for the first time in this study. *L. pentosus* LA 0445 showed a very low MIC of 0.032 µg/mL against clindamycin, while all the other *L. pentosus* isolates showed a higher MIC which needs to be investigated further (Table 3.4). The resistance against clindamycin for some isolates is slightly above the EFSA proposed cutoff of 2 µg/mL (Table 3.4). However, various cutoff values were proposed for *L. plantarum/pentosus* against clindamycin including 32 µg/mL (Danielsen and Wind 2003), 12 µg/mL (Flórez *et al.* 2006) and 4 µg/mL (Ammor *et al.* 2007). Even within *L. plantarum/pentosus* group the MIC range for a given antibiotic is widely distributed, for example, the MIC for clindamycin was reported between 0.03-16 µg/mL for *L. plantarum* (Danielsen and Wind 2003; Klare *et al.* 2007). A broad range of MIC of various antibiotics by *L. pentosus* strains isolated from olive fermentation such as 0.002-2 µg/mL for ampicillin, chloramphenicol, clindamycin and 0.002-16 µg/mL for erythromycin and tetracycline indicating variability even within species (Casado Muñoz *et al.* 2014). The test medium (broth vs. agar) also has an impact on the MIC values which explains variable values compared to other reports (Klare *et al.* 2007). Mechanisms such as reduced cell wall permeability and antibiotic efflux contribute to a slightly higher resistance than the suggested MIC even though there are no dedicated proteins/enzymes for antibiotic resistance. Even though phenotypic resistance was observed against some antibiotics, no transferable antibiotic resistance gene elements were identified in *L. pentosus* from table olives (Casado Muñoz and others 2014). Thus, their mode of action was attributed to efflux mechanisms or other intrinsic mechanisms which is most likely the situation with the intermediately resistant isolates in this study (Casado Muñoz and others 2014). Thus, the isolates in this study can be used as starter cultures for commercial cucumber

fermentation as their MIC values within the proposed cutoff values. However, the possibility of acquiring antibiotic resistance in the long run cannot be ignored. So, future studies should be directed towards understanding the recipient nature of these isolates when present in conjunction with other donor bacteria that possess transferable antibiotic resistant gene elements.

In conclusion, the isolates in this study show a variable carbohydrate utilization pattern and the *L. pentosus* isolates here do not ferment D-xylose. No production of biogenic amines was detected in these isolates both phenotypically and genotypically, the isolates are able to decarboxylate malic acid, produced a variable ratio lactic acid isomers and EPS depending on the growth medium. No phenotypic trait of bacteriocin production was observed even though they possessed the associated genes and the MIC values of the antibiotics within the cut-off values. The features observed in the eight isolates studied here, including one *L. plantarum* and seven *L. pentosus*, should not preclude their utilization as starter cultures in commercial fermentation.

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TABLES

Table 3.1: Sugar utilization in API[®] 50CHL test strips by the isolates of interest and type strains

Isolate ID	Control	Glycerol	Erythritol	D-Arabinose	L-Arabinose	D-Ribose	D-Xylose	L-Xylose	D-Adonitol	Methyl-βD-Xylopyranoside	D-Galactose	D-Glucose	D-Fructose	D-Mannose	L-Sorbose	L-Rhamnose	Dulcitol	Inositol	D-Mannitol	D-Sorbitol	Méthyl-αD-Mannopyranoside	Méthyl-αD-Glucopyranoside	N-Acetylglucosamine	Amygdaline	Arbutine	Esculine	Salicine	D-Cellobiose	D-Maltose	D-Lactose	D-Melibiose	D-Saccharose	D-Trehalose	Inulin	D-Melezitose	D-Raffinose	Amidon Starch	Glycogen	Xylitol	Gentiobiose	D-Turanose	D-Lyxose	D-Tagatose	D-Fucose	L-Fucose	D-Arabitol	L-Arabitol	K-gluconate	K-2-Ketogluconate	K-5-Ketogluconate								
<i>Lactobacillus pentosus</i>																																																										
1.2.11																									C																																	
1.2.13																									C																																	
1.8.6																									C																																	
1.8.9																									C																																	
3.8.24																									C																																	
3.2.37																									C																																	
LA0445																									C																																	
ATCC 8041																									C																																	
<i>Lactobacillus plantarum</i>																																																										
ATCC 14917																									C																																	
3.2.8																									C																																	

Green colored boxes indicate positive and red colored boxes indicate negative for utilization of sugars

C- Control

Table 3.2: Production of EPS by the isolates on MRS supplemented with glucose, fructose, sucrose and equimolar glucose+fructose and cucumber juice medium supplemented with the ruthenium red dye

Isolates	Sugar Source				Cucumber Juice
	Glucose	Fructose	Sucrose	Glu+Fru	
	<i>Lactobacillus pentosus</i>				
1.2.11	-	-	-	-	+
1.2.13	-	-	-	-	+
1.8.6	+	-	+	+	+
1.8.9	+	-	-	+	+
3.8.24	-	-	-	-	+
3.2.37	-	-	-	-	+
LA 0445	+	-	+	+	+
	<i>Lactobacillus plantarum</i>				
3.2.8	+	+	+	+	+
WCFS1	+	+	+	+	+
	<i>Lactobacillus rhamnosus</i>				
ATCC 53103	+	+	+	+	+

(-) indicates formation of pink colored colonies on plates suggesting lack of EPS production

(+) indicates the observation of a ropy EPS phenotype

Table 3.3: Growth rate (h^{-1}) of indicator bacteria against the three culture supernatants derived from the isolates of interest

Isolates	†SN1 ^a	SN2 ^b	SN3 ^c	SN1 ^a	SN2 ^b	SN3 ^c	SN1 ^a	SN2 ^b	SN3 ^b	SN1 ^a	SN2 ^b	SN3 ^b
	<i>L. brevis</i>			<i>L. buchneri</i>			<i>L. mesenteroides</i>			<i>W. paramesenteroides</i>		
1.2.11	0.03 ± 0.02	0.08 ± 0.01	0.07 ± 0.02	0.02 ± 0.00	0.08 ± 0.00	0.1 ± 0.00	0.03 ± 0.01	0.09 ± 0.00	0.07 ± 0.01	NG	0.13 ± 0.00	0.17 ± 0.01
1.2.13	0.01± 0.00	0.08 ± 0.01	0.09 ± 0.03	0.02 ± 0.00	0.08 ± 0.00	0.1 ± 0.00	0.02 ± 0.01	0.14 ± 0.00	0.17 ± 0.00	NG	0.12 ± 0.01	0.17 ± 0.00
1.8.6	0.05 ± 0.03	0.09 ± 0.01	0.11 ± 0.02	0.02 ± 0.00	0.08 ± 0.00	0.09 ± 0.00	0.04 ± 0.00	0.1 ± 0.01	0.11 ± 0.05	NG	0.13 ± 0.01	0.17 ± 0.00
1.8.9	0.02 ± 0.00	0.1 ± 0.04	0.07 ± 0.02	0.02 ± 0.00	0.08 ± 0.00	0.11 ± 0.00	0.02 ± 0.00	0.11 ± 0.01	0.11 ± 0.02	NG	0.13 ± 0.00	0.17 ± 0.01
3.8.24	NG	0.13 ± 0.00	0.16 ± 0.00	0.03 ± 0.00	0.08 ± 0.00	0.1 ± 0.01	0.02 ± 0.00	0.11 ± 0.01	0.15 ± 0.01	NG	0.06 ± 0.00	0.09 ± 0.01
3.2.8	0.01 ± 0.00	0.15 ± 0.00	0.17 ± 0.00	0.02 ± 0.01	0.08 ± 0.00	0.1 ± 0.00	NG	0.12 ± 0.01	0.15 ± 0.00	NG	0.05 ± 0.01	0.09 ± 0.00
3.2.37	NG	0.14 ± 0.01	0.17 ± 0.00	0.02 ± 0.00	0.08 ± 0.00	0.1 ± 0.00	NG	0.11 ± 0.01	0.15 ± 0.01	NG	0.04 ± 0.00	0.09 ± 0.01
LA 0445	NG	0.13 ± 0.01	0.17 ± 0.00	0.02 ± 0.00	0.08 ± 0.00	0.1 ± 0.01	NG	0.11 ± 0.00	0.14 ± 0.01	NG	0.05 ± 0.01	0.07 ± 0.00
WCFS1	NG	0.13 ± 0.00	0.16 ± 0.00	0.03 ± 0.00	0.08 ± 0.00	0.1 ± 0.00	NG	0.1 ± 0.00	0.14 ± 0.00	NG	0.05 ± 0.02	0.08 ± 0.01

†SN1- untreated supernatant; SN2- supernatant neutralized to pH 7; SN3- supernatant neutralized to pH 7 and treated with catalase to 1mg.mL^{-1} .

^{a, b and c} Specific growth rate of supernatants with same alphabets (a, b and c) indicate that they are not significantly different from each other and supernatants with different alphabets in superscription indicate that they are statistically different

*NG- No growth

Table 3.4: Minimum Inhibitory Concentrations (MIC $\mu\text{g/mL}$) of antibiotics against the isolates

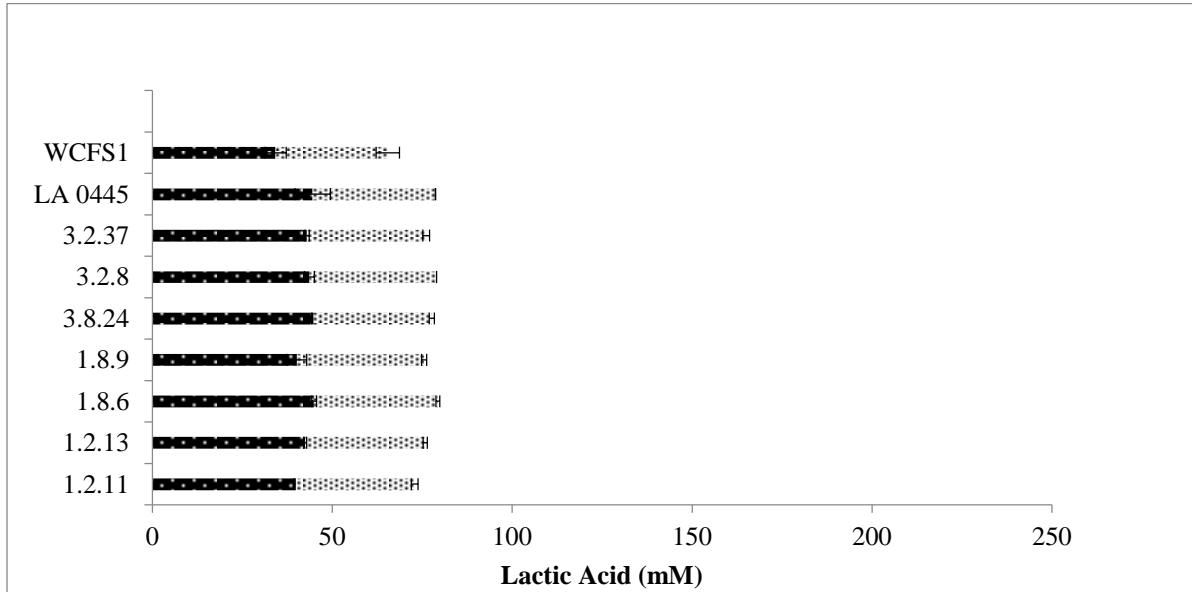
Isolates	MIC ($\mu\text{g/mL}$)					
	Ampicillin (2)*	Chloramphenicol (8)	Clindamycin (2)	Erythromycin (1)	Gentamicin (16)	Tetracycline (32)
1.2.11	2	7	2	1	10	16
1.2.13	1	6	2	2	12	10
1.8.6	1	7	4*	2	6	18
1.8.9	2	7	4	2	7	16
3.8.24	1	8	4	2	14	28
3.2.37	1	8	3	1	7	20
LA 0445	2	5	0	1	3	14
3.2.8	1	6	1	1	5	16
ATCC 14917	1	8	2	2	11	6
ATCC 8041	2	8	1	1	4	11
Cutoff value	2	8	2	1	16	32

*MIC values above the cutoff value are highlighted in bold indicating intermediate-resistant

FIGURES

Figure 3.1: Amounts of D/L- lactic acid produced by the isolates of interest in (a) D/L-lactic cucumber juice medium (b) MRS broth (Dotted black and white bars represent D-lactic acid and L-lactic acid concentrations (mM) measured, respectively)

(a)



(b)

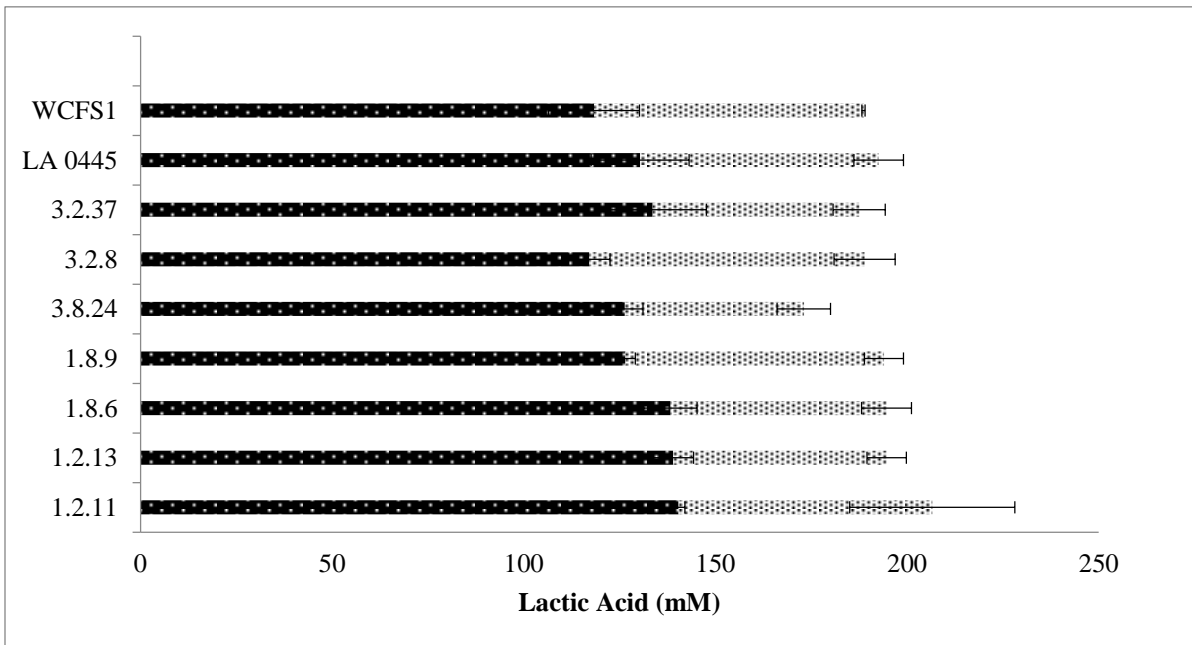
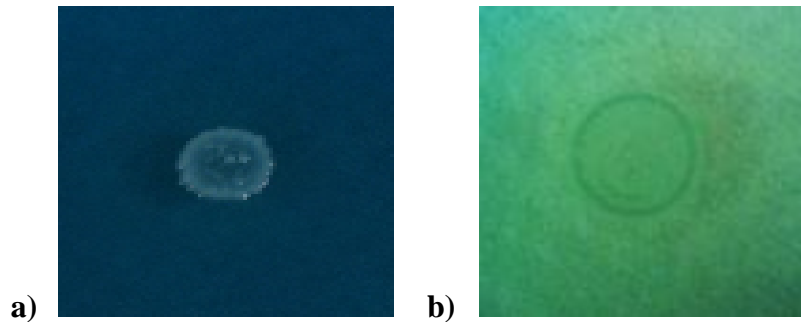


Figure 3.2: MD agar plates inoculated with a) *Lactobacillus pentosus* 1.8.9 (MDC⁺) and b) *Lactobacillus pentosus* LA 0081 (MOP3-M6) (MDC⁻).

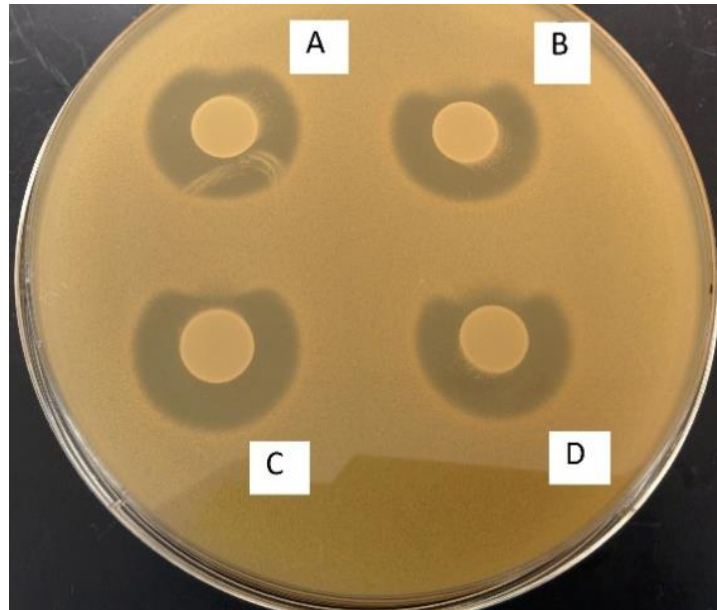


Inability to decarboxylate malic acid induces a change in the color of the medium from blue (a) to yellow (b)

Figure 3.3: Ropy phenotype by exopolysaccharide producing culture *Lactobacillus plantarum* 3.2.8 on cucumber juice agar supplemented with ruthenium red dye



Figure 3.4: Antimicrobial activity of the positive control *Lactobacillus johnsonii* NCK 468 against *Lactobacillus delbrueckii* NCK235



The curvatures around the zone of inhibition were formed due to the localized addition of the following enzymes/reagents (A) Papain (B) Pepsin (C) Proteinase K and (D) α -Chymotrypsin.

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CHAPTER 4

Use of Autochthonous Lactobacilli as Starter Cultures in Jar and Commercial Cucumber Fermentation with Calcium Chloride Brine

ABSTRACT

Most starter cultures commercially available in the US for food fermentations are developed for dairy products. Starter cultures that are native and specifically designed for commercial cucumber fermentation are currently unavailable. Adoption of sodium chloride (NaCl) free cucumber fermentation by the pickling industry in the US makes it imperative to identify appropriate starter cultures for such process. Autochthonous lactobacilli from commercial cucumber fermentation were used in this study to evaluate as starter cultures in sodium free fermentation in one gallon jars at 30 °C and 15 °C and on a commercial scale in approximately 10,000 gallon tanks at ambient weather conditions. Seven *Lactobacillus pentosus* and one *Lactobacillus plantarum* isolates that are autochthonous to commercial cucumber fermentation were used in this study. *L. plantarum* WCFS1 (of human origin) was used as an allochthonous starter culture and an uninoculated jar was used as an experimental comparative control for wild fermentation. pH and microbial counts were monitored in all the fermentations and production of metabolites and changes in the sugar contents were measured from the brine in jar fermentation studies. In the industrial tanks, lactobacilli counts reached a maximum of ~ 8 log CFU/mL and pH of 3.52 ± 0.15 by day 3. In the jar fermentation studies, pH of the brine was reduced to 2.97 ± 0.03 by the end of fermentation at both 30 °C and 15 °C incubation. Lactobacilli counts in the brine increased to a maximum by day 2 at 30 °C jars but on day 4 in jars at 15 °C. A wide range of residual glucose and fructose contents were measured in the autochthonous, allochthonous and wild fermented jars at both 30 °C and 15 °C incubation temperatures. The average lactic acid produced by autochthonous, allochthonous and uninoculated wild fermented jar at 30 °C by day 14 were 159.86 ± 17.22 , 157.89 ± 5.42 and 111 mM, respectively. Whereas at 15 °C the average lactic acid produced by autochthonous,

allochthonous and uninoculated wild fermented jar by day 14 were 68.70 ± 9.09 , 40.14 ± 23.57 and 11 mM, respectively. Acetic acid in brine from all the starter culture inoculated jars was close to zero after 14 days at 15 °C incubation whereas 8.03 ± 0.38 mM at 30 °C incubation, indicating an activity of heterofermentation. After day 7, acetic acid started increasing in the uninoculated control jar at 30 °C incubation suggesting heterofermentative spoilage. Bloating was observed in all the jars at both 30 °C (28-97%) and 15 °C (31-83%) incubated jars. The malic acid in fresh cucumber juice was 14.22 ± 3.33 mM and estimated to be equilibrated greater than 7 mM. Malic acid in brine reduced to ~ 1.5 mM at both the temperatures and remained constant throughout day 14 indicating that it was utilized to produce CO₂ to cause bloating. While no significant differences were observed in instrumental texture measurements between the two fermentation temperatures, incidence of bloaters in jars incubated at 30 °C was higher than in jars incubated at 15 °C.

Keywords: Autochthonous, allochthonous, starter cultures, cucumber fermentation

INTRODUCTION

Autochthonous bacteria are isolated from specific ecological niche whereas allochthonous bacteria belong to the same species as autochthonous bacteria but are of a different ecological origin (Di Cagno and others 2009). Most commercial starter cultures are allochthonous and they are generally used to ferment a variety of food products (Di Cagno and others 2013). Parameters such as adaptation to food matrix, microbial diversity and metabolic pliability differ between auto- and allochthonous bacteria (Di Cagno and others 2013; Di Cagno and others 2009). Autochthonous bacteria are tailored to grow in their specific origin and thus offer competitive growth and adaptive advantage over allochthonous bacteria (Di Cagno and others 2013; Di Cagno and others 2009; Hurtado and others 2010; Rao and others 2013). Longer lag phases were observed with allochthonous starter cultures in vegetable fermentations (Di Cagno and others 2008; Di Cagno and others 2009). The reduction of pH was more rapid and microbial growth was quicker and higher in vegetables fermented with autochthonous starter cultures compared to allochthonous starter cultures. In addition, autochthonous bacteria have an ability to produce metabolites such as bacteriocins and exopolysaccharides that are tailored specifically for their establishment during fermentation and thus have a greater chance of survival when used as starter cultures (Di Cagno and others 2013). Cell numbers of autochthonous starter bacteria were at least 1 log CFU/unit more than the allochthonous starter cultures and 4 log CFU/unit higher than spontaneous fermentation systems in vegetable fermentations such as carrots, French beans and marrows (Di Cagno and others 2008). Quality and nutritional attributes including color, sensorial and rheological properties, vitamins and antioxidant activity of fermented vegetables are favorably impacted and retained when

autochthonous starter cultures were used (Di Cagno and others 2008; Di Cagno and others 2009; Di Cagno and others 2013).

Sodium chloride free fermentation is currently gaining popularity due to the negative health effects and environmental impact associated with NaCl rich brine disposal into fresh waters by pickle industries (discussed more in the literature review). However, NaCl provides the expected salty flavor to the final product brine while also favoring the proliferation of desired lactic acid bacteria (LAB) (Chavasit and others 1991; Fleming and others 1985). Consequently, growth of LAB promotes the conversion of glucose and fructose naturally present in the cucumbers to lactic acid, concurrent acidification to pH 3.5-3.0 and formation of other secondary metabolites and antimicrobial compounds such as peroxides and bacteriocins (Ivey and others 2013). A desalting step is performed at the end of fermentation to bring down the salt concentration from 6% to 2% which generates a brine waste (Breidt and others 2013). Approximately 45 million L of effluent waste rich in NaCl (~2800 ppm chlorides) is generated every year from pickle industries (Pérez-Díaz and others 2015). Harmful effects of chloride rich brine disposal are pressurizing pickle industries to reduce or eliminate salt from processing (McFeeters and Pérez-Díaz 2010). Thus, the current practice of recycling brine in some industries that may carry over undesirable flavors, enzymes and bacterial load can be eliminated if the brine can be discharged without impacting the environment (Pérez-Díaz and others 2015).

However, a selective advantage of the desired LAB to dominate is compromised if NaCl is reduced or removed from cucumber fermentations (McFeeters and Pérez-Díaz 2010; Pérez-Díaz and others 2015). In salt free fermentations, intrinsic desirable LAB may have difficulties dominating a spontaneous fermentation during the initiation phase which promotes undesirable microorganisms to proliferate and thus impacting the quality of final product. Therefore, it is

necessary to develop starter cultures specifically selected for the NaCl free system while assuring the efficiency and consistency of the fermentation.

Low salt fermentation studies with starter culture addition were performed in sterile cucumber juice and jars, but the results may not be directly applicable at a commercial scale due to numerous confounding variables involved (Chavasit and others 1991; McFeeters and Pérez-Díaz 2010). Calcium chloride based cucumber fermentation was attempted in closed jars and industrial scale (McFeeters and Pérez-Díaz 2010; Pérez-Díaz and others 2015). CaCl_2 can be added as an alternative to NaCl to achieve the desirable carbohydrate utilization as well as eliminating the spoilage Gram negative bacteria (Pérez-Díaz and others 2015). Calcium chloride also helps in firmness retention; it is less toxic to plants and can be used at a much lower concentration than NaCl (Pérez-Díaz and others 2015; McFeeters and Pérez-Díaz 2010). In fact, the chemical changes and texture differences during industrial storage were not significantly different between salt and salt-free fermentation including the consumer preference of the final product (Pérez-Díaz and others 2015). The residual sugars by the end of NaCl-free fermentation were low or absent, pH and lactic acid concentration remained constant during the storage and indicated no detectable propionic/butyric acid formation that might otherwise indicate growth of spoilage microorganisms (McFeeters and Pérez-Díaz 2010).

The amount of available fermentable sugars present in cucumbers has an impact on acid production and thus the pH reduction that impacts the safety and quality of the final product. Mechanical stress can induce changes in the sugar constituents of fruit from the action of cell wall degrading enzymes such as cellulose, polygalactouranase and pectin methylesterase. For example, mannose and galactose were significantly reduced in mechanically stressed cucumbers (Miller 1989). Other sugars such as rhamnose, arabinose and xylose levels slightly decreased in

mechanically stressed fruits after 48 h (Miller 1989). Additionally, an ideal starter culture(s) for cucumber fermentation should not perform malic acid decarboxylation or possess inducible malolactic fermenting genes that produces CO₂ (Breidt and others 2013; McFeeters and others 1984). Processing defect called bloating occurs due to CO₂ accumulation in the fruit. The events combining- natural respiration of cucumbers, production of CO₂ by malic acid decarboxylating (MDC⁺) bacteria and heterofermentors cause serious bloating in cucumbers (Etchells and others 1975; Perez-Diaz and others 2014). Air purging is done intermittently during fermentation to minimize bloating but it involves a heavy ongoing capital investment and the purging may also support the growth of spoilage oxidative yeasts (Franco and others 2012).

Adoption of low/no salt fermentation at an industrial scale, inadequate inhibition of spoilage and/or pathogenic microorganisms, unpredictable flavor quality and capital investments for air purging to minimize bloating are the main reasons for a demand for starter cultures in cucumber fermentation industry (Pérez-Díaz and others 2015). Although there have been successful studies conducted to determine the applicability of starter cultures for a salt free cucumber fermentation, a thorough understanding of their feasibility, efficiency and safety was not determined especially at an industrial scale (Pérez-Díaz and McFeeters 2011; Lindgren and others 1990; Reina and others 2005). Understanding the metabolic products of starter cultures such as acid production and sugar utilization during fermentation along with their interaction with the food matrix is currently sought-after and allows for a more scientifically valid selection of starter cultures (Hansen 2002).

The objective of this study was to evaluate the potential of various starter cultures to serve as starter cultures for a low salt fermentation containing 100 mM CaCl₂ and 6 mM potassium sorbate. These starter bacteria are autochthonous to commercial cucumber

fermentation and were selected based on their ability to rapidly acidify sterile cucumber juice (Anekella and others, unpublished). Fermentations in one gallon jars and commercial scale open top tanks of 10,000 gallons were performed to understand the practicality of starter culture implementation in a CaCl₂ brined commercial fermentation. Small scale fermentations were performed at 30 and 15 °C and an allochthonous starter *L. plantarum* WCFS1 was also included. Sugars, pH, microbial counts and fermentation acid metabolites were monitored throughout the jar fermentation. Incidence of bloaters and texture of the pickles from jars were recorded after 14 days of fermentation.

MATERIALS AND METHODS

Preparation of Bacterial Cultures:

Isolates *L. pentosus* 1.2.11, 1.2.13, 1.8.6, 1.8.9, 3.2.37, 3.8.24 and LA 0445 and *L. plantarum* 3.2.8 were used as autochthonous starter cultures. *L. plantarum* WCFS1 was used as an allochthonous starter culture. All the isolates were collected from USDA Food Science Research Unit Culture Collection, NC, USA (Anekella and others, unpublished). The frozen stocks of these cultures were grown in deMan, Rogosa and Sharpe medium (MRS; Difco, MD, USA) overnight at 30 °C under static aerobic conditions. Pure colonies of these overnight cultures were further isolated on MRS plates incubated at 30 °C for 48 h. Commercial fresh pack Kosher spear pickle jars were obtained (Mt. Olive Pickle Company, NC, USA) and their pH was adjusted to 5.2 (Acumet[®] Research 25, Fischer Scientific, CA, USA) using 5N NaOH (Spectrum Chemicals, NJ, USA) (Pérez-Díaz and McFeeters 2011). The pH was measured again after a three day refrigerated storage to ensure that there were no deviations from an equilibrated pH of 5.2 ± 0.1 . After equilibration, these jars were inoculated with pure colonies of the cultures individually as described previously (Pérez-Díaz and McFeeters 2011). The jars were incubated

for four days at ambient room conditions prior to using their cover liquor as inoculum for subsequent fermentations.

Cucumber Fermentations in One Gallon Jars:

The brine composition used for fermentation included 200 mM anhydrous CaCl₂ and 12 mM potassium sorbate prior to equilibration. Size 3A cucumbers (diameter 1¹/₂- 2 inch) were used for this study. A 50:50 w/w pack out ratio (brine: cucumbers) was used in one gallon jars. The jars were tightly sealed and a rubber septum was inserted on top of the lid to allow for samples collection using a 10 mL syringe. A metal screw with gas outlet tube was also inserted on the lid to release gas produced during fermentation. The other end of the tube is inserted into a 0.2 µm sterile filter to prevent external contamination through air into the jars (Appendix Figure A.4.1). Cultures enriched in brine from above were inoculated in these jars at a level of 5 log CFU/mL. Each culture was inoculated in duplicate jars except control that underwent wild fermentation. The jars were incubated at either 30 °C or 15 °C for 14 days. Brine samples were aseptically collected on days 1, 2, 5, 7 and 14 using a syringe through the rubber septum after vigorously shaking the jars. One mL aliquots of the cover brine samples were spun at 2655xg for 5 min (Eppendorf Centrifuge, Model 5810, Hamburg, Germany) and the supernatant was stored at -20 °C until further analysis. Additional brine samples were collected on the same day to measure the pH and microbiological analysis. Lactobacilli counts were determined throughout the fermentation. Yeast and mold counts (on YM agar) were determined on days 2, 7 and 14.

Pickle Quality Assessment:

All the jars were opened on day 14 to visually inspect for the presence of bloaters by cutting the pickle longitudinally in half. The % amount of bloated cucumbers was recorded from

all the jars. Mechanical firmness was determined by punch test (at a rate of 2.5mm/sec) on mesocarp of 6 mm thick slices (n>15) from each jar using the TA-XT2 Texture Analyzer with a 3 mm probe (Texture Technologies Corp, N.Y./Stable Micro Systems, Surrey, UK) (Thompson and others 1982).

Analysis of Sugars and Acids:

Residual sugars including glucose and fructose and acids including lactic acid, acetic acid and malic acid were measured from the jar brine fermentation samples on days 1, 2, 5, 7 and 14 using High Performance Liquid Chromatography (HPLC; Shimadzu Model CBM-20A, OR, USA). Fresh cucumber juice was also included to estimate the sugars and malic acid naturally present. Sample components were eluted with 0.02 N sulfuric acid at a flow rate of 0.6 mL/min on Bio-Rad column (Aminex® HPX-87H, CA, USA). The sugars and acetic acids were detected using differential refractometric detector (RID-10A, Shimadzu) and the acids and potassium sorbate were detected on UV-Vis detector (SPD-20A, Shimadzu).

Industrial Tank Fermentation

Salt free fermentation with starter cultures as performed at an industrial scale with 200 mM and 12 mM potassium sorbate CaCl_2 brine prior to equilibration. Inocula were prepared as described above in one gallon jars. Commercial open-top fiberglass tanks (~10,000 gallons) were inoculated at levels of 5 log CFU/mL in triplicate. pH, and microbiological counts of lactobacilli on MRS agar; yeasts and molds on YM agar from tank brine samples on days 0, 3, 7 and 14. Plate counts of aerobic bacteria on PC agar; Enterobacteriaceae on violet red bile glucose agar and Clostridial counts on differential reinforced clostridial agar were determined on days 0 and 3.

RESULTS

Fermentation in Jars:

pH:

The pH of brine in all the jars inoculated with various starter cultures started decreasing from 5.5 on day 1 at 30 °C incubation (Figure 4.1a). By the end of day 2 all the starter culture inoculated jar brines (except LA 0445 and uninoculated control) reached pH 3.4 or lower. The jar inoculated with isolate LA 0445 was slower than the rest of isolates in reducing the pH until day 4. This strain was used as a standard starter culture in the past studies for its rapid acidifying property (McDonald and others 1993; Pérez-Díaz and McFeeters 2011; McFeeters and Pérez-Díaz 2010; Pérez-Díaz and others 2015). Control jar undergoing wild fermentation reduced the pH slower compared to all the inoculated jars. However, the control jar with wild fermentation reached 3.3 by day 5 with the rest of the starter culture inoculated jars (Figure 4.1a and Table 4.1a). By day 14 all jar brines measured approximately 2.98 ± 0.02 which was consistent with previous observations (Figure 4.1a and Table 4.1a) (McFeeters and Pérez-Díaz 2010; Pérez-Díaz and others 2015). A wide range of pH observed in the starter culture inoculated jar brine incubated at 15 °C by the end of day 2 between 4.5 and 5.3 (Figure 4.1b). By day 4 the pH of brine in all the jars was reduced to < 3.5 . However, the allochthonous starter culture *L. plantarum* WCFS1 reduced the pH quicker than all the isolates by day 2 to the lowest value did not reduce pH any further compared to the autochthonous starter culture inoculated jars. All the jar brines except uninoculated control reached to a stable pH of approximately 3.5 by day 4 compared to day 2 observed at 30 °C indicating a longer fermentation (Figures 4.1a and b). By day 14 all the brines in jars incubated at 15 °C measured approximately 3.06 ± 0.04 which were

higher than the jars incubated at 30 °C (Tables 4.1a and b). The control jar brine's pH remained at 3.72 by the end of day 14 (Figure 4.1b).

Microbial Analyses:

The lactobacilli counts in brines of all the starter culture inoculated jars reached at least 7.9 log CFU/mL while the uninoculated control jar reached 7.42 log CFU/mL by day 2 at 30 °C fermentation (Figure 4.2a). However, the bacterial numbers of brine in autochthonous inoculated jars started decreasing after day 7 and cell numbers after day 14 was around 6.51 ± 0.36 log CFU/mL which is in consistent with previous studies (Table 4.1a and Figure 4.2a) (Pérez-Díaz and others 2015). Uninoculated control jar's brine bacterial counts reached a maximum of 8.1 log CFU/mL after day 7 (Figure 4.2a). However, at 15 °C the brine of jar inoculated with allochthonous *L. plantarum* WCFS1 reached the highest count of 7.9 log CFU/mL by day 2 similar to its equivalent jar at 30 °C (Figures 4.2a and b). All the jar brines reached maximum counts (> 8.2 log CFU/mL) by day 4 unlike by day 2 at 30 °C fermentation indicating a slower fermentation in the former (Figures 4.2a and b). The brine counts after day 14 ranged between 7.89 ± 0.10 showing a slight decrease in counts from day 7. Uninoculated control jar brine had the lowest counts on all the days with a maximum of 7.8 log CFU/mL on day 4 and a final count of 7.2 log CFU/mL after day 14 (Figure 4.2b). There were no colonies detected on YMA plates in any of the brines from jars fermented at 15 °C and 30 °C (< 2 log CFU/mL).

Acids and Sugars:

Fresh cucumber juice contained approximately 18.98 ± 3.96 mM of glucose and 23.70 ± 5.19 mM of fructose. The residual glucose in the brine at 30 °C varied between 0 and 2 mM on day 2 (Figure 4.3a). There was slight increment by day 2 but it remained constant throughout the

fermentation (Table 4.1). A similar trend was observed in brines of the jars incubated at 15 °C where the residual glucose was constant throughout the 14 days of fermentation but with a slight increment after day 4 (Figure 4.3b). The final residual glucose present in all the jar brines at both the fermentation temperatures is presented in Tables 4.1 and 4.2. The brine from the jar inoculated with allochthonous starter *L. plantarum* WCFS1 had the highest residual glucose at any given time point at both 30 °C and 15 °C (Figures 4.3a and b). The concentrations of residual glucose in uninoculated control jar brine was in the similar range of starter culture inoculated jars but by the day 14 it reached as low as 0.08 mM.

The residual fructose trend increased until day 2 and decreased after day 7 in most starter culture inoculated jars fermented at 30 °C (Figure 4.4a). Uninoculated control jar brine incubated at 30 °C displayed the highest fructose concentration in the brine until day 7 and gradually reduced to almost 0 by day 14. A wide range of residual fructose was seen by the end of day 14 similar to residual glucose. The brine from uninoculated control jar at 15 °C contained more residual fructose in brine than at 30 °C by the end of day 14 (Tables 4.1 and 4.2). However, at 15 °C the residual fructose increased after day 4 and remained constant throughout the end of fermentation (Figure 4.4b). Residual fructose concentration after day 14 was higher than residual glucose in brine of jars fermented at 15 °C (Figures 4.3b and 4b). However, at 30 °C fermentation both the residual glucose and fructose concentrations in brines after day 14 were approximately between 1 and 3 mM (Figures 4.3a and 4a). All the jars produced lactic acid at a higher rate at 30 °C fermentation than at 15 °C by day 2 (Figures 4.5a and b). In fact at 15 °C a prominent increase in lactic acid concentration in brine was not seen in all the jars until day 4 suggesting a slower fermentation. Both autochthonous and allochthonous inoculated jars fermented at 30 °C, produced lactic acid > 150 mM (Table 4.1). The brine from uninoculated

starter jar at 30 °C measured only 111 mM of lactic acid after day 14 indicating a much slower fermentation rate. The average lactic acid produced by autochthonous and allochthonous in brines of the starter culture inoculated jars incubated at 15 °C was > 40mM. Uninoculated control jar brine incubated at 15 °C produced at least 10 times lower lactic acid (~11 mM) than all the jars (Tables 4.1 and 4.2).

A wide range of acetic acid was detected on day 2 in all the jar brines incubated at 30 °C (Figure 4.6a). However, it plummeted immediately and reached to as low as < 10 mM. At 15 °C, acetic acid levels increased by day 2, however, the concentration gradually decreased similar to the jars incubated at 30 °C fermented jars to < 5 mM after day 14 (Figure 4.6b). Interestingly, acetic acid levels in the brine from uninoculated control jar at 30 °C increased after day 7 to 34.89 mM (Figure 4.6a). Similar to acetic acid a wide range of malic acid levels were detected in the jar brines fermented at 30 °C on day 2, however, the levels remained constant throughout the fermentation (Figure 4.7a). Fresh cucumber juice contained 14.22 ± 3.34 mM of malic acid and thus the equilibrated malic acid was estimated to be > 7 mM. Even though malic acid levels increased slightly by day 2 in all the jars fermented at 15 °C, they remained constant during the remainder of fermentation (Figure 4.7b). The uninoculated control jar brine had the highest malic acid at 30 °C which actually increased by day 2 before rapidly decreasing to almost 0 by the end of day 14. Uninoculated control jar brine's malic acid levels increased until day 5 and reduced to ~ 1 mM by day 14 at 15 °C fermentation (Figures 4.7a and b).

Texture:

Punch tests on pickle slices and visual inspection for bloating were used to characterize the texture of final products. The texture obtained through punch test of pickles at 30 °C and 15 °C did not vary notably between the jars and between the same isolates at different temperatures

(Figure 4.8). At 30 °C, the highest peak force was 13.15 ± 2.6 N measured in the jar inoculated with *L. pentosus* 1.2.11 and the lowest was 10.8 ± 1.9 N in the jar with *L. pentosus* 1.8.6. At 15 °C, the highest peak force was observed in the jar inoculated with isolate *L. plantarum* 3.2.8 with 13.1 ± 2.8 N and the lowest was 11.3 ± 2.0 N in the jar with isolate *L. pentosus* 3.8.24.

Bloating was observed in all the jars at both 30 °C and 15 °C (Figure 4.9). Percentage bloating by each isolate from both the jars was calculated. Bloating in general was less frequently seen at 15 °C fermented jars compared to 30 °C (Table 4.1). All the jars showed moderate to extreme bloating at 30 °C and as high as 97% in the jar inoculated with *L. pentosus* 1.2.13 (Table 4.1). Jar with isolate *L. pentosus* 1.8.6 showed the least bloating of 30% at 30 °C. Moderate bloating was observed in most of the jars incubated at 15 °C. Jars inoculated with the autochthonous isolates *L. pentosus* 1.8.6 and 1.2.13 had the least amount of bloating 30% and uninoculated control had the highest amount of bloating at 83% at 15 °C (Table 4.2).

Fermentation in Tanks:

The pH of brine in all the tanks reduced to < 4.0 by day 3 and remained stable throughout 14 days (Figure 4.1c). The lactobacilli counts varied between 6 and 7 log CFU/mL at the initiation of fermentation (Figure 4.2c). The counts in brine reached a maximum by day 3 in all the tanks with 3.8.24 and reached 8.2 log CFU/mL. However, the numbers started declining from day 5 and by day 14. The pH and lactobacilli count trends are in agreement with previous study (Pérez-Díaz and others 2015). Yeasts and mold counts in brine varied between 1.8 and 3.3 log CFU/mL on day 0 and after that they were not detected throughout the fermentation (data not shown). Similarly aerobic plate counts were detected in all the tanks up to 7 log CFU/mL until day 3 and no counts were detected after that (data not shown). The enterobacteriaceae and clostridial counts were not detected throughout the fermentation (< 2 log CFU/mL).

DISCUSSION

The ambient weather conditions play a key role in fermentation especially in the Midwestern US where the average ambient temperature is usually $< 20\text{ }^{\circ}\text{C}$ limiting the fermentation periods. So, the fermentation process which includes complete utilization of sugars and production of lactic acid must be quick before temperatures drop to freezing levels (Etchells and others 1975; Pérez-Díaz and others 2015). Thus, one of the foremost criteria for cucumber fermentation is rapid production of acid at various temperatures. This is the rationale of current study to understand fermentation patterns at two different temperatures ($30\text{ }^{\circ}\text{C}$ and $15\text{ }^{\circ}\text{C}$). Even though the isolates used in this study were selected initially based on their rapid acid production in sterile cucumber juice medium, the fermentation dynamics is complex in an actual cucumber fermentation jar or tanks (Anekella and others, unpublished). Hence, a careful evaluation of the sugar concentrations, metabolites production and final product quality must be performed to validate their use as starter cultures commercially (Pérez-Díaz and others 2015).

All the starter culture inoculated jars at $30\text{ }^{\circ}\text{C}$ and $15\text{ }^{\circ}\text{C}$ reached terminal pH of at least 3.5 that is required to attain microbial stability throughout the shelf life (Fleming and others 1996; Lu and others 2002). Pre-adaptation of bacteria in the environment to be fermented allows these starter cultures to induce and adapt to specific metabolic pathways that otherwise may not be activated (Di Cagno and others 2013). Hence, these starter cultures were grown in pH adjusted pickle jars prior to their inoculation. This method of propagation also allows pickle industries to adapt the process easily because it is technologically feasible with minimal personnel training and adheres to Kosher guidelines (Pérez-Díaz and McFeeters 2011). The size of cucumbers has an impact on fermentation because larger fruits have greater amount of sugars available for fermentation (Lu and others 2002). Cucumbers naturally contain ~2% fermentable

sugars and bigger cucumbers have higher amount of sugars (Etchells and others 1975; Fleming and others 1973b; Lu and others 2002; Miller 1989). Glucose and fructose are the primary sugars available for fermentation at a concentration of 58 mM and 63 mM of the whole cucumbers, respectively (Lu and others 2001). In mesocarp and endocarp glucose and fructose are 8.6 and 10.3 mg per 100 g of fresh cucumber respectively (Handley and others 1983). However, we detected approximately 18.99 ± 3.96 mM and 23.70 ± 5.19 mM of glucose and fructose, respectively. The sugar concentrations can vary depending on the processing as discussed previously (Miller 1989). Amount of initial sugar has a role in the completion of primary fermentation (Lu and others 2001). Higher initial sugar concentration may lead to higher residual glucose by the end of fermentation if they are not utilized completely which promotes secondary fermentation leading to spoilage (Lu and others 2002).

Lu and others determined the effect of cucumber matrix on fermentation and found that the lactic acid concentration towards the end of process was similar for any particular cucumber size regardless of the fermentation being performed in juice, blanched or unblanched whole cucumbers (Lu and others 2002). The residual sugars measured in this study are most likely the left over sugars after fermentation indicating a rapid utilization of sugars from day 0 (Figures 4.3 and 4.4). The residual sugar concentration was <10 mM in CaCl_2 jar fermentation compared to NaCl fermentation (McFeeters and Pérez-Díaz 2010). Diffusion of sugars begun right from day 0 due to acid production and perhaps determining the sugar contents in cucumbers along with brine may give more insights into the actual changes during the process.

Glucose and fructose are utilized at a different rate and to different extents depending on the starter strain and sugar availability. When glucose is present in enough quantities, fructose is used as an electron transporter while glucose is used for production of energy. Fructose

fermentation yields lactate, mannitol and ATP using phosphoketolase (PK) pathway. Even though glucose is the preferred fermentative sugar in cucumber fermentation, there was residual glucose left by the end of primary fermentation which was seen in this study too (Lu and others 2001; Franco and Pérez-Díaz 2013). The optimal activity of enzymes involved in glucose metabolism is at a higher pH than the fructose metabolism (Lu and others 2001). Thus, glucose metabolism is inhibited as lactic acid is accumulated during the progression of fermentation due to decrease in pH which explains the residual glucose leftover in this study. It is also possible that fructose-1-phosphate (metabolic product of fructose) may compete for ATP with fructose-6-phosphate (metabolic product of glucose) and thus favor fructose metabolism at low pH. Lactic acid is excreted via facilitated diffusion at the end of metabolism (Axelsson 2004). Most sugars are utilized during primary fermentation by lactobacilli and any residual sugars left out after the decline of predominating microbiota (*L. plantarum*/*L. pentosus*) triggers secondary fermentation by spoilage microbes such as *L. buchneri* and yeasts such as *Pichia manshurica* and *Issatchenkia occidentalis* contributing to off flavors and more bloating (Franco and Pérez-Díaz 2013; Franco and others 2012). Complete sugar utilization and thus lower residual sugars by the end of fermentation is desirable to prevent the growth of spoilage microorganisms (Franco and Pérez-Díaz 2013).

The fermentation of cucumbers occurs inside and outside the fruit. Lactic acid is produced from the sugar fermentation by natural microbiota and also from decarboxylation of malic acid by lactobacilli predominantly (Lu and others 2001; McFeeters and others 1984). In the current study, lactic acid was still being produced by the end of day 14 in the jars indicating an active microbial metabolism by lactobacilli (Figure 4.5a and 5b). The fermentation patterns in regard to chemical changes (pH, sugars and acids) and texture are similar to previous studies

reported where an increase in lactic acid is seen even without NaCl (McFeeters and Pérez-Díaz 2010; Pérez-Díaz and others 2015). Fermentation is usually completed in 14-21 days depending on cucumber size, microbial load and brine composition (Etchells and others 1975; McFeeters and Pérez-Díaz 2010; Pérez-Díaz and McFeeters 2011; Pérez-Díaz and others 2015). However, in CaCl₂ fermentation with starter cultures the process was more rapid and complete by 7 days determined by a rapid decrease in sugar (Pérez-Díaz and others 2015). We observed a similar rate of sugar decrement and lactic acid production in all the jars with starter cultures. In fact, some brine samples had greater reduction in sugars than others and a corresponding higher lactic acid production (Figures 4.3, 4.4 and 4.5).

Similar to a previous study, lactic acid production was greater than 40 mM by day 1 at 30 °C fermentation when isolate *L. pentosus* LA 0445 (named *L. plantarum* at the time of study) was used in NaCl free fermentation studies (McFeeters and Pérez-Díaz 2010; Breidt and Fleming 1992; Passos and others 1994; Pérez-Díaz and others 2015). This strain has been used as an acid resistant starter in various cucumber fermentation studies (Pérez-Díaz and McFeeters 2011; Pérez-Díaz and others 2015). The final lactic acid concentration was approximately 130 mM in CaCl₂ fermentation while the current experiment measured approximately 173.03 ± 6.12 mM (Table 4.1) (McFeeters and Pérez-Díaz 2010). In addition, this isolate *L. pentosus* LA 0445 decreased the pH of brine to 4.2 by day 1 in NaCl free jar fermentation as reported previously (Figure 4.1a) (McFeeters and Pérez-Díaz 2010). The same isolate reduced the pH to only 5.1 by day 3 in a previous tank fermentation study with CaCl₂ brine but < 3.6 in this study (Pérez-Díaz and others 2015). Even after initiation, the pH reduced to only 3.7 by day 14 which is slightly higher than the expected pH of 3.5 in pickles for microbial stability (Fleming and others 1996; Lu and others 2002). This variability in fermentation efficiency is likely due to slower diffusion

process which affects sugar equilibration between the brine and cucumber. Also, the brine composition is different compared to previous studies where acetic acid was not used in this study which may have otherwise served as a buffer for enhanced sugar utilization (McFeeters and Pérez-Díaz 2010; Etchells and others 1975). The fermentation was most likely not complete by day 14 and lactic acid concentration may increase further in all the starter culture inoculated jars (Figures 4.5a and b). Brine from uninoculated control jar incubated at 15 °C showed no reduction in pH until day 6 indicating a longer adaptation of native microbiota to such low temperature to initiate fermentation.

In the current study there was acetic acid present in all the brine from jars fermented at 30 °C and showed a slight increase after day 7 (Figure 4.6a). Uninoculated jar's brine showed a rapid increase indicating growth of heterofermentative bacteria. However, at 15 °C acetic acid levels remained constant in all the jars including the uninoculated control indicating that these starter cultures did not switch to heterofermentation and also there is little to no activity of other heterofermentative bacteria (Figure 4.6b). Under glucose limiting conditions, growth rate of *L. plantarum* is similar at both aerobic and anaerobic conditions; however, the cell yields are higher under aerobic condition. But under high glucose and aerobic condition growth rate may be reduced due to accumulation of reactive oxygen species and it can also increase acetate kinase activity producing more acetate (Tseng and Montville 1993). Pentoses can also be metabolized through PK pathway by both homo and heterofermentors but there is no CO₂ formed and instead substrate level phosphorylation occurs with the formation of equimolar lactate and acetate and ATP (Axelsson 2004). Facultative heterofermentors such as *L. plantarum* and *L. pentosus* have inducible PK enzyme that is used to ferment pentose sugars (Tseng and Montville 1993). Acetate kinase has an optimal activity at a higher pH than other enzymes and thus there is an increased

acetate production in PK/pentose phosphate pathway in low acid conditions. These strategies help LAB to produce more ATP and also maintain acid homeostasis at low pH (Tseng and Montville 1993). Acetic acid is produced by *L. plantarum* group during late stationary phase since more ATP is generated when pyruvate is converted to acetyl phosphate and then acetic rather than lactate (Quatravaux and others 2006). Acetic acid formation also helps the cell to maintain pH homeostasis and survival (Guidone and others 2013; Quatravaux and others 2006). Acetate formation from pyruvate is catalyzed by pyruvate oxidase and acetate kinase and it happens only under aerobic and glucose depriving conditions (Guidone and others 2013; Quatravaux and others 2006). Acetic acid was seen in previous studies with whole cucumbers where lactobacilli switched to heterofermentation under glucose depleting conditions (Lu and others 2002). Hence, it is beneficial to have an oxygen insensitive enzyme machinery of acetate synthesis for more ATP generation (Tseng and Montville 1993). The inducible heterofermentative pathway is important with respect to formation of metabolites such as acetate as well as CO₂ that causes bloating (discussed below).

The CO₂ produced inside the fruit causes hollow spaces called bloaters during fermentation (Lu and others 2001; McFeeters and others 1984). The bloater damage was classified as slight, moderate or extreme (Etchells and others 1974). During CO₂ accumulation in cucumber, water is released from the cucumber tissues which cause a raise in the volume of the brine (Fleming and others 1973a). The solubility of CO₂ in brine is reduced at higher solute concentration and temperatures and thus bloating is seen inside the fruit (Fleming and others 1973a). Bloater damage is a more serious issue in larger cucumbers and it is seen even during primary fermentation. It can be minimized through air purging but it would be ideal to use a starter culture that does not enhance bloating to avoid costs involved in air purging (Fleming

1979). Heterofermenting LAB such as *L. brevis* and *L. buchneri* can also contribute to bloating during fermentation (Etchells and others 1968; Etchells and others 1975). During nutrient depletion in stationary phase, homofermentative lactobacilli switch to heterofermentation which produces acetate and/or ethanol and CO₂ (Thomas and others 1979). Under low glucose conditions, inorganic phosphate (P_i) and 3-phosphoglycerate are increased in concentration while fructose diphosphate decreased. Inorganic phosphate is a potent inhibitor of Ldh and thus these LAB switch to heterofermentation to produce other products such as acetic acid, butyric acid and CO₂. Due to the change in concentration of these products, inhibition of pyruvate kinase is seen which activates the alternative heterofermentative pathway (Thomas and others 1979). Pyruvate formate lyase is involved in the production of ATP under such glucose deprived condition which cleaves pyruvate to formate-acetate and ethanol switching from homo to heterolactic fermentation. It was also demonstrated that these strains switched back to homofermentation immediately when supplied with excess glucose in the chemostat (Borch and others 1991; Thomas and others 1979). Bloating seen in most of the jars in this study may be attributed partly to the starter culture that may have switched to heterofermentation during glucose depletion. However, a corresponding increase in acetic acid levels was not observed in any starter culture inoculated jars at 15 °C except the uninoculated control (Figures 4.6a and b).

Malic acid decarboxylation (MDC) by lactobacilli also yields lactic acid and CO₂ which contribute towards bloating. Even in a non-gas forming *L. plantarum*, the inducible malic acid decarboxylase enzyme can metabolize malic acid to lactate and CO₂ when the cucumbers have sufficient amounts of malic acid (McFeeters and others 1984). Fresh cucumber juice had approximately 14.22 ± 3.39 mM of malic acid which after equilibration reduced rapidly at both 30 °C and 15 °C fermented jars (Figures 4.7a and b). Malic acid in brine was less than 2 mM

regardless of the fermentation performed with or without salt whereas the malic acid inside cucumbers varied between 8 and 13 mM (McFeeters and Pérez-Díaz 2010). Increase in CO₂ was parallel to the decrease in malic acid which confirms that the malic acid decarboxylation by homofermentors was responsible for CO₂ production in cucumber juice fermentation along with heterofermenting bacteria (McFeeters and others 1984). This mechanism is most likely responsible for bloating observed here too. Uninoculated control jar showed this pattern where the decrease in malic acid corresponded to a high bloating % (Figures 4.7a and b). Malic acid contents of all the starter culture inoculated jars remained almost constant throughout the fermentation after it was equilibrated between brine and cucumbers to 1-2 mM at 30 °C after 5 days (McFeeters and Pérez-Díaz 2010). Also, sugar was completely utilized in cucumber juice medium in the presence of malic acid due to the buffering capacity of malic acid. Malolactic and heterofermentation pathways are usually activated during the sugar depletion by homofermenting LAB which may have occurred in this study especially at 30 °C incubated jars (Thomas and others 1979). In addition, MDC⁺ cultures increase the pH by producing lactic acid whereas the MDC⁻ cannot decarboxylate malic acid and thus maintains the external medium more acidic. Lactic acid produced during malolactic fermentation acts as a buffer and thus the drop in final pH is lower compared to fermentation without malic acid or lower malic acid concentration. The pH linearly increased with an increase in lactic acid concentration and a decrease in residual malic acid concentration. This suggests that the lactate formed during the malic acid fermentations acts as a buffer and thus the sugar utilization is complete under a higher malic acid concentration (120 μM/mL) (McFeeters and others 1982). The pH of the cucumber juice medium dropped to 4.1 at 135 mM concentration of malic acid whereas it dropped to 2.1 in 13 mM of malic acid which remained unknown (McFeeters and others 1982). Studies also show that

bigger cucumbers have lower malic acid content which is seen in our current study (Lu and others 2002).

All the starter cultures used in this study have an ability to decarboxylate malic acid and produce lactic acid and CO₂ (Anekella and Perez-Diaz, unpublished). The % bloating in these jars resonates with the malic acid utilization because bloating as high as 94% was recorded in the uninoculated control jar which had a greater amount of equilibrated malic acid during the initial phase of fermentation than other jars. However, other autochthonous *L. pentosus* isolates also had a high bloating rate as compared to control even at lower malic acid levels (Figures 4.6a and b). Even though it may be logical to assume that bloating in those cucumbers could be from heterofermentation which produces acetic acid and CO₂, the corresponding acetic acid levels were low in par with other inoculated jars especially at 15 °C (Figures 4.5a and b). So, it is possible that CO₂ from malic acid decarboxylation in combination with natural respiration of fruits contributed towards bloating in these jars. An MDC⁻ strain *L. plantarum* MOP3-M6 was developed for starter culture purposes to minimize bloating (Daeschel and others 1984). This strain dominated the fermentation performed with heat treated cucumbers and salt (4%) leaving a high residual malic acid towards the end of process (Breidt and Fleming 1992). There was no significant difference seen in the sugar metabolism of malolactic fermenting strain and its mutant. This is important because malolactic fermenting strain contribute to increased lactic acid during the fermentation (Lu and others 2001; McFeeters and others 1984). However, an increased concentration of acetic acid was observed indicating that the strain may have undergone heterofermentation (Lu and others 2002). An initial heat treatment was suggested in commercial plants to ensure elimination of naturally occurring undesirable microbiota. Blanched cucumbers exhibit less malic acid utilization than unblanched due to reduction of natural

microbiota of cucumbers by the heat treatment (Lu and others 2002). In addition, softening enzymes from microorganisms on smaller fruits are easily inactivated by blanching than the larger fruits and thus firmer texture is seen in the smaller fruits (Lu and others 2002). However, this is not practical in the current commercial scale scenario with tanks of 8000-10,000 gallon capacity (Breidt and others 2013; Pérez-Díaz and others 2015).

A precise correlation between pH, lactobacilli counts, residual sugars and lactic acid production at lab fermentations (both at 15 °C and 30 °C) and industry tank could not be obtained in this study; for example, isolate *L. plantarum* 3.2.8 rapidly reduced the pH at 15 °C incubation and correspondingly had higher lactobacilli counts in jar fermentations but not at 30 °C and industrial tank fermentation. It was expected to produce higher lactic acid concentrations and more complete sugar utilization. On the contrary, a higher concentration of residual sugars and lower lactic acid levels were observed at both the temperatures in brine with *L. plantarum* 3.2.8 compared to the other starter culture inoculated jars. This indicates that the fermentation ability is dependent at a strain level and can vary drastically depending on the fermentation conditions. However, uninoculated control jars at 30 °C and 15 °C consistently had a slower pH reduction, higher residual sugars, lower lactic acid levels and lactobacilli counts, higher acetic acid production, and higher incidences of bloating indicating a slower and undesirable fermentation compared to all the starter culture inoculated jars. To our knowledge this is the first time in literature where cucumber fermentation was attempted at a controlled lower temperature of 15 °C. The fermentation in jars was still in progress even by the end of day 14 incubated at 15 °C. This was expected because lactobacilli are mesophilic organisms with an optimal temperature for growth between 25 and 30 °C.

The average firmness of 1.1% CaCl₂ brine pickled cucumbers was found to be approximately 9.4 N after 9 months of storage and had an acceptable texture (Rosenberg 2013). Commercial scale CaCl₂ fermentation with starter cultures showed a lower firmness than the traditional NaCl fermented pickles. These differences may be attributed to a bulk and long storage life (Pérez-Díaz and others 2015). Even though there were perceivable differences in texture of pickles in CaCl₂ vs. NaCl fermentation, there was no consumer preference of one over the other (Pérez-Díaz and others 2015). Punch test on pickle slices revealed that even within the same isolates at different incubation temperatures there was no consistent peak force pattern observed. It should also be noted that the standard deviation was high in all the jars due to the large number of samples from each jar in duplicates (Figure 4.8). Pickles obtained by fermentation at various concentration of CaCl₂ showed that the texture was firm even during long term storage (McFeeters and Pérez-Díaz 2010). The peak force of the pickle slices in this current sodium free fermentation is higher than previous studies and anticipated to follow similar trend during storage. Thickness of skin and tissue texture plays a major role in determining the firmness and crispiness of pickles. The decrease in instrumental firmness was not observed until two months after bulk storage (Rosenberg 2013).

An interesting visual observation was made in starter culture inoculated jars at 30 °C where the brine increased in thickness that was not seen in the uninoculated control jar. The starter cultures used in this study were documented for their production of exopolysaccharides (EPS) which may potentially increase the thickness of brine by the end of 14 days (Anekella and Perez-Diaz, unpublished). In addition, white spots are occasionally seen on final pickle products which were assumed to be yeast cells (communication with local pickle processors; Appendix Figure A.4.2). However, in this current study we noticed that these spots were present only on

the pickles from jars with thicker brine by the end of day 14. This suggests that the spots might be cell masses adhered to cucumber surface through production of EPS. Evidently, the jar inoculated with isolate *L. plantarum* 3.2.8 had higher brine viscosity and larger white spots on pickles than all other jars by the end of fermentation. This isolate routinely displayed a sticky appearance during its growth on MRS plates and it was documented for presence of EPS biosynthesis genes and phenotypic production of EPS on various nutrient media including cucumber based medium (Anekella and Perez-Diaz, unpublished).

Fermentation ecology is very complex and interconnected variables such as cucumber harvest season, fruit surface area, fermentation conditions (pH and temperature), prevalence and diversity of natural microbiota on cucumbers, acidification, buffering capacity and presence of salt are the major factors in determining the fermentation of capabilities of these potential starter bacteria (Etchells and others 1975; Di Cagno and others 2008; Buckenhüskes 1993). In conclusion, a finer picture of fermentation capabilities, metabolite production and pickle quality in CaCl₂ brine at two controlled laboratory scale fermentations and industrial scale were evaluated this study. There were differences in trends such as residual glucose and fructose contents between autochthonous and allochthonous inoculated starter jars. Overall, starter culture inoculated jar performed superior to uninoculated control jar at both the temperatures tested here. However, a thorough understanding on sugar utilization and acids production at an industrial scale must be performed prior to implementing these starter cultures. Depending on the priorities of the manufacturing process such as complete sugar utilization, rapid acidification, higher lactic acid production and fermentation at lower temperatures, manufacturer could choose from various isolates in this study. Future studies should focus on understanding the change in microbiota during fermentation to better assess the metabolic changes.

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Table 4.1: End point analysis of cucumber fermentation in 1 gal jars incubated at 30 °C after day 14

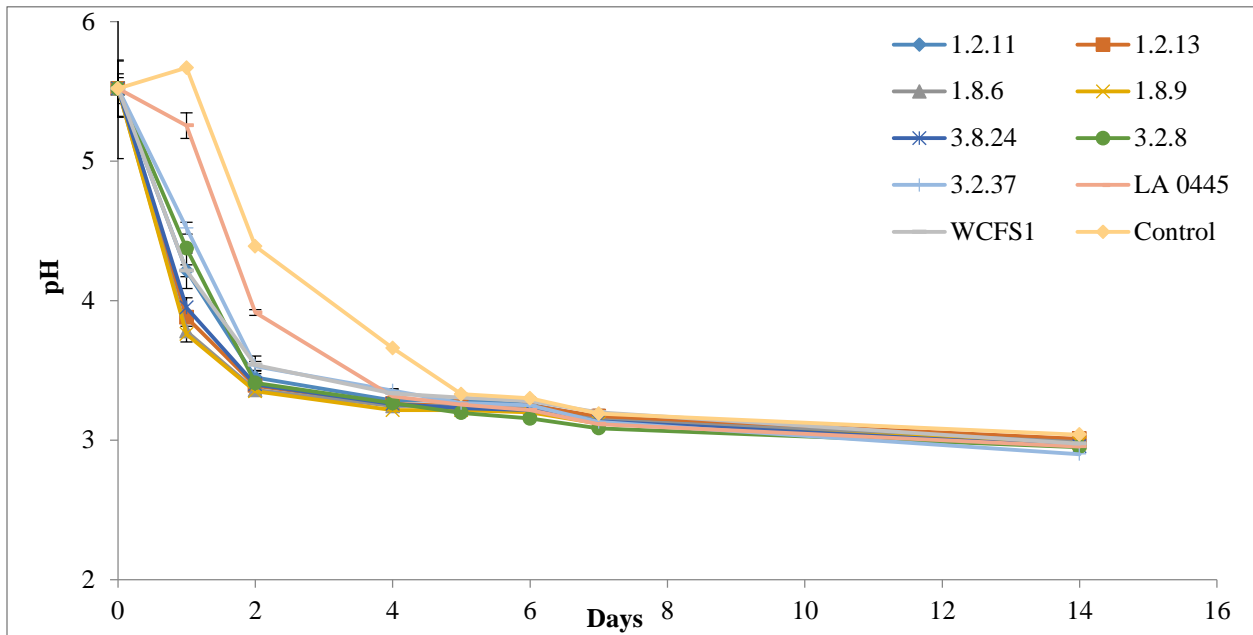
Fermentation Type	Residual Glucose (mM)	Residual Fructose (mM)	Lactic Acid (mM)	Malic Acid (mM)	pH	Lactic Acid Bacteria (Log CFU/mL)	Bloating
Autochthonous:							
<i>L. plantarum</i> 3.2.8	2.54 ± 0.18	2.22 ± 0.12	130.19 ± 7.94	1.15 ± 1.15	2.95 ± 0.01	5.83 ± 0.32	71%
<i>L. pentosus</i> 1.2.11	0.46 ± 0.04	1.01 ± 0.68	147.93 ± 11.99	2.76 ± 0.58	2.99 ± 0.05	6.28 ± 0.19	28%
<i>L. pentosus</i> 1.2.13	0.44 ± 0.01	2.02 ± 1.08	150.64 ± 2.95	1.36 ± 0.09	3.01 ± 0.01	6.65 ± 0.03	97%
<i>L. pentosus</i> 1.8.6	0.5 ± 0.02	0.54 ± 0.43	174.31 ± 12.10	1.36 ± 0.26	2.99 ± 0.04	6.64 ± 0.08	30%
<i>L. pentosus</i> 1.8.9	0.53 ± 0.06	0.74 ± 0.00	175.36 ± 17.18	1.26 ± 0.15	2.98 ± 0.00	6.52 ± 0.08	74%
<i>L. pentosus</i> 3.8.24	0.58 ± 0.01	1.36 ± 0.02	174.68 ± 5.50	1.26 ± 0.63	2.96 ± 0.02	6.63 ± 0.21	92%
<i>L. pentosus</i> 3.2.37	0.56 ± 0.02	1.46 ± 0.63	168.25 ± 3.04	0.87 ± 0.23	2.90 ± 0.01	6.41 ± 0.16	94%
<i>L. pentosus</i> LA 0445	2.4 ± 0.38	2.70 ± 0.43	173.03 ± 6.12	2.03 ± 1.54	2.96 ± 0.04	7.10 ± 0.04	79%
Average:	1.00 ± 0.91	1.51 ± 0.75	159.86 ± 17.22	1.51 ± 0.60	2.97 ± 0.03	6.51 ± 0.36	71 ± 27%
Allochthonous:							
<i>L. plantarum</i> WCFS1	1.71 ± 0.44	2.22 ± 0.48	157.89 ± 5.42	1.29 ± 1.10	2.98 ± 0.02	7.33 ± 0.09	68%
Wild:							
Uninoculated control	0.08	0.45	111	0.23	3.04	6.64	94%

Table 4.2: End point analysis of cucumber fermentation in 1 gal jars incubated at 15 °C after day 14

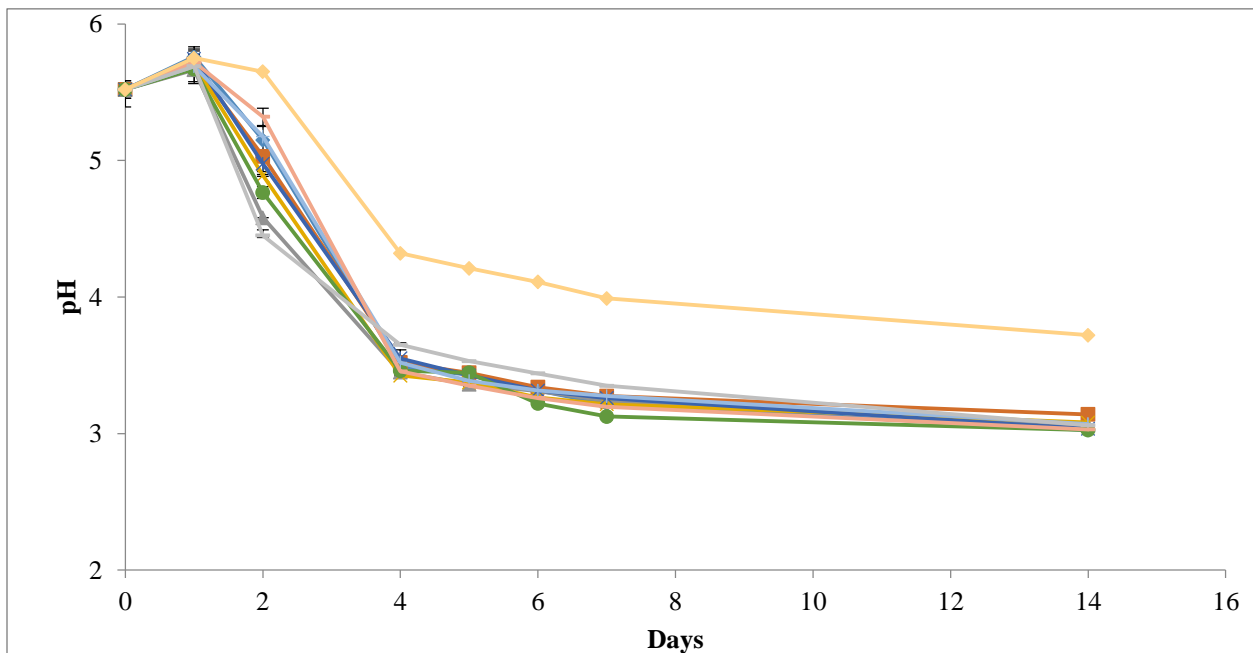
Fermentation Type	Residual Glucose (mM)	Residual Fructose (mM)	Lactic Acid (mM)	Malic Acid (mM)	pH	Lactic Acid Bacteria (Log CFU/mL)	Bloating
Autochthonous:							
<i>L. plantarum</i> 3.2.8	2.31 ± 0.36	2.19 ± 0.29	75.61 ± 1.23	0.49 ± 0.02	3.03 ± 0.01	7.85 ± 0.08	64%
<i>L. pentosus</i> 1.2.11	2.35 ± 0.27	6.15 ± 0.50	62.86 ± 1.09	1.35 ± 0.06	3.03 ± 0.02	8.12 ± 0.56	67%
<i>L. pentosus</i> 1.2.13	1.51 ± 1.44	4.38 ± 0.90	63.74 ± 6.93	1.09 ± 0.09	3.14 ± 0.03	7.84 ± 0.03	29%
<i>L. pentosus</i> 1.8.6	0.53 ± 0.09	4.60 ± 1.05	71.63 ± 9.85	1.16 ± 0.07	3.06 ± 0.01	7.83 ± 0.07	31%
<i>L. pentosus</i> 1.8.9	0.35 ± 0.07	1.73 ± 0.29	55.74 ± 0.87	0.55 ± 0.19	3.08 ± 0.03	7.87 ± 0.03	44%
<i>L. pentosus</i> 3.8.24	1.35 ± 1.17	5.22 ± 0.90	70.55 ± 1.91	1.27 ± 0.01	3.04 ± 0.05	7.85 ± 0.03	20%
<i>L. pentosus</i> 3.2.37	0.51 ± 0.03	3.59 ± 0.25	64.26 ± 1.27	1.11 ± 0.00	3.07 ± 0.01	7.8 ± 0.07	39%
<i>L. pentosus</i> LA 0445	2.71 ± 0.03	3.51 ± 0.84	85.21 ± 6.47	0.65 ± 0.28	3.03 ± 0.00	7.96 ± 0.07	64%
Average:	1.45 ± 0.93	3.92 ± 1.48	68.70 ± 9.09	0.96 ± 0.34	3.06 ± 0.04	7.89 ± 0.10	45 ± 18%
Allochthonous:							
<i>L. plantarum</i> WCFS1	2.72 ± 0.10	4.24 ± 0.76	40.14 ± 23.57	1.25 ± 0.05	3.06 ± 0.00	7.89 ± 0.04	83%
Wild:							
Uninoculated Control	1.07	3.06	11.11	0.94	3.72	7.2	69%

Figure 4.1: Changes in pH of brine in jars during fermentation at a) 30 °C and b) 15 °C and c) industrial tanks

(a)



(b)



(c)

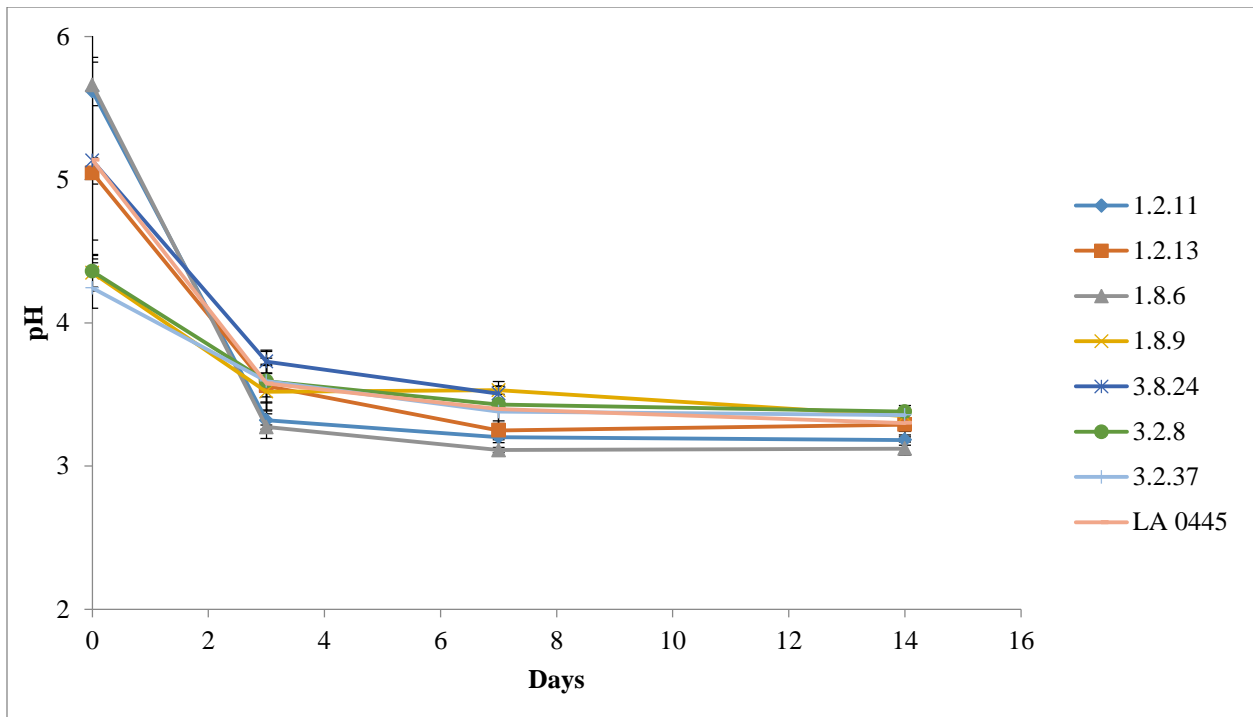
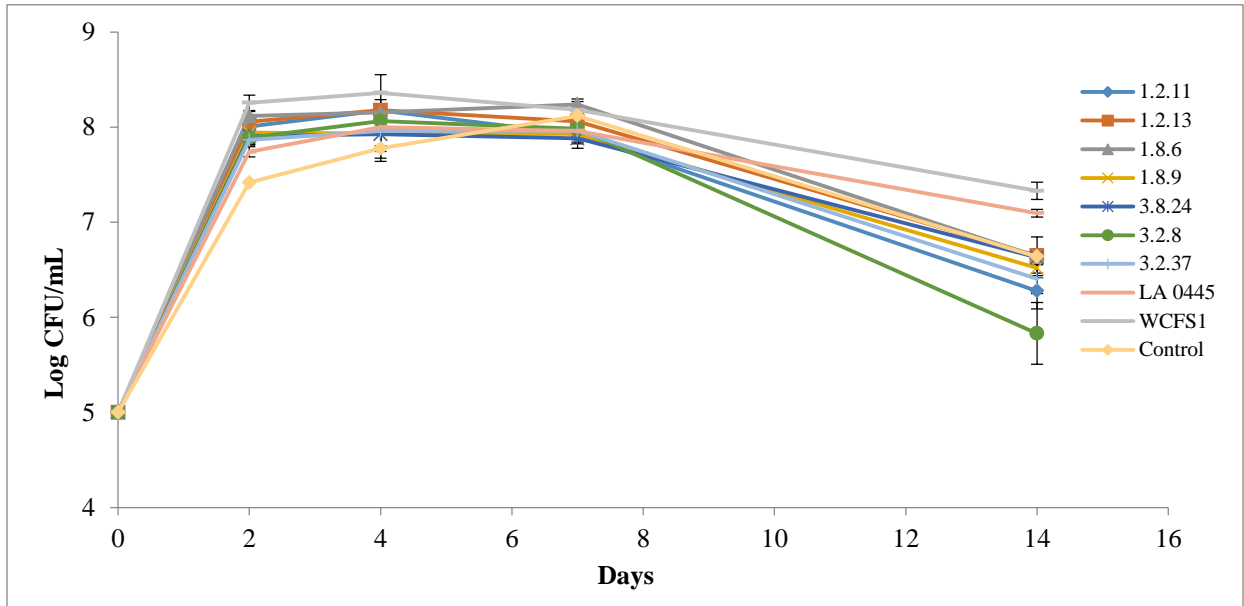
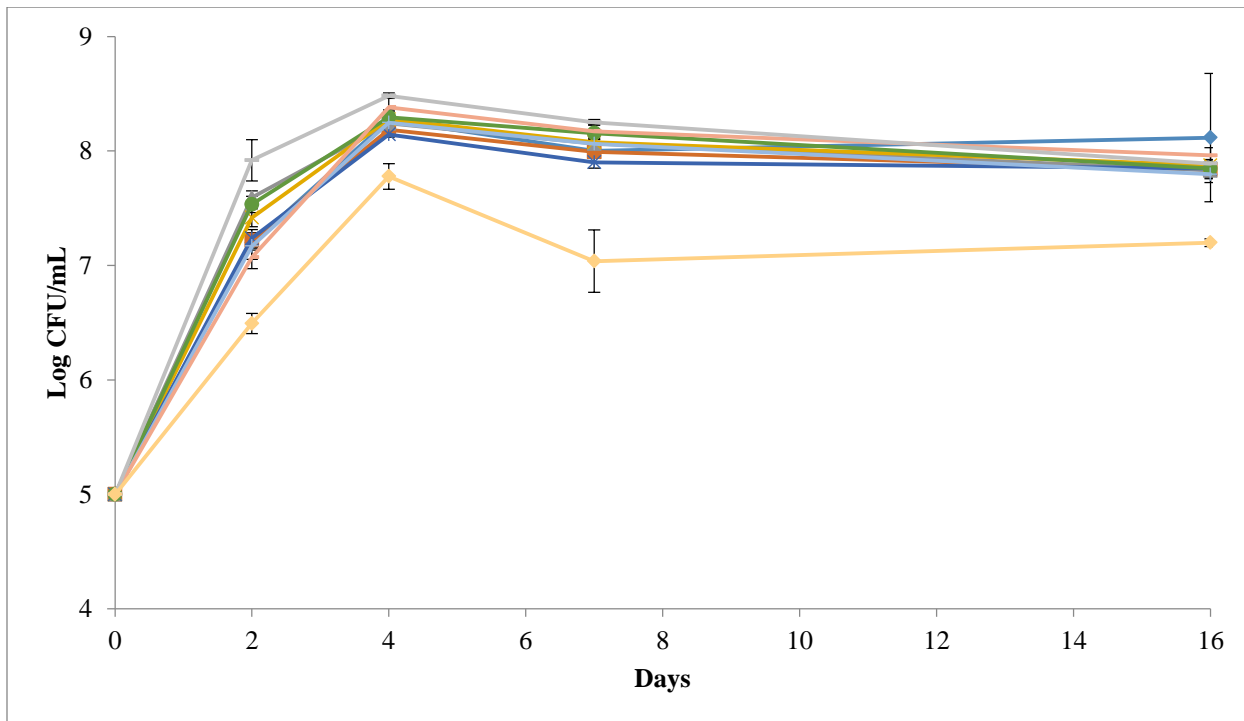


Figure 4.2: Changes in lactobacilli counts (log CFU/mL) in brine during fermentation at a) 30 °C and b) 15 °C and c) industrial tanks

(a)



(b)



(c)

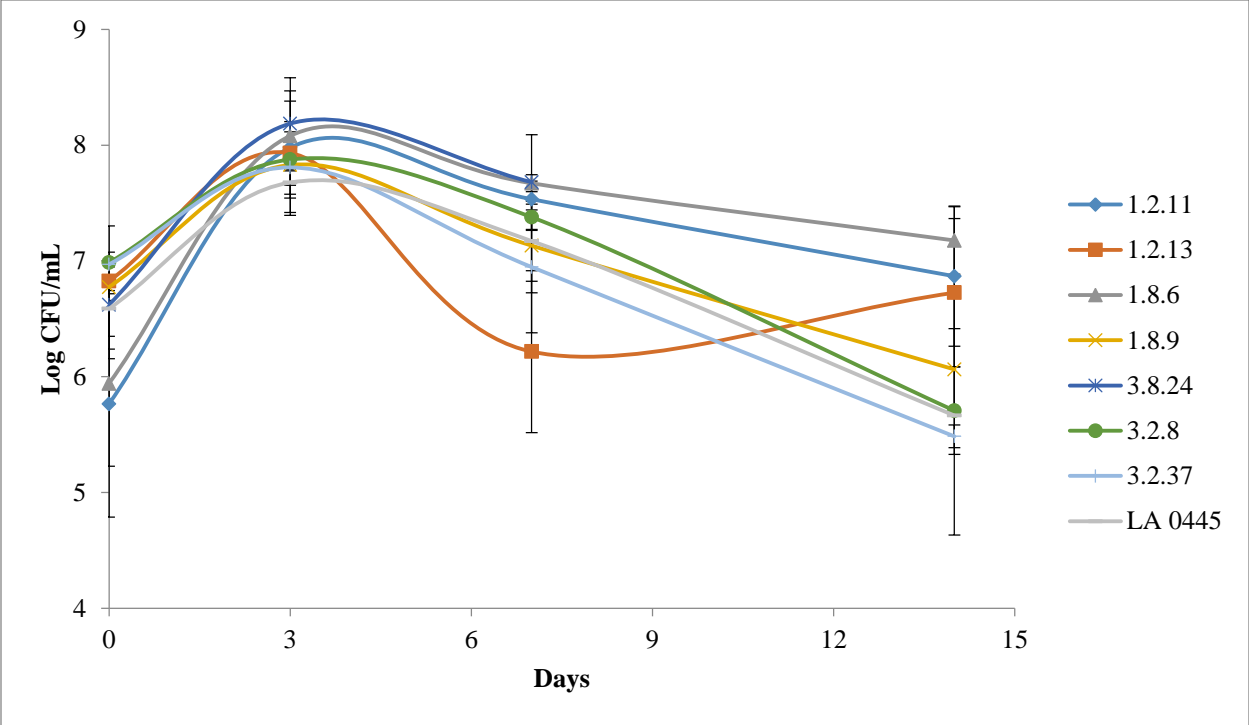
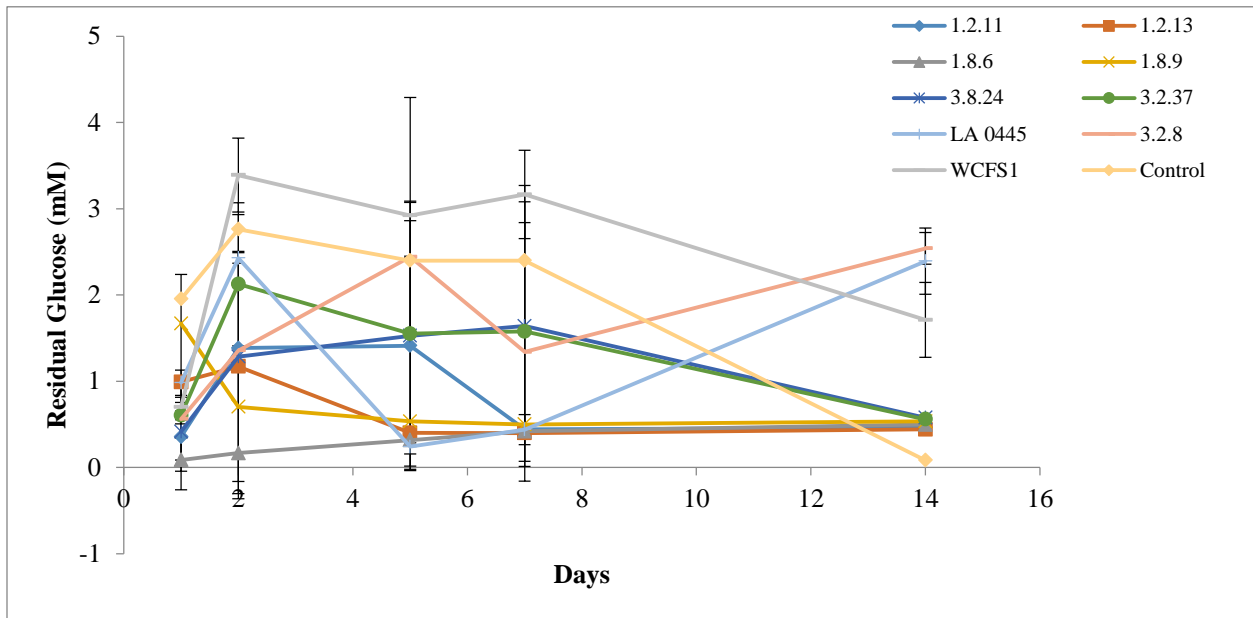


Figure 4.3: Changes in residual glucose in brine during fermentation at a) 30 °C and b) 15 °C

(a)



(b)

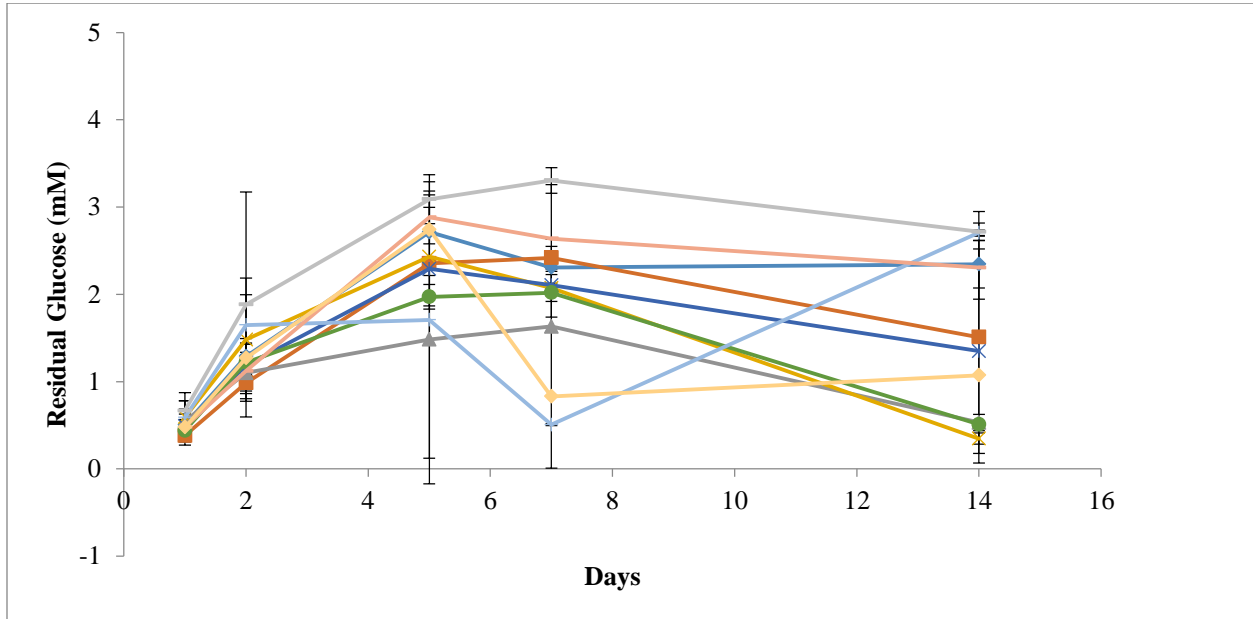
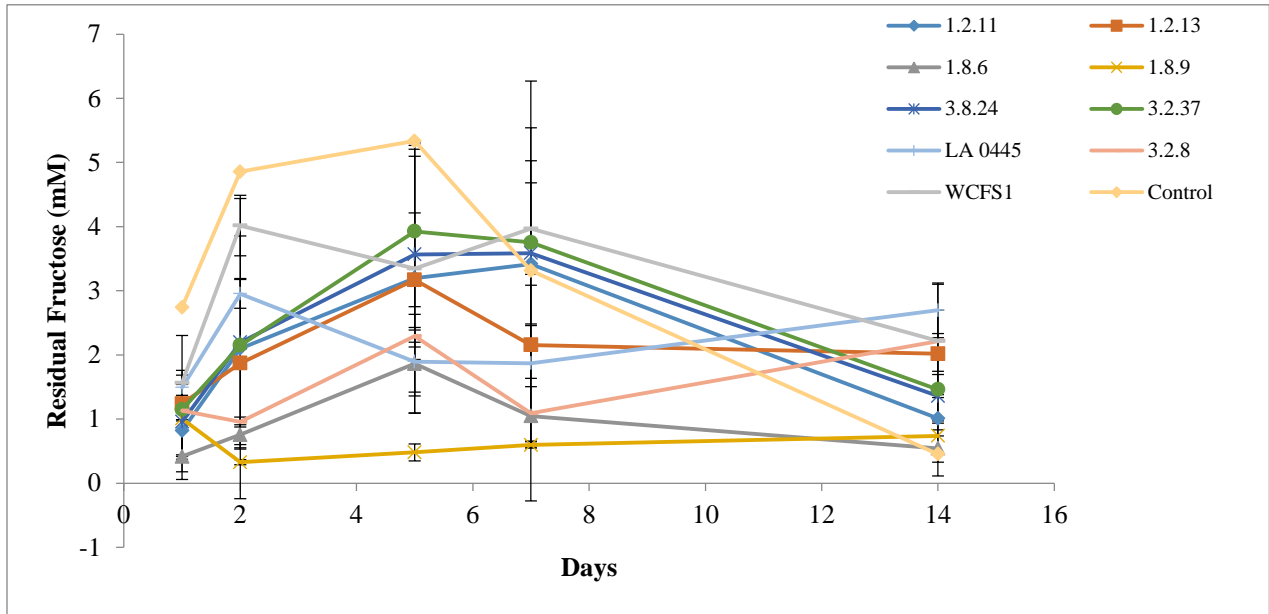


Figure 4.4: Changes in residual fructose in brine during fermentation at a) 30 °C and b) 15 °C

(a)



(b)

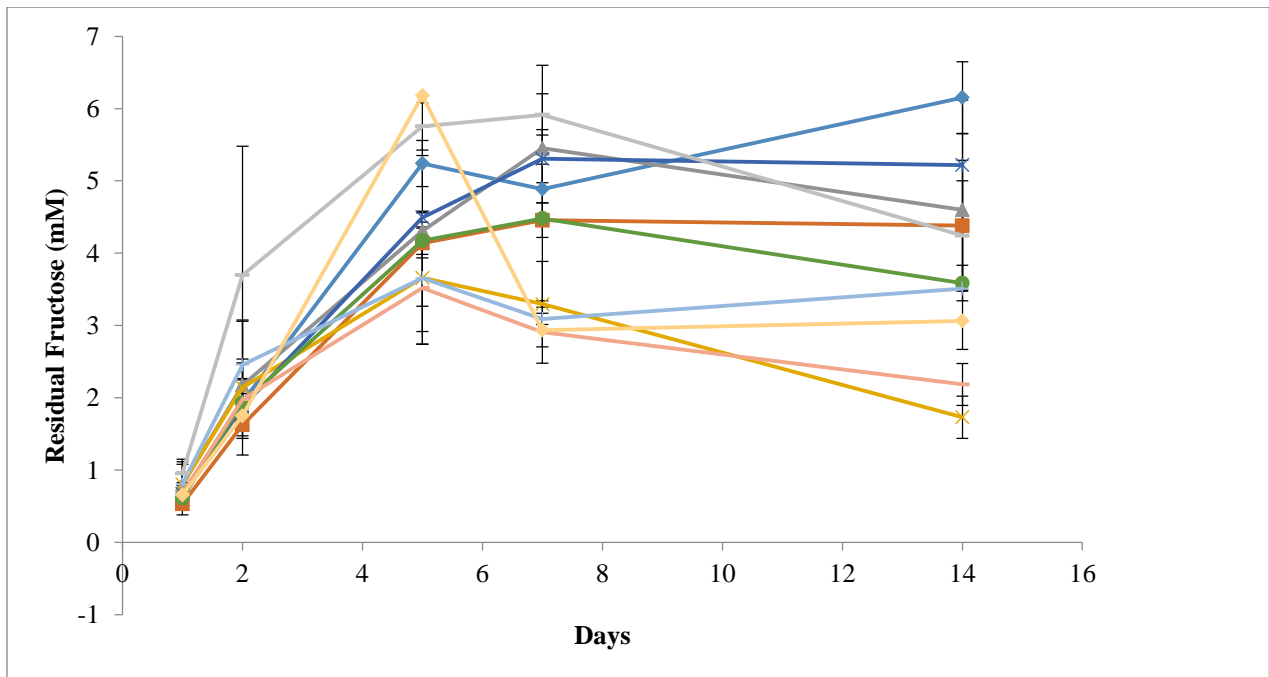
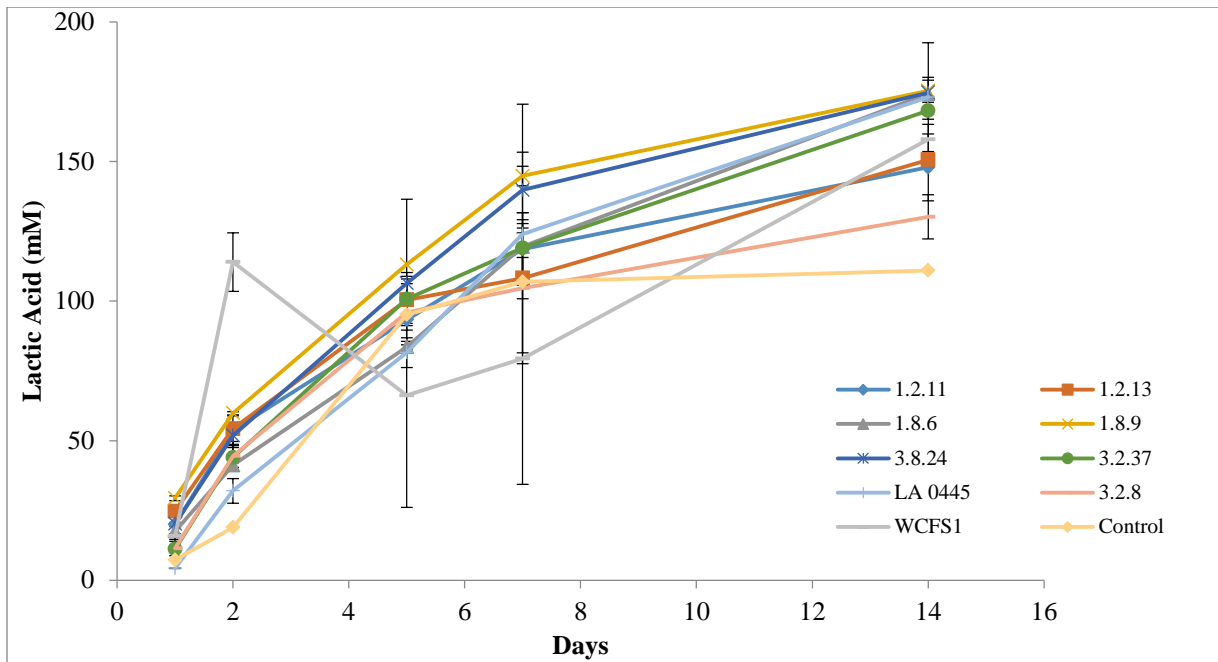


Figure 4.5: Changes in lactic acid in brine during fermentation at a) 30 °C and b) 15 °C

(a)



(b)

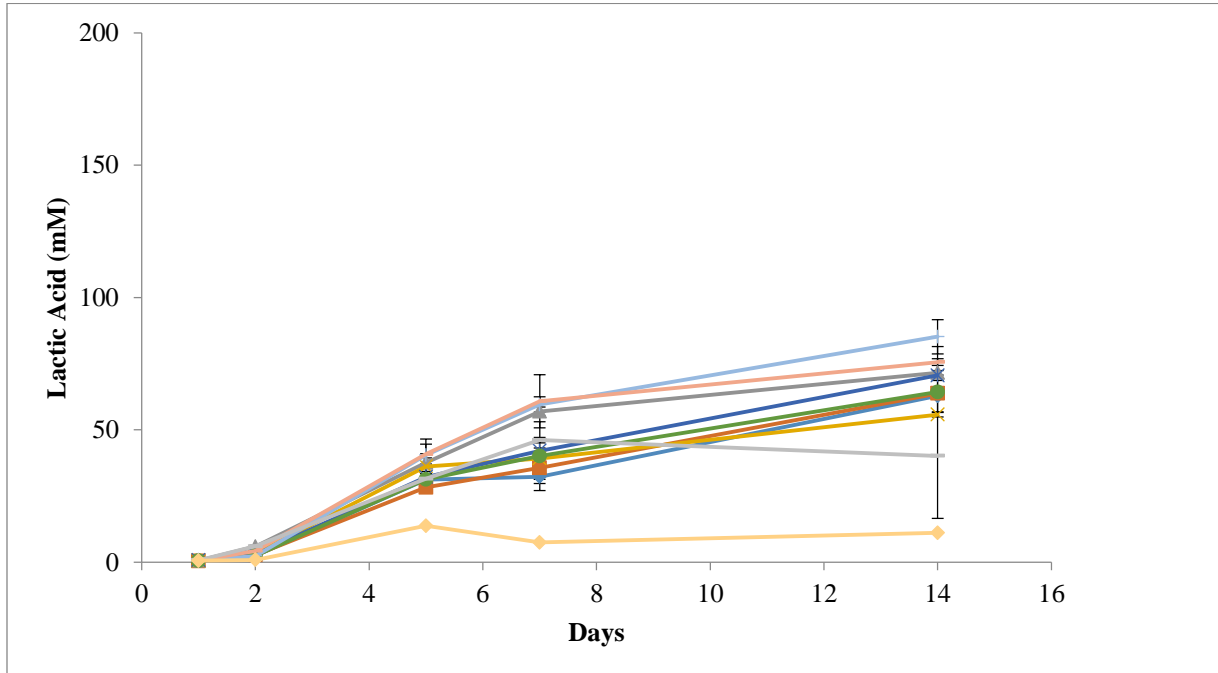
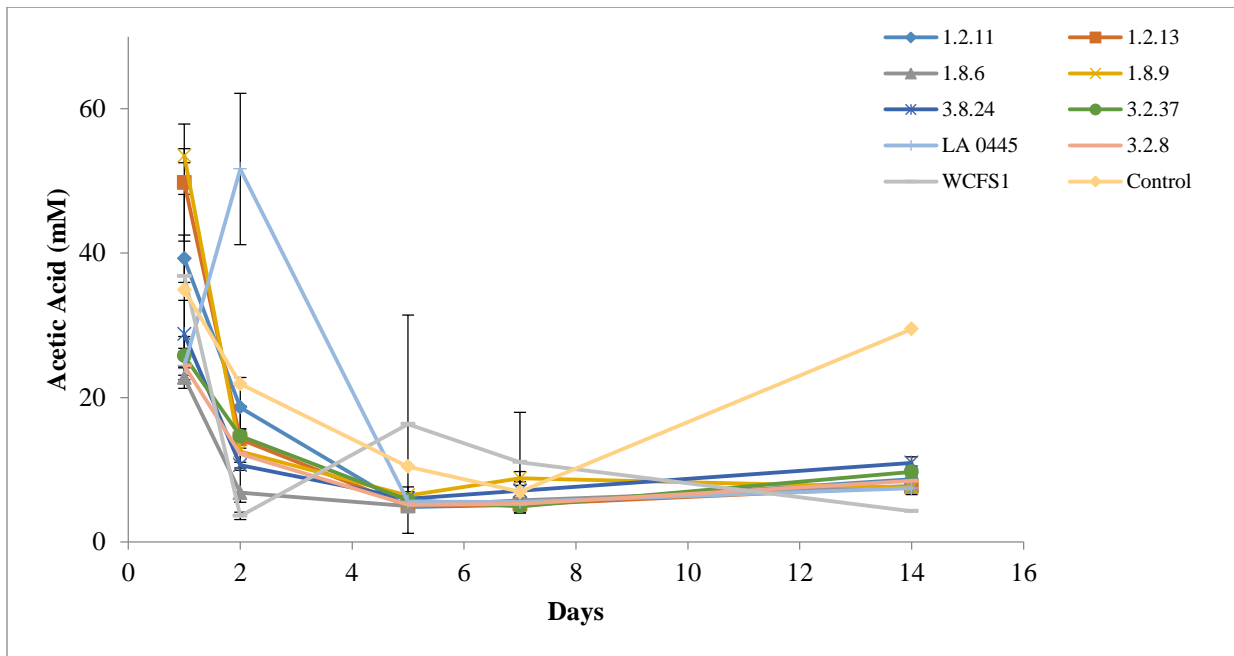


Figure 4.6: Changes in acetic acid in brine during fermentation at a) 30 °C and b) 15 °C

(a)



(b)

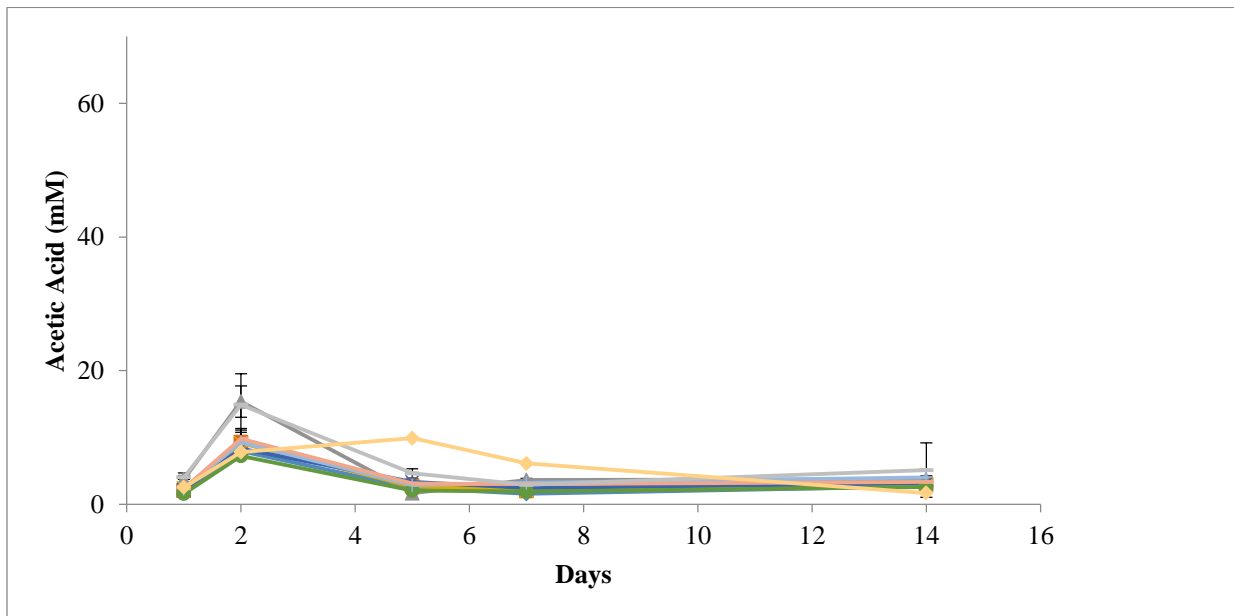
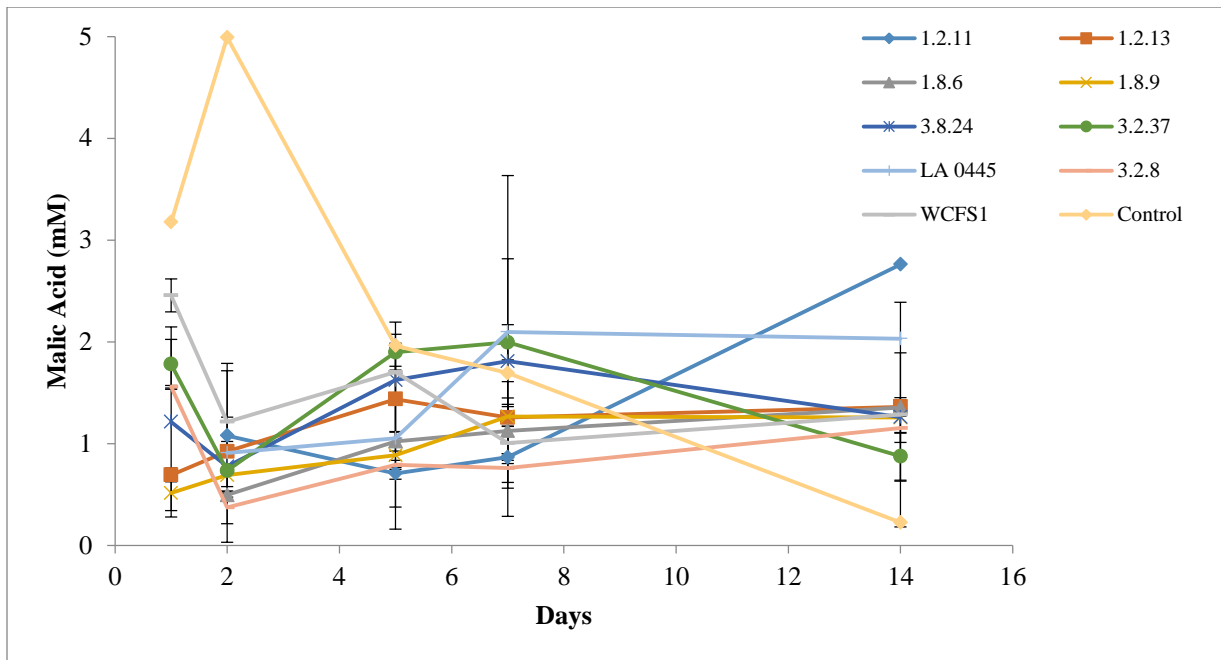


Figure 4.7: Changes in malic acid in brine during fermentation at a) 30 °C and b) 15 °C

(a)



(b)

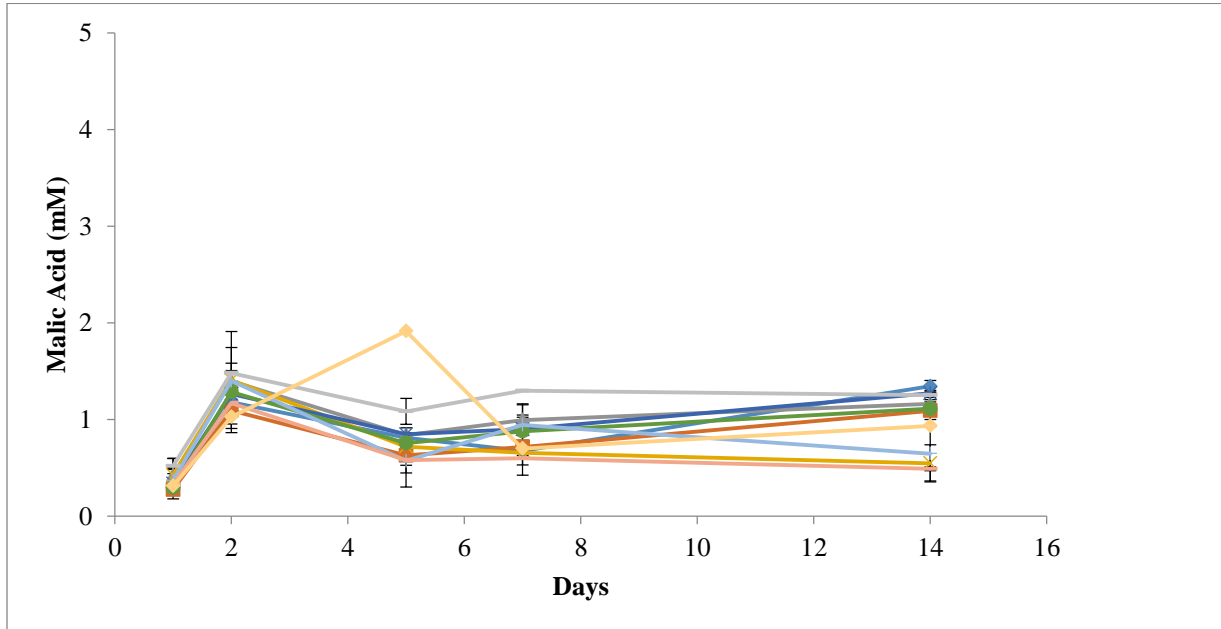


Figure 4.8: Peak force (N) determined by punch test on fermented cucumber slices (n≥14) after 14 days of fermentation at 30 °C and 15 °C

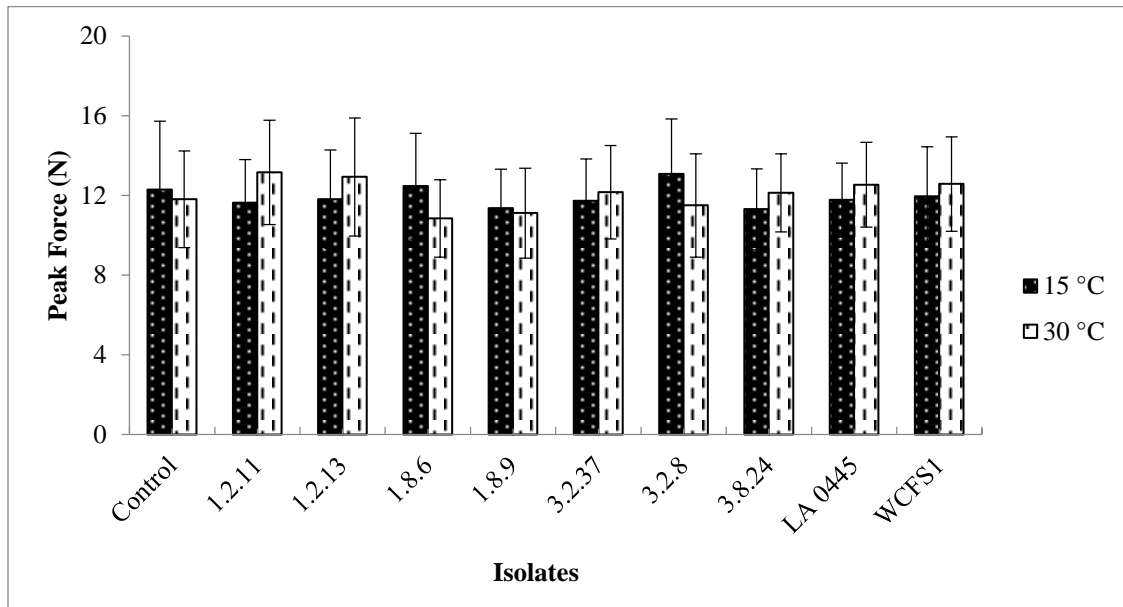


Figure 4.9: Bloating defects on fermented cucumbers after 14 days of fermentation



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CHAPTER 5

Screening of Lactic Acid Bacteria from Fresh Produce and Pickle Fermentations for Heavy Metal, Disinfectant and Antibiotic Resistance and Conjugative Acquisition of Antimicrobial Resistance Genes

ABSTRACT

Fermented foods and fresh produce containing live cultures of lactic acid bacteria (LAB) pose potential threats for disseminating antibiotic resistance genes through horizontal gene transfer from LAB to gut commensals and pathogens. In this study, various species of LAB from two different origins (fresh cantaloupe and commercial cucumber fermentations) were screened for resistance to the antibiotics erythromycin and tetracycline, as well as to the heavy metals cadmium (Cd) and arsenic (As) and the quaternary ammonium disinfectant benzalkonium chloride (BC). The LAB isolates (n=94) screened in this study belonged to diverse genera and species based on 16S rRNA sequencing, including *Leuconostoc paramesenteroides*, *Enterococcus* spp., *Exiguobacterium* spp., *Weissella* spp., *Lactococcus lactis*, *Lactobacillus plantarum* and *Lactobacillus pentosus*. Along with LAB, certain Gram-negative isolates (n=19) isolated on MRS media from fresh cantaloupe were also screened. As resistance (500 µg/mL) was detected in *Leuconostoc* spp. BW2BY and *Exiguobacterium* spp. NC42N. Cd resistance (75 µg/mL) was detected in *Leuconostoc* spp. BW2BY and BW1DZ. All the isolates were sensitive to BC. Intermediate tetracycline resistance (≤ 8 µg/mL) was detected in isolates *Weissella* spp. BW1AY and NC2Y while erythromycin resistance (8 µg/mL) was detected only in *Enterococcus* spp. M1BR. Co-resistance to heavy metals and antibiotics was not detected. The potential for conjugative transfer of antibiotic resistance genes *erm*(B) and *tet*(M) (resistance to erythromycin and tetracycline, respectively) was studied in six selected isolates (three *Leuconostoc pseudomesenteroides* from fresh cantaloupe, two *L. pentosus* and one *L. plantarum* from commercial cucumber fermentation). Conjugative transfer was tested at two different donor: recipient ratios, 1:1 and 1:10. Donors for *erm*(B) and *tet*(M) were *Enterococcus faecalis* JH2-2 (harboring plasmid pAMβ1) and *Listeria monocytogenes* N057F1/2.FB, respectively. Two

isolates of *L. pseudomesenteroides* (BW2CY and M15Y) were able to acquire *erm*(B) at transfer frequencies of approximately 10^{-7} per recipient while *L. pseudomesenteroides* M15Y acquired *tet*(M) at a frequency of approximately 10^{-9} per recipient. PCR- based confirmation of *erm*(B) and *tet*(M) in transconjugants along with absence of spontaneous mutants of the parental cultures on double selection media further indicated that the colonies obtained were true transconjugants derived from the recipient isolates. *L. plantarum* and *L. pentosus* did not yield transconjugants, suggesting that the frequency of conjugative transfer may be species and origin-dependent.

Significance: This study examined incidence of various types of resistance (heavy metal, disinfectant and antibiotics) in a population of LAB from cucumber fermentations and fresh produce (cantaloupe). *L. pseudomesenteroides* could participate as recipient in conjugations with other Gram-positive bacteria and receive antibiotic resistance genes that may subsequently disseminate to other pathogens or commensal gut bacteria.

INTRODUCTION

For a long time antibiotic resistance gene transfer risk was focused primarily on clinical pathogens and settings. However, it is now recognized that the food chain is one of the most common vectors for the spread of antibiotic resistance between environment, humans and animals (Toomey and others 2009b; Rossi and others 2014). Bacteria with acquired antibiotic resistance have a potential to horizontally disseminate these genes through mobile elements such as plasmids, conjugative transposons and phages (Teuber and others 1999; Rossi and others 2014). Therefore, it is recommended that bacteria intended for use as starter cultures or live probiotic cultures do not harbor transferable antibiotic resistance genes (Casado Muñoz and others 2014; Rossi and others 2014).

The antibiotics tetracycline and erythromycin have been widely used as growth promoters in animal agriculture and for treatment of human infections, which has led to widespread resistance to these antibiotics (Devergiliis and others 2013). There is high risk of exchange of antibiotic resistance determinants between environment, humans and animals even in materials that are used on a daily basis such as cutting boards and cleaning towels (Kruse and Sørum 1994). *L. plantarum* isolates from various food origins including dairy, meat and fermented vegetables were examined for presence of *erm*(B) and *tet*(M) for erythromycin and tetracycline resistance, respectively (Feld and others 2009; Nawaz and others 2011; Devergiliis and others 2013). Antibiotic resistance genes can be transferred between LAB and pathogens, with the resulting resistant LAB having the potential to subsequently transfer the resistant genes to pathogens as new donors (Pucci and others 1988; Rossi and others 2014).

In LAB the resistance genes *erm*(B) and *tet*(M) are commonly found on plasmids and conjugative transposons, posing a risk of horizontal transfer to others, including pathogens

(Ammor and others 2007). *L. plantarum* strains harboring plasmids encoding *tet(M)* and *erm(B)* were able to participate in conjugative transfer with *E. faecalis in vivo* in mice demonstrating their natural ability of horizontal gene transfer (discussed more in the literature review) (Jacobsen and others 2007). Nawaz and others demonstrated that *L. plantarum* from various foods including dairy and fermented vegetables possessed genes for *erm(B)* and *tet(M)* and in fact a strain of *L. plantarum* from fermented vegetables was able to donate *tet(M)* to *E. faecalis* at a high frequency of 1.39×10^{-5} transconjugants per recipient (Nawaz and others 2011). Dairy based strain of *L. plantarum*'s conjugative transfer of *erm(B)* via plasmid pLFE1 was demonstrated in *Enterococcus faecalis*, *Lactococcus lactis*, *Bacillus* spp. and *Listeria* spp. at frequencies in the range of 10^{-10} to 10^{-6} (Feld and others 2009). Feld and others also demonstrated that *L. plantarum* from raw milk was able to transfer *erm(B)* harbored on a plasmid to *E. faecalis in vitro* and *in vivo* in mice (Feld and others 2008). The transconjugants obtained in this study were not able to subsequently donate the antibiotic resistant genes to *E. faecalis* (Feld and others 2008). However, transconjugant *Lactobacillus* spp. and *L. monocytogenes* that received pAM β 1 harboring *erm(B)* were subsequently able to transfer the plasmid to other bacteria such as *E. faecalis* acting as new donors at frequencies of approximately 10^{-8} to 10^{-9} transconjugants per recipients (Tannock 1987; Flamm and others 1984). *L. monocytogenes* was also able to transfer multiple drug resistance genes (mediating resistance to antibiotics such as chloramphenicol, erythromycin, streptomycin and tetracycline) to LAB and other bacteria including *Staphylococcus* spp., *Enterococcus* spp. and *Streptococcus* spp. (Charpentier and Courvalin 1999; Allen and others 2016; Bertsch and others 2013). Metagenomic analysis of fresh foods, ready-to-eat foods and food processing environments revealed that *Listeria* is often present in the microbiota and a reservoir of antibiotic resistance genes in these ecosystems

(Bertsch and others 2013). Thus, the transferability of antibiotic resistance genes between pathogens and LAB is of significant interest and therefore explored in this study.

Co-resistance of antibiotic with heavy metals and disinfectants is also commonly seen when co-transcription of heavy metal and antibiotic resistance genes and similarities in resistance mechanisms between heavy metals and antibiotics (Baker-Austin and others 2006; Chapman 2003). Currently, heavy metal contamination is more rapidly spreading and long lasting than antibiotics and there is a greater chance of evolution and dissemination of antibiotic resistance genes if the microorganisms possess co-resistance (Baker-Austin and others 2006; Berg and others 2005). Cantaloupes are frequent vehicles foodborne outbreaks involving pathogens such as *Salmonella* and *Listeria* (Bowen and others 2006; CDC outbreak updates, 2012; Walsh and others 2014). However, little is known about LAB populations from the surface of fresh cantaloupe and their potential to participate in horizontal gene transfer (HGT) with other bacteria including pathogens. Additionally, resistance of cantaloupe-derived LAB to antibiotics, heavy metals and disinfectant is poorly understood, and we currently lack information about HGT of resistance genes between LAB from cantaloupe and other bacteria, including pathogens.

To address some of these knowledge gaps, the objective of this study was to screen LAB isolated from fresh cantaloupe and from commercial cucumber fermentations for resistance to heavy metals (As and Cd), BC and antibiotics (erythromycin and tetracycline). Conjugative transfer of the resistance genes *erm(B)* and *tet(M)* to LAB from *E. faecalis* and *L. monocytogenes*, respectively, was performed to understand the capacity of LAB from these sources to receive these resistance genes.

MATERIALS AND METHODS

Bacterial Strains and Growth Media:

The strains isolated and characterized in this study are listed in Table 5.1. The *L. monocytogenes* strains were from the *Listeria* strain collection of the Kathariou laboratory at North Carolina State University. *L. monocytogenes* N057F1/2.FB is a tetracycline-resistant strain harboring *tet*(M) and isolated from animal feces (C. Parsons and S. Kathariou, unpublished), while the streptomycin-resistant strain *L. monocytogenes* 10403S was originally obtained from Dr. D. Portnoy (University of California, Berkeley). *L. monocytogenes* strains 2858, H1F1, F8027, H7550 and F2365 were from the Kathariou *Listeria* strain collection of the Kathariou laboratory at North Carolina State University and were used as positive and negative controls in antimicrobial susceptibility determinations. *E. faecalis* was obtained from the USDA-ARS Culture Collection, Raleigh, NC, USA. *L. plantarum* and *L. pentosus* strains were obtained from the USDA-ARS Culture Collection, Raleigh, NC, USA and were isolated from commercial cucumber fermentation tanks in North Carolina. Lactic acid bacteria and other bacterial cultures from fresh cantaloupe rind were isolated as described below. The cultures were purified and grown in Lactobacilli Susceptibility Media (LSM) overnight at 30 °C in all subsequent experiments. The composition of LSM is described elsewhere (Flórez and others 2006). All reagents including disinfectants, heavy metals and antibiotics were purchased from Sigma-Aldrich, MO, USA unless otherwise mentioned. Stock cultures of *L. monocytogenes* were preserved at -80 °C in 20% glycerol in Brain Heart Infusion, while all others were preserved at -80 °C in 30% glycerol in LSM.

Isolation of LAB from Cantaloupes:

Cantaloupes were obtained from a farm participating in a cover-crop investigation (Principal Investigator, Dr. Eduardo Gutierrez, North Carolina State University). Three plots at the same farm were designated NC (no cover crop), S (sun hemp cover crop), BW (Buckwheat cover crop) and M (mustard cover crop). The cantaloupes from each plot were harvested when ripe using a scheme that ensured representation of the various sites in the plot, placed individually in large sterile whirl-paks, and transported on ice to the laboratory of Dr. Eduardo Gutierrez, Dept of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, who kindly made the melons available for the study. In the Kathariou laboratory, a 2x2 cm rind fragment with a thickness of no more than 2-3 mm (to minimize amount of fruit flesh) was excised from each cantaloupe using a flame-sterilized knife and immersed in 20 mL saline in 50 mL conical tubes (Corning[®], NY, USA). The samples were vortexed and 50 μ L of each suspension was spiral-plated (Eddy Jet 2, IUL Instruments, Barcelona, Spain) on deMan, Rogosa and Sharpe (MRS; Difco[™], MI, USA) medium with cycloheximide (0.01%) and MRS with streptomycin (128 μ g/mL) and cycloheximide (0.01%). Colonies were randomly chosen from the plates based on varying colony appearance and pure cultures were grown overnight in liquid MRS medium. Frozen stocks were prepared from these pure cultures in 30% glycerol LSM liquid culture and stored at -80 °C until further use. The codes NC, BW, M and S in the isolate designations (Table 5.1) indicate the type of plot for the cantaloupes yielding the respective isolates (NC, no cover crop; BW, buckwheat cover crop; M, mustard cover crop; and S, sun hemp cover crop).

Putative Taxonomic Designations for Selected Isolates from Cantaloupe:

Selected isolates were randomly chosen for identification using partial sequencing of 16S rRNA (Amann and others 1995) with primers 8F (5'- AGA GTT TGA TCC TGG CTC AG - 3') and U1492r (5'- GGT TAC CTT GTT ACG ACT T - 3'). The following conditions were used in PCR: 94 °C for 3 min, 25 cycles of 94 °C for 1 min, 57 °C for 2 min and 72 °C for 2 min and a final extension cycle of 72 °C for 7 min.

Antimicrobial Susceptibility Determinations:

Antimicrobial resistance was determined on LSM plates with the following concentrations of the indicated compounds: arsenic (As), 100 and 500 µg/mL; cadmium (Cd), 35 and 70 µg/mL; benzalkonium chloride (BC), 10 µg/mL; tetracycline (Tet), 4, 8 and 16 µg/mL; erythromycin (Erm), 1, 4 and 8 µg/mL. The concentrations were determined based on the resistance levels of strains used as positive controls. The isolates were grown in LSM broth overnight in 96 well plates and flame-sterilized stainless steel replicating device was used to transfer from the 96 well plate to LSM media with the various antimicrobials. The plates were incubated at 30 °C for 48 h and visually examined for growth. Isolates yielding confluent growth were considered resistant (+), those with weak growth or with separate colonies were considered as intermediate (i) and those with no detectable growth were considered susceptible (-). The positive controls included *L. monocytogenes* F8027, for As and Cd (35 µg/mL); *L. monocytogenes* H7550 for Cd (70 µg/mL) and BC; *L. monocytogenes* 2858 for tetracycline and *L. monocytogenes* H1F1 for erythromycin. *L. monocytogenes* F2365 was used as negative control for Cd, Tet and BC and *L. monocytogenes* H7550 for Erm and As (Table 5.1; Mullapudi and others 2010; Katharios-Lanwermeier and others 2012).

PCR Screening for Heavy Metal Resistance Determinants, *erm(B)* and *tet(M)*:

The isolates that displayed resistance towards Cd and As were further screened by PCR for the presence of resistance genes known to mediate resistance in *L. monocytogenes* (Lee and others, 2013). PCR was employed with primers for genes *arsA1* (LMOSA_2220F and LMOSA_2220R) and *arsA2* (pLI37_F and pLI37_R) for As resistance and *cadA1* (*cadA*-Tn5422F and *cadA*-Tn5422R), *cadA2* (*cadA*-pLM80F and *cadA*-pLM80R), *cadA3* (*cadC*-EGDeF and *cadC*-EGDeR) and *cadA4* (*cadA4*F and R) for Cd resistance (Lee and others, 2013; Parsons and Kathariou, unpublished). The positive controls for *cadA1*, *cadA2*, *cadA3* and *cadA4* were *L. monocytogenes* LWA131, *L. innocua* CLIP11262, *L. monocytogenes* EGDe and *L. monocytogenes* F8027, respectively. F8027 was also a positive control for PCR targeting *arsA1* and *arsA2*. The negative controls used were the same as described above.

tet(M) detection in conjugative transfer studies was performed using the primers TetMF (5'-GGG GCA ATT CTA CTG ATT TC-3') and TetMR (5'-TTT GCA CAA TAT TTG GGA GC-3') using the following PCR conditions: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 56 °C for 1 min and 72 °C for 1.5 min and a final extension cycle of 72 °C for 7 min (Parsons and Kathariou, unpublished). Detection of *erm(B)* in conjugation studies by PCR was performed using the primers and conditions described by (Wang and others 2014). Colony PCR was performed using GoTaq[®] green DNA master mix (Promega, WI, USA) with 0.5 µM of each primer pair and PCR conditions described previously (Mullapudi and others 2010). All PCR screening tests were performed in duplicate.

Conjugative Transfer of Antibiotic Resistance:

L. pseudomesenteroides strains BW2CY, M15Y and NC47Y were selected from the cantaloupe isolate collection for their vancomycin resistance (van^{R}) and used as recipients in conjugations. Additional van^{R} strains tested as potential recipients in these conjugations were *L. pentosus* 1.2.11, *L. pentosus* LA 0445 and *L. plantarum* 3.2.8, from commercial cucumber fermentations. The erythromycin and tetracycline Minimum Inhibitory Concentrations (MIC) of these isolates were determined previously (Table 5.1). The donor strain used for *erm*(B) transfer was *E. faecalis* JH2-2 with plasmid pAM β 1 harboring *erm*(B) while *L. monocytogenes* N057F.1/2FB harboring *tet*(M) was employed as the donor of *tet*(M).

Conjugation was performed following previously described methods with slight modifications (Lampkowska and others 2008; Toomey and others 2009c). Cultures were grown overnight in LSM broth at 30 °C (LAB) or 37 °C (*E. faecalis* JH2-2 and *L. monocytogenes* strains), and 1:1 and 1:10 donor: recipient mixtures were prepared in one mL tubes. Each donor and recipient was also included individually as controls. The tubes were centrifuged at 2655g for 2 min (Eppendorf centrifuge 5415D, Brinkmann Instruments, NY, USA). The supernatant was discarded and the pellet was reconstituted in 100 μL of Maximum Recovery Diluent (MRD- 1 g/L peptic digest and 8.5 g/L NaCl). The mixture was spotted in duplicate (50 μL each spot) on LSM plates and allowed to dry in a laminar flow biosafety cabinet for 20 min. The single cultures of each donor and recipient were similarly centrifuged and reconstituted in 100 μL MRD, and 25 μL was spotted on separate LSM plates. The plates were incubated for 24 h at 30 °C and spots were transferred to 1 mL MRD using sterile cotton swabs. The cell suspensions were plated on the double selective media at appropriate dilutions.

The double-selective medium for *erm(B)* transconjugants contained vancomycin (64 µg/mL) and erythromycin (10 µg/mL). The double-selective medium for *tet(M)* transconjugants of *L. pseudomesenteroides* contained vancomycin (64 µg/mL) and tetracycline (8 µg/mL) while for *L. plantarum/pentosus* recipients the double selective medium contained vancomycin (64 µg/mL) and tetracycline (40 µg/mL). Putative transconjugants were PCR-tested for the presence of *erm(B)* or *tet(M)*. Single cultures of parent donors and recipients used in all the conjugations were plated on the appropriate double-selective medium to detect any spontaneous mutants. *L. monocytogenes* 10403S (serotype 1/2a) was used as positive recipient control for both *erm(B)* and *tet(M)* conjugative transfers. The double-selective medium for *erm(B)* transconjugants of *L. monocytogenes* 10403S contained streptomycin (600 µg/mL) and erythromycin (8 µg/mL), while *tet(M)* transconjugants were selected on streptomycin (600 µg/mL) and tetracycline (8 µg/mL). Multiplex PCR (Doumith and others 2004) was employed to determine whether *tet(M)*-harboring transconjugants had the serotype of the recipient (serotype 1/2a) and were not streptomycin-resistant mutants of the donor, which had serotype 4b. Conjugation transfer frequency was calculated as the number of transconjugants formed per recipient CFU in the conjugation mating spot at the end of the conjugation. If no transconjugants were detected, the transfer frequency was represented as lower than one transconjugant per recipient CFU in the mating spot. LAB recipients were enumerated on vancomycin (64 µg/mL), while *L. monocytogenes* 10403S was enumerated on streptomycin (600 µg/mL). Selected transconjugants were purified on the appropriate double-selective media at least twice before their frozen stocks were prepared. All conjugations were performed in duplicate.

RESULTS AND DISCUSSION

A total of 52 isolates with different morphologies were selected from MRS plates with streptomycin (128 µg/mL), while 34 isolates were randomly selected from MRS plates without antibiotics (Table 5.1). Of the latter, 19 were found to be Gram-negative bacteria tentatively identified as *Kosakonia cowanii* (M31P) and *Cronobacter dublinensis* (M31T) based on 16S rRNA sequencing (Table 5.1). Gram-positive isolates were tentatively identified as *Lactococcus lactis* (M21Z, NC45Z and BW1CR), *Leuconostoc pseudomesenteroides* (BW2CY, M15Y, NC45Y, NC47Y, BW1CQ and M31Q), *Leuconostoc mesenteroides* (BW2BY), *Exiguobacterium* spp. (NC42N), *Enterococcus* spp. (M1BR) and *Weissella* spp. (NC2Y and BW1AY) (Table 5.1).

Absence of Co-resistance to Antibiotics, Heavy Metals and Disinfectant:

Two Gram-positive isolates from cantaloupe, *Leuconostoc mesenteroides* BW2BY and *Exiguobacterium* spp. NC42N, tested positive for As resistance (at both 100 and 500 µg/mL) while two isolates, BW1DZ and *Leuconostoc mesenteroides* BW2BY, tested positive for Cd resistance (35 and 70 µg/mL) (Tables 5.1). *Weissella* spp. BW1AY and NC2Y tested positive for intermediate tetracycline resistance as they were able to grow at 4 and 8 µg/mL but not at 16 µg/mL. Only one isolate, *Enterococcus* spp. M1BR, tested positive for erythromycin resistance, displaying confluent growth at 1, 4 and 8 µg/mL (Table 5.1). All the Gram-positive isolates were sensitive to BAC and did not display co-resistance among the antimicrobial agents tested (Table 5.1). However, all the Gram-negative isolates displayed co-resistance for at least two or more of the tested compounds. Most of these Gram-negative isolates were concomitantly resistant to BC, Cd and erythromycin at all the concentrations tested, and more than half were also resistant to As at both 100 and 500 µg/mL. However, all but one of the Gram-negative isolates were sensitive to

tetracycline with no confluent growth beyond 4 µg/mL; the resistant isolate was BW2BSP, which grew at 4 and 8 µg/mL but not at 16 µg/mL (Table 5.1).

PCR testing of As and Cd-resistant LAB isolates with primers derived from genes known to be associated with As and Cd resistance in *L. monocytogenes* failed to yield the expected PCR products (the positive controls yielded the expected product in these PCRs) (Figure 5.1). In *Listeria* spp., resistance quaternary ammonium disinfectants is frequently encountered together with Cd resistance and can be co- transferred to other pathogenic and non-pathogenic *Listeria* strains upon selection for Cd resistance, indicating co-selection (Katharios-Lanwermyer and others 2012). However, none of the Gram-positive isolates in this study displayed co-resistance. The resistance to individual heavy metals could be mediated by innate nonspecific mechanism such as efflux through protein pumps or to genes other than those targeted by the primers used here. The LAB here that were naturally found on fresh cantaloupes and cucumber fermentation displayed intermediate to low/no resistance to antibiotics and thus using them as donors was not feasible. Hence, the recipient nature of these LAB to participate in horizontal gene transfer acquire antibiotic genes was tested.

Conjugative Transfer is Dependent on the Recipient Species:

Conjugations between *E. faecalis* JH2-2 (donor) and the positive control *L. monocytogenes* 10403S (recipient) in 1:1 and 1:10 (donor: recipient) ratios yielded transconjugants on the streptomycin-erythromycin double selective plates at a rate of $1.68 \pm 2.15 \times 10^{-6}$ and $4.43 \pm 1.35 \times 10^{-7}$, respectively (Table 5.2). Individual cultures of recipients did not yield any spontaneous mutants on the double-selective medium indicating that the transconjugants obtained were from the recipient parent. This was confirmed by microscopic examination of selected transconjugants, which indicated that conjugative transfer of *erm*(B) was

much less common among the LAB tested as recipients. However, transconjugants on individual *E. faecalis* were seen on the double selective medium indicating spontaneous mutants of streptomycin at a frequency of $3.29 \pm 4.67 \times 10^{-6}$. Transconjugants were obtained on the erythromycin-vancomycin double-selective plates only with two *L. pseudomesenteroides* isolates (BW2CY and M15Y), at frequencies between $1.13 \pm 1.5 \times 10^{-6}$ and $1.75 \pm 1.12 \times 10^{-7}$, respectively (Table 5.2). Transconjugants for *L. plantarum* and *L. pentosus* isolated from cucumber fermentation were not obtained at either donor: recipient ratio (1:1 and 1:10), suggesting transfer frequency $< 1.48 \times 10^{-9}$ (Table 5.2). The transconjugants obtained on the double-selective medium from the control recipient 10403S and the *Leuconostoc* isolates were further tested by PCR for the presence of *erm*(B). The expected PCR product (~400 bp) was obtained in all cases (Figure 5.2).

Several earlier studies demonstrated successful conjugative transfer of pAM β 1 from the donor *E. faecalis* into LAB such as *L. lactis*, *L. plantarum*, *Lactobacillus reuteri*, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, and *Leuconostoc* spp. (Shrago and others 1986; Sasaki and others 1988; Gibson and others 1979; Pucci and others 1988; West and Warner 1985; Tannock 1987; Vescovo and others 1983; Soeding and others 1993). However, none of the *L. plantarum* and *L. pentosus* in the current study produced *erm*(B)-harboring transconjugants. It is also possible that these isolates may yield transconjugants with other *erm*(B) donors. However, *E. faecalis* JH2-2 is a well-established promiscuous donor of pAM β 1 to a broad range of LAB (Soeding and others 1993; Shrago and others 1986; Tannock 1987). So, it is likely that the *L. plantarum/pentosus* in this study may be unable to acquire the plasmid via conjugative transfer, for reasons that remain unknown.

Conjugations of *L. monocytogenes* 10403S with the *tet(M)* harboring *L. monocytogenes* N057F.1/2FB yielded transconjugants on the tetracycline-streptomycin double-selective plates, with a frequency of 10^{-7} at both tested ratios (1:1 and 1:10) (Table 5.2). The expected PCR product for *tet(M)* was obtained from these transconjugants and serotype determinations by multiplex PCR indicated that they had serotype 1/2a, as did the recipient strain. This indicated that these transconjugants were not spontaneous streptomycin-resistant mutants of the donor strain, which had serotype 4b (Figure 5.3). The conjugation transfer frequency was in the range of 10^{-7} at both donor: recipient ratios (1:1 and 1:10) tested here. Similarly to what was observed with transfer of *erm(B)*, conjugative transfer of *tet(M)* to LAB was much less efficient than observed with *L. monocytogenes* as the positive control (Table 5.2). Among the LAB isolates tested as recipients, transconjugants were obtained on tetracycline-vancomycin double-selective media only with *L. pseudomesenteroides* M15Y, at low frequency (1.35×10^{-9}) and using 1:1 donor: recipient ratio (Table 5.2). All tested transconjugants were PCR-positive for *tet(M)*, yielding the expected product of ~1400 bp (Figure 5.4). No spontaneous mutants of individual donors and recipients were seen on the double-selective medium.

Multiple reasons may account for the observed poor ability of the LAB strain to acquire *erm(B)* or *tet(M)* via conjugative transfer. Biotic and abiotic factors including pH, temperature, incubation time, donor to recipient ratio and presence of sublethal amounts of the antibiotics may impact frequency of transfer (discussed more in the literature review) (Fernandez-Astorga and others 1992). The origin of isolates and matrix of conjugation also can have an impact on the involvement of cells in conjugation. After conjugation, the stability of pAM β 1 in *L. brevis* after a series of propagations was higher in vegetable juices (cabbage and potato) than milk highlighting the importance of matrix (Soeding and others 1993). Different matrices have been tested for their

impact on the frequency of conjugation among LAB. For example, rumen fluid, meat, whole milk, water, sprouts and cheese were used as matrices to assess the transfer capability of LAB as both donors and recipients (Toomey and others 2009a; Toomey and others 2009b; Tamanai-Shacoori and others 1995).

Mating medium has an impact on conjugation and perhaps these isolates from high acid cucumber fermentation need similar environment for interaction and conjugation of DNA material from other bacteria (Toomey and others 2009a; Toomey and others 2009b). Conjugative transfer of *erm*(B) and *tet*(M) was higher in sprouts ($> 10^{-4}$) compared to rumen fluid ($< 10^{-6}$), possibly due to intimate contact and restricted mobility on the surface of the sprouts (Toomey and others 2009a). Lactic acid bacteria that produce biofilms and exopolysaccharides are known to have higher probabilities of horizontal gene transfer (Rachid and others 2000; Harrison and others 2005; Angles and others 1993; Kajiura and others 2006; Madsen and others 2012). Even though *L. plantarum* and *L. pentosus* isolates used in this study have been documented for their exopolysaccharide production they were not able to participate in conjugative transfer of antibiotic resistance genes tested here (Anekella and others, unpublished). Haug and others proved that *E. faecalis* was able to transfer antibiotic resistant genes to *L. monocytogenes* in infant feces and subsequently from these transconjugants to commensal bacteria in colonic fermentation model (Haug and others 2011). Thus, the transconjugant *L. pseudomesenteroides* formed in this study may have similar potential when they are closely in contact with other LAB and pathogens. Pucci and others also demonstrated transconjugant leuconsotocs were able to act as new donors and donate pAM β 1 at frequencies between 10^{-3} and 10^{-5} per donor (Pucci and others 1988). Other factors that may impact conjugation are discussed in the literature review.

In conclusion, heavy metal and antibiotic resistance was only infrequently detected among LAB isolates from cantaloupe and co-resistance to multiple antimicrobials was not observed in any of the LAB isolates tested here. Only two of the cantaloupe-derived LAB isolates appeared able to acquire *erm*(B) or *tet*(M) via conjugation, and conjugative acquisition of *tet*(M) was especially uncommon. Current work is in progress to study the transconjugant recipients as the new donors to transfer the previously acquired antibiotic resistant genes. Further studies are needed to determine whether such infrequently obtained transconjugants have the potential to transfer these genes to other bacteria.

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TABLES:

Table 5.1: Isolates from fresh cantaloupe and cucumber fermentations tested against antibiotics, heavy metals and disinfectants

16S rRNA Identification	Isolates	Concentration (µg/mL)										
		Erythromycin			Tetracycline			As		Cd		BAC
		1	4	8	4	8	16	100	500	35	70	10
Controls												
NA	<i>L. monocytogenes</i> F8027							+	+	+		
	<i>L. monocytogenes</i> 2858				+	+	+					
	<i>L. monocytogenes</i> H1F1	+	+	+								
	<i>L. monocytogenes</i> H7550	-	-	-				-	-		+	+
	<i>L. monocytogenes</i> F2365				-	-	-				-	-
Isolates from commercial cucumber fermentation												
NA	<i>L. plantarum</i> 3.2.8*	-	-	-	+	+	+	+	-	-	-	-
	<i>L. pentosus</i> 1.2.11	-	-	-	+	+	i	+	-	-	-	-
	<i>L. pentosus</i> LA 0445	-	-	-	+	+	+	+	-	-	-	-
	<i>L. pentosus</i> 1.2.13	-	-	-	+	+	i	+	i	-	-	-
	<i>L. pentosus</i> 1.8.6	-	-	-	+	+	+	+	i	-	-	-
	<i>L. pentosus</i> 1.8.9	-	-	-	+	+	+	+	-	-	-	-
	<i>L. pentosus</i> 3.8.24	-	-	-	+	+	+	+	-	-	-	-
	<i>L. pentosus</i> 3.2.37	-	-	-	+	+	+	+	-	-	-	-

Table 1 continued

16S rRNA Identification	Isolates	Concentration ($\mu\text{g/mL}$)										
		Erythromycin			Tetracycline			As		Cd		BAC
		1	4	8	4	8	16	100	500	35	70	10
Isolates from cantaloupe on MRS with streptomycin (128 $\mu\text{g/mL}$)												
<i>L. pseudomesenteroides</i>	BW2CY	-	-	-	-	-	-	i	-	-	-	-
<i>L. pseudomesenteroides</i>	M15Y	-	-	-	-	-	-	i	-	-	-	-
<i>L. pseudomesenteroides</i>	NC4-7Y	-	-	-	-	-	-	i	i	-	-	-
<i>L. pseudomesenteroides</i>	NC4-5Y	-	-	-	i	-	-	i	-	-	-	-
<i>W. cibaria</i>	BW1AY	-	-	-	+	i	+	+	-	i	i	-
<i>L. lactis</i>	M21Z	-	-	-	-	-	-	-	-	-	-	-
<i>L. lactis</i>	NC4-5Z	-	-	-	-	-	-	-	-	-	-	-
ND	BW1BY	-	-	-	-	-	-	i	i	-	-	-

*Isolates in bold were selected for conjugative transfer studies

NA- Not Applicable, ND- Not Determined

i= intermediate; + denotes positive and – denotes negative

Table 1 Continued

16S rRNA Identification	Isolates	Concentration (µg/mL)												
		Erythromycin			Tetracycline			As		Cd		BAC		
		1	4	8	4	8	16	100	500	35	70	10		
Isolates from cantaloupe on MRS with streptomycin (128 µg/mL)														
ND	BW1BZ	-	-	-	-	-	-	-	i	i	-	-	-	
	BW1CY	-	-	-	-	-	-	-	i	i	-	-	-	
	BW1CZ	-	-	-	-	-	-	-	-	-	-	-	-	
	BW1DY	-	-	-	-	-	-	-	i	i	-	-	-	
	BW1DZ	-	-	-	-	-	-	-	i	i	+	+	-	
	BW2AY	-	-	-	-	-	-	-	i	i	+	-	-	
	BW2BY	-	-	-	-	i	-	-	+	+	+	+	-	
	BW3AY	-	-	-	-	-	-	-	i	i	-	-	-	
	BW3BY	-	-	-	-	i	-	-	+	-	i	-	-	
	BW3CY	-	-	-	-	i	-	-	+	-	+	-	-	
	BW3CZ	-	-	-	-	-	-	-	i	i	-	-	-	
	ND	M1AY	-	-	-	-	i	-	-	+	-	+	-	-
		M1BY	-	-	-	-	i	-	-	+	-	i	-	-
M1CY		-	-	-	-	i	-	-	+	-	+	-	-	
M1DY		-	-	-	-	i	-	-	+	-	+	-	-	
M2AY		-	-	-	-	i	-	-	+	-	+	-	-	
M2BY		-	-	-	-	i	-	-	+	-	i	-	-	
M2CY		-	-	-	-	i	-	-	i	-	i	-	-	
M2DY		-	-	-	-	i	-	-	+	-	i	-	-	
M3AY		-	-	-	-	i	-	-	+	-	+	-	-	
M4AY		-	-	-	-	i	-	-	+	-	+	-	-	
M4BZ		-	-	-	-	-	-	-	-	-	-	-	-	
M31Y		-	-	-	-	i	-	-	+	-	+	-	-	
Weissella spp.		M31Z	-	-	-	-	-	-	-	-	-	-	-	-
	NC2Y	-	-	-	-	+	+	i	+	-	-	-	-	
	NC2Z	-	-	-	-	-	-	-	-	-	i	i	-	
	NC4-1Y	-	-	-	-	i	-	-	-	-	+	-	-	
ND	NC4-2Y	-	-	-	-	i	-	-	+	-	+	-	-	
	NC4-3Z	i	-	-	-	-	-	-	i	-	+	+	-	
	NC4-4Y	-	-	-	-	i	-	-	i	-	-	-	-	

ND- Not Determined

i= intermediate; + denotes positive and – denotes negative

Table 1 Continued

16S rRNA Identification	Isolates	Concentration (µg/mL)										
		Erythromycin			Tetracycline			As		Cd		BA C
		1	4	8	4	8	16	10	50	3	7	10
Isolates from cantaloupe on MRS with streptomycin (128 µg/mL)												
ND	NC4-4Z	-	-	-	-	-	-	-	-	-	-	-
	NC4-6Y	-	-	-	-	-	-	i	-	+	i	-
	NC4-8Z	i	-	-	-	-	-	i	-	i	i	-
	NC3-AY	-	-	-	-	-	-	i	-	i	-	-
	NC3-BY	-	-	-	-	-	-	+	-	+	-	-
	S1AZ	-	-	-	-	-	-	-	-	i	-	-
	S1BY	i	-	-	-	-	-	i	-	-	-	-
	S2AY	-	-	-	-	-	-	i	-	-	-	-
	S2AZ	-	-	-	-	-	-	i	-	-	-	-
	S2BY	-	-	-	-	-	-	+	-	i	-	-
	S3Y	-	-	-	-	-	-	i	i	-	-	-
	S4AZ	-	-	-	-	-	-	-	-	i	i	-
	S4BY	-	-	-	-	-	-	i	-	i	-	-
	S4BZ	-	-	-	-	-	-	-	-	-	-	-
Isolates from cantaloupe on MRS												
<i>L. pseudomesenteroides</i>	BW1CQ	-	-	-	i	-	-	i	-	-	-	-
<i>L. pseudomesenteroides</i>	NC2R	+	-	-	-	-	-	i	-	-	-	-
<i>L. lactis</i>	BW1CR	-	-	-	-	-	-	-	-	-	-	-
ND	S4BQ	-	-	-	-	-	-	+	-	+	-	-
	S4BR	i	-	-	-	-	-	-	-	i	-	-
	BW3B	-	-	-	i	-	-	+	-	i	-	-
	M1BQ	-	-	-	i	-	-	i	-	i	-	-
<i>Enterococcus</i> spp.	M1BR	+	+	+	i	-	-	i	-	i	-	-
	M3BR	-	-	-	-	-	-	i	-	-	-	-
ND	M31Q	-	-	-	i	-	-	i	-	i	-	-
	NC2Q	-	-	-	-	-	-	i	-	-	-	-
<i>Exiguobacterium</i> spp.	NC4-2N	-	-	-	-	-	-	+	+	+	+	-
	NC4-2Q	-	-	-	-	-	-	+	-	i	-	-
	NC4-4Q	-	-	-	i	-	-	i	-	i	-	-
	NC4-4R	i	-	-	-	-	-	i	-	i	-	-

ND- Not Determined

i= intermediate; + denotes positive and – denotes negative

Table 1 Continued

16S rRNA Identification	Isolates	Concentration (µg/mL)										
		Erythromycin			Tetracycline			As		Cd		BAC
		1	4	8	4	8	16	100	500	35	70	10
Gram-negative isolates from cantaloupe on MRS												
<i>Kosakonia cowanii</i>	M31P	+	+	+	+	-	-	+	+	+	+	+
<i>Cronobacter dublinensis</i>	M31T	+	+	+	-	-	-	-	-	+	+	+
	BW2BR	+	+	+	-	-	-	+	i	+	i	+
	BW2BSP	+	+	+	+	+	-	+	+	+	+	+
	BW4P	+	+	+	i	-	-	-	-	+	+	+
	BW4R	+	+	+	-	-	-	+	+	+	+	+
	BW4T	+	+	+	-	-	-	+	+	+	+	+
	M1CR	+	+	+	-	-	-	+	i	+	+	-
	M1CSP	+	+	+	+	-	-	+	+	+	+	+
	M15P	+	+	+	+	-	-	+	i	+	+	+
	M15SP	+	+	+	+	-	-	+	+	+	+	+
	M31SP	+	+	+	+	i	-	i	-	+	+	+
	NC2P	+	+	+	i	-	-	+	i	+	+	+
	NC4-2P	+	+	+	+	-	-	+	+	+	+	+
	NC4-2R	+	+	+	i	-	-	+	-	+	-	+
	S1BP	+	+	+	i	-	-	+	-	+	+	+
	S1BR	+	+	+	-	-	-	+	i	+	+	-
	S1BSP	+	+	+	+	-	-	+	-	+	+	+
	S2BQ	+	+	+	+	-	-	+	-	+	+	i

ND- Not Determined

i= intermediate; + denotes positive and – denotes negative

Table 5.2: Frequency of *erm*(B) and *tet*(M) conjugative transfer per recipient

	<i>erm</i> (B)		<i>tet</i> (M)	
	1:1*	1:10	1:01	1:10
<i>L. monocytogenes</i> 10403S [§]	$1.68 \pm 2.15 \times 10^{-6}$	$4.43 \pm 1.35 \times 10^{-7}$	$4.71 \pm 6.5 \times 10^{-7}$	$6.24 \pm 8.8 \times 10^{-7}$
<i>L. pseudomesenteroides</i> BW2CY	$1.13 \pm 1.5 \times 10^{-6}$	$6.92 \pm 8.58 \times 10^{-7}$	$< 4.17 \times 10^{-10}$	$< 4.38 \times 10^{-10}$
<i>L. pseudomesenteroides</i> M15Y	$1.94 \pm 1.46 \times 10^{-7}$	$1.75 \pm 1.12 \times 10^{-7}$	1.35×10^{-9}	$< 3.09 \times 10^{-9}$
<i>L. pseudomesenteroides</i> NC47Y	$< 4.52 \times 10^{-10}$	$< 4.18 \times 10^{-10}$	$< 3.61 \times 10^{-10}$	$< 6.81 \times 10^{-10}$
<i>L. pentosus</i> 1.2.11	$< 1.48 \times 10^{-9}$	$< 5.67 \times 10^{-9}$	$< 2.53 \times 10^{-9}$	$< 2.45 \times 10^{-9}$
<i>L. pentosus</i> LA 0445	$< 1.41 \times 10^{-10}$	$< 3.53 \times 10^{-10}$	$< 1.20 \times 10^{-9}$	$< 1.61 \times 10^{-9}$
<i>L. plantarum</i> 3.2.8	$< 1.40 \times 10^{-10}$	$< 4.49 \times 10^{-10}$	$< 1.15 \times 10^{-9}$	$< 1.33 \times 10^{-9}$

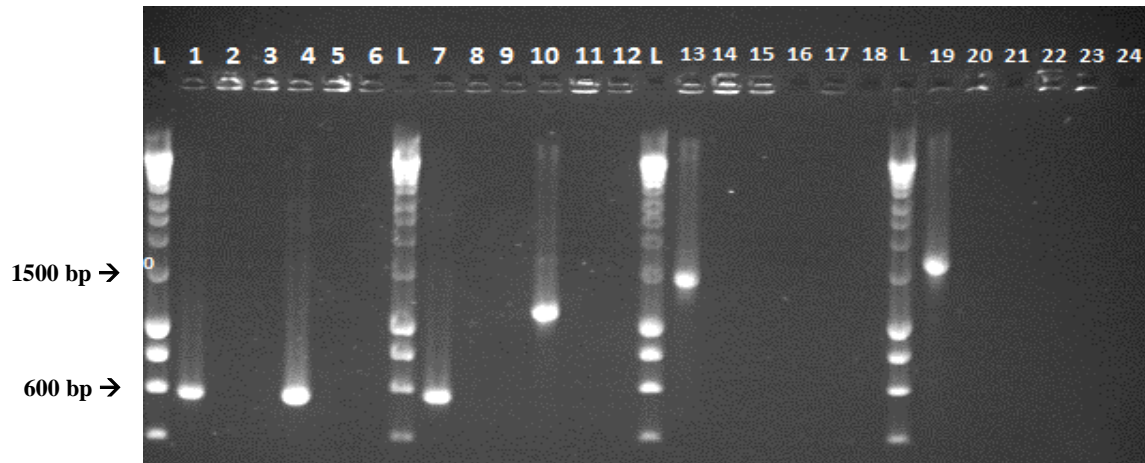
[§]Positive control for the conjugation.

The donors of *erm*(B) and *tet*(M) were *E. faecalis* JH2-2 and *L. monocytogenes* N057F.1/2FB, respectively

*1:1 and 1:10 indicate the donor: recipient ratio

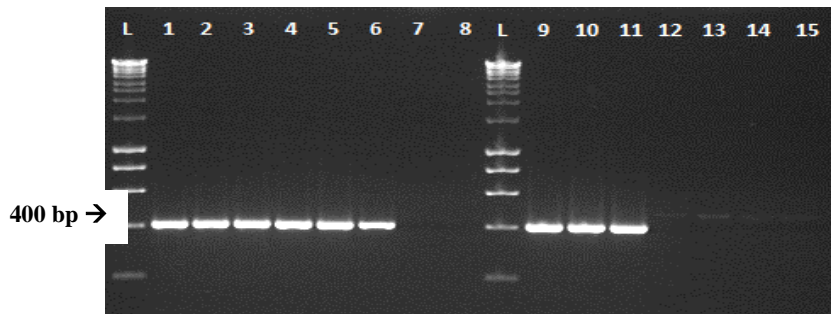
FIGURES

Figure 5.1: Detection of genes involved in As and Cd resistance



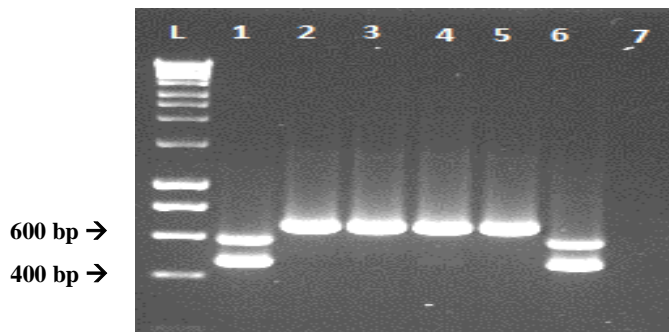
Lanes: L: DNA size markers (HyperLadder™ 1kb, Bioline); 1-3: *cadA1* PCR of *L. monocytogenes* LWA131, BW1DZ and BW2BY; 4-6: *cadA2* PCR of *L. innocua* CLIP11262, BW1DZ and BW2BY; 7-9: *cadA3* PCR of *L. monocytogenes* EGDe, BW1DZ and BW2BY; 10-12: *cadA4* PCR of *L. monocytogenes* F8027, BW1DZ and BW2BY; 13-18: *arsA1* PCR of *L. monocytogenes* F8027, BW2BY, S3Y, NC42N, *L. monocytogenes* H7550 and nuclease free water; 19-24: *arsA2* PCR of *L. monocytogenes* F8027, BW2BY, S3Y, NC42N, *L. monocytogenes* H7550 and nuclease-free water.

Figure 5.2: Detection of *erm*(B) PCR products



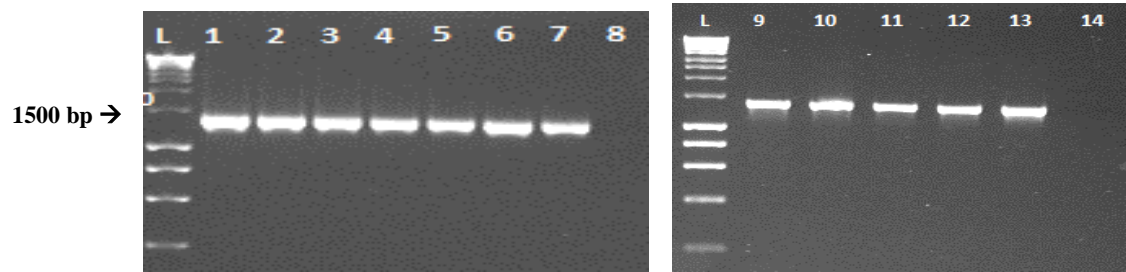
Lanes: L: DNA size markers (HyperLadder™ 1kb, Bioline); 1-4: transconjugants of *L. monocytogenes* 10403S; 5-6: Donor strain *E. faecalis* JH2-2; 9-11: transconjugants of *L. pseudomesenteroides* NC47Y; 12-15: Recipient *L. pseudomesenteroides* NC47Y

Figure 5.3: Serotypic differentiation of strains of *L. monocytogenes* 10403S (type 1/2a) and N057F.1/2FB (type 4b)



Lanes: L: DNA size markers (HyperLadder™ 1kb, Bioline); 1: parent donor of *L. monocytogenes* N057F.1/2FB; 2: Recipient *L. monocytogenes* 10403S; 3-5: transconjugants of *L. monocytogenes* 10403S from 1:1 conjugation spots; 7: *E. faecalis* JH2-2 (negative control)

Figure 5.4: Detection of *tet(M)* PCR products



Lanes: L: DNA size markers (HyperLadder™ 1kb, Bioline); 1-2 and 6-7: transconjugants of *L. monocytogenes* 10403S from 1:1 conjugation spots; 3-4, 9: parent donor *L. monocytogenes* N057F.1/2FB from the conjugation spot; 8: Recipient *L. monocytogenes* 10403S from the conjugation spot with no *tet(M)* PCR product; 10-13 transconjugants of *L. pseudomesenteroides* M15Y from 1:1 conjugation spots and 14: Recipient *L. pseudomesenteroides* M15Y.

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Chapter 6

Conclusions and Future Directions

Efforts to study starter cultures for commercial cucumber fermentation began as early as the 1950s. However, industry has not adopted starter cultures due to lack of aseptic inoculation in industrial open-top tanks, costs involved and lack of comprehensive study on safety of utilization of starter cultures. Further understanding of autochthonous bacteria in commercial cucumber fermentations is needed to guide development of starter culture applications. A major objective of this study was to enhance understanding of such autochthonous bacteria. A collection of lactic acid bacteria from an industrial fermentation tank in two locations (NC and MN) were obtained including but not limited to *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Pediococcus* spp. and *Weissella* spp. Based on *16S rRNA* sequencing. Seven tentative *L. plantarum* isolates that were rapidly able to ferment in cucumber juice and coexist with *L. brevis* while inhibiting *L. buchneri* were studied for their starter culture properties relevant to cucumber fermentation. Genotypic detection tools including MLST and analysis of *16S rRNA* and housekeeping genes such as, *recA*, *pheS*, *rpoA* and *dnaK* identified seven isolates were *L. pentosus* and only one *L. plantarum*. Randomly Amplified Polymorphic DNA (RAPD) procedure was optimized in this study to differentiate the *L. pentosus* and *L. plantarum* isolates at a strain level. The carbohydrate utilization pattern based on API 50CH[®] strips gave an atypical fermentation profile for all the *L. pentosus* and only confirmed the identity of *L. plantarum*. The *L. pentosus* isolates in this study are metabolically atypical given their inability to utilize pentose sugar D-xylose. Based on the partial sequence of *recA* PCR product (218 bp), more than 95% isolates from NC tank and more than 70% isolates from MN were identified as *L. pentosus* suggesting that *L. pentosus* was in fact the predominant bacteria in commercial cucumber fermentation. This is the first study in literature demonstrating that *L. pentosus*, and not *L. plantarum*, was the predominant bacterium during cucumber fermentation.

This also indicates that geographical location and prevailing weather conditions are key variables contributing towards variations in other fermentation microbiota.

Starter culture properties relevant to commercial cucumber fermentation were also developed and explored in detail throughout this study. Properties including- fermentation in NaCl-free brine, carbohydrate utilization, malic acid decarboxylation, amounts of D- and L- lactic acids produced, production of biogenic amines, exopolysaccharides and bacteriocins and incidence of antibiotic resistance were studied in depth. The isolates obtained in this study were capable of malic acid decarboxylation yielding CO₂, which is expected to cause bloating. Therefore, air purging would still be required even with the use of these starter cultures. D- and L- lactic production was equimolar in cucumber juice but a higher amount of D- lactic acid was produced in MRS medium. Exopolysaccharide (EPS) production was seen in all isolates in a cucumber juice-based medium but only by few isolates on medium with individual sugars such as glucose, fructose and sucrose indicating that growth matrix has an impact on EPS production. Bacteriocin activity was not detected phenotypically in any of the isolates even though genes for plantaricin production were found. Even under conditions simulating native cucumber fermentations such as growth in cucumber juice in the presence of heterofermentative bacteria and yeasts, did not display any bacteriocin activity indicating that the genes are either inactive or regulated in such a way that they are produced only under specific conditions not included in the current study.

Antibiotic resistance is widely being investigated in various foods and especially in fermented foods that contain live cultures. The minimum inhibitory concentrations (MIC) of antibiotics ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin and tetracycline were within the cut-off limits proposed by European Food Safety Authority indicating that there

is no antibiotic resistance genes in these isolates that may potentially disseminate to other bacteria. However, their potential to acquire antibiotic resistance genes and subsequently transfer them to other bacteria remains poorly characterized.

Fermentation studies in one gallon jars revealed that these isolates do not have a consistent pattern of fermentation. Some isolates performed superior in utilizing sugars to produce lactic acid while others did not. Similarly some isolates rapidly reduced the pH by producing lactic acid and increased in counts quicker at 15 °C than others. Industrial-scale NaCl-free fermentation with these starter cultures displayed a quick fermentation where the pH reached the lowest and lactobacilli counts reached a maximum within 3 days unlike the usual 7-14 days. Unfortunately, a definite starter culture that is a “one size fits all” could not be identified in this study. Depending on the manufacturer’s priority there is a wide choice of starter cultures available from this study. More studies on metabolite production and sugar utilization are necessary prior to implementing use of these starters on a commercial scale.

Co-resistance of antibiotics with heavy metals and disinfectants in diverse LAB was tested. Lactic acid bacteria isolated from fresh cantaloupe rinds belonged to the genera *Leuconostoc* and *Lactococcus* based on 16S rRNA sequencing. Co-resistance of LAB isolates from fresh cantaloupe and commercial cucumber fermentation with heavy metals arsenic and cadmium, benzalkonium chloride and antibiotics erythromycin and tetracycline was not observed. However, resistance towards individual heavy metals and antibiotics was detected in few LAB from cantaloupes. Conjugative transfer of erythromycin and tetracycline genes *erm(B)* and *tet(M)*, respectively were tested in three cantaloupe (*L. pseudomesenteroides*) and three cucumber fermentation isolates (two *L. pentosus* and one *L. plantarum*). Conjugative transfer of *erm(B)* was observed in two isolates of *L. pseudomesenteroides* from *Enterococcus faecalis* as

the donor. Similarly, conjugative transfer of *tet(M)* was observed in one isolate of *L. pseudomesenteroides* from *L. monocytogenes* as the donor. Isolates from commercial cucumber fermentation did not participate in conjugative transfer of both *tet(M)* and *erm(B)*. This study highlighted the inability of the cucumber fermentation isolates to participate in conjugation with a low potential of antibiotic resistance gene acquisition which may be attributed to their source of isolation. However, these isolates may participate in conjugative exchanges with other bacteria. Thus, their antibiotic resistance must be carefully monitored in the future if they are implemented as starter cultures commercially.

There is a need for clear guidelines on “ideal starter culture properties” since there have been no documentation of these comprehensive recommendations since the 1980s. New information is increasingly obtained on autochthonous bacteria of the cucumber fermentation ecosystem, safety and quality concerns in fermented foods and lifestyle choices of consumers which have hindered the adoption of starter cultures by the pickle industries. Future studies should be directed towards finding autochthonous bacteria that are incapable of malic acid decarboxylation naturally. Physiological and metabolic aspects including the bacteriocin ‘pentocin’ production from *L. pentosus*, quorum sensing signals that stimulate bacteriocin production must be well explored. Similarly, the role of EPS production in these starter cultures in cucumber fermentation needs to be explored. The antibiotic resistant transconjugants obtained in this study may have a potential to subsequently transfer these acquired genes to other pathogens or to commensal bacteria; this must be investigated further. Manufacturers may benefit from implementing these starter cultures for rapid fermentation at lower temperatures especially in the Midwestern US where the fermentation is usually limited to only summer months. However, before any of the isolates are commercialized, comprehensive industrial scale

trials are necessary to check the consistency of the process and products at the end of fermentation and during storage. The starter culture properties characterized in this study can be used as guidelines for future studies of starter cultures for cucumber fermentation. Due to the growing interest in probiotic live cultures; these isolates should also be studied further for potential health benefits to assess their probiotic prospective. This would not only increase revenue for the manufacturers but also help the consumers to achieve better health through consumption of fermented food products.

APPENDIX

Table A.2.1: Distribution of the antimicrobial activity presented by the *L. plantarum*/*L. pentosus* cultures against *L. brevis* and *L. buchneri* indicator strains[§]

Grouping Based on Response Patterns	Indicator strains			Fermentation age (d) at the time of isolation			
	<i>L. brevis</i> LA0036	<i>L. buchneri</i> LA1147	<i>L. buchneri</i> LA1149	1	3	7	14
No. of Cultures Found Incompatible for Mixed Starter Cultures							
Pattern 1	+	+	+	15	17	16	15
Pattern 2	+	-	+	2	2	2	5
Pattern 3	+	+	-	1	ND [¥]	ND	1
Pattern 4	+	-	-	1	ND	ND	ND
No. of Cultures Found Compatible for Mixed Starter Cultures							
Pattern 5	-	-	+	2	1	ND	ND
Pattern 6	-	+	-	ND	ND	ND	ND
Pattern 7	-	+	+	ND	2	ND	ND
Pattern 8	-	-	-	ND	ND	ND	1

[§]Table compiled by Dr. Ilenys Pérez-Díaz

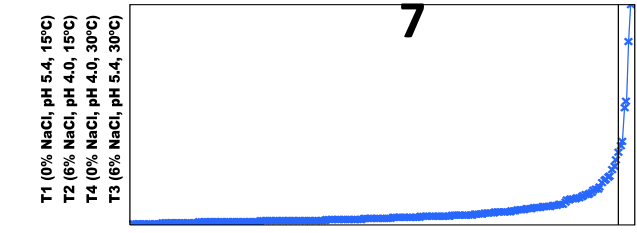
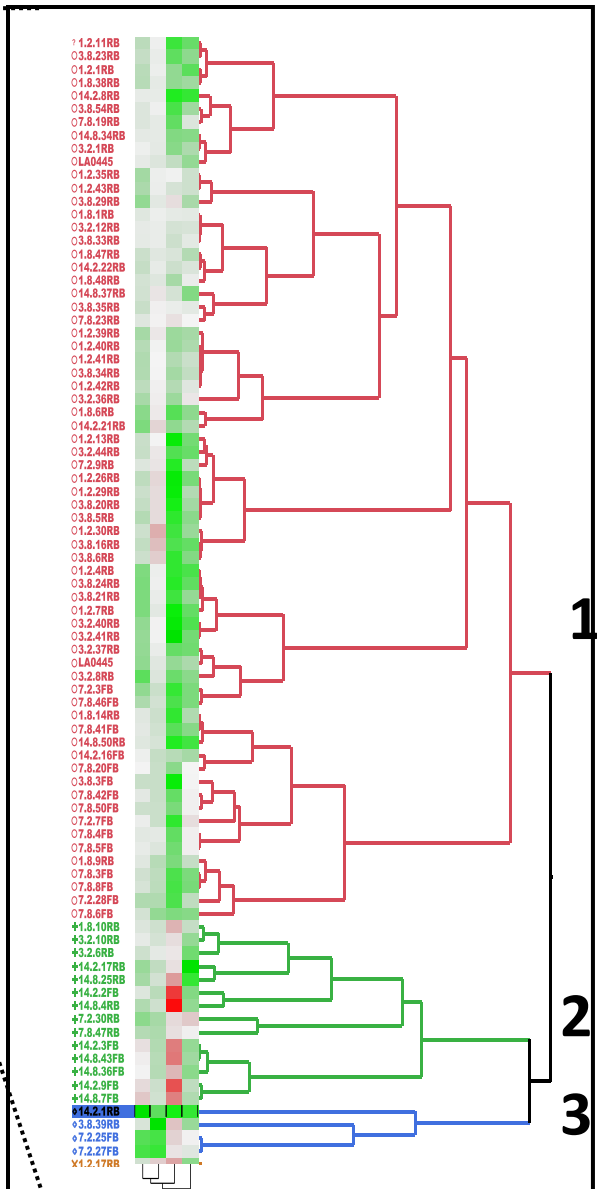
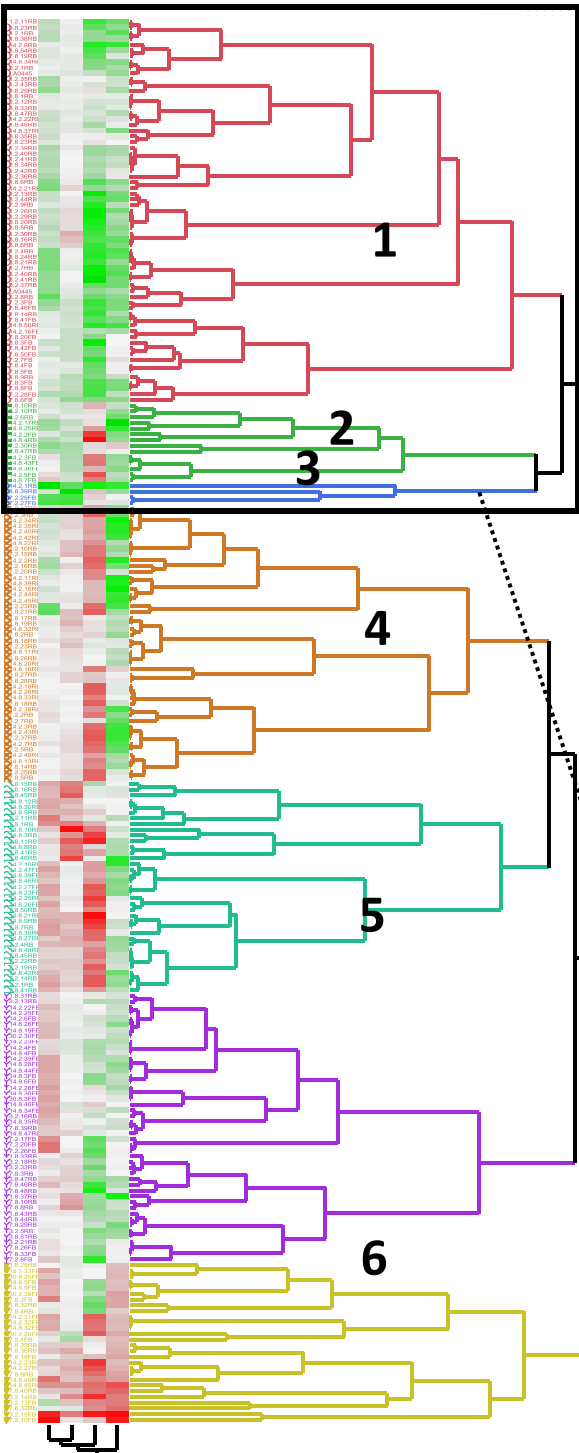
[¥] ND: not detected

Table A.3.1: Accession numbers of nucleotide sequences in GenBank

Isolate ID	Accession Numbers for the Partial Gene Sequences Obtained							
	<i>xyIA</i>	<i>plnD</i>	<i>plnE</i>	<i>plnEF</i>	<i>plnG</i>	<i>bip1</i>	<i>ebp1</i>	<i>eclD1</i>
1.2.11			KT281855		KT281846	KT215180	ND	KT215171
1.2.13					KT281847	KT215186	KT215168	KT215172
1.8.6			ND		KT281848	KT215181	KT215169	KT215173
1.8.9		ND		ND	KT281849	KT215185		KT215174
3.8.24					KT281850	KT215182	ND	KT215175
3.2.37	ND*		KT281856		KT281851	KT215187		KT215176
LA 0445	KT124139		KT281857		KT281852	KT215183	KT215170	KT215177
3.2.8		KT281844	KT281858	KT281860	KT281853	ND		KT215178
<i>L. plantarum</i> WCFS1	ND	KT281845	KT281859	KT281861	KT281854	KT215184	ND	KT215179
<i>L. pentosus</i> ATCC 8041	KT124140							

*ND- Not Determined

Figure A.2.1: Hierarchical Cluster Analysis (HCA) of *L. plantarum/pentosus* isolates from commercial cucumber fermentations. Two-way clustering of isolates based on 48 h fermentation pH under varying conditions of salt, initial pH, and temperature (Figure generated by Dr. Suzanne Johanningsmeier)



T1 (0% NaCl, pH 5.4, 15°C)	T2 (6% NaCl, pH 4.0, 15°C)
4.02	3.4
4.1752	3.4939
4.3305	3.5878
4.4857	3.6817
4.6409	3.7756
4.7962	3.8695
4.9199	3.9206
5.0437	3.9717
5.1675	4.0228
5.2912	4.0739
5.415	4.125

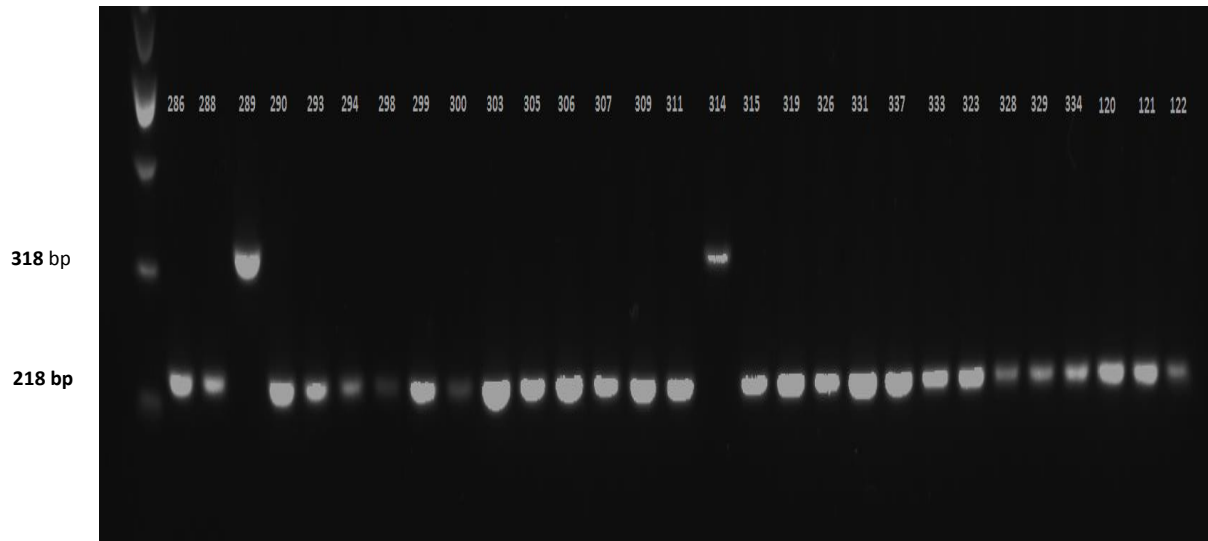
T4 (0% NaCl, pH 4.0, 30°C)	T3 (6% NaCl, pH 5.4, 30°C)
3.03	3.21
3.1318	3.2851
3.2336	3.3603
3.3354	3.4354
3.4372	3.5106
3.539	3.5857
3.6392	3.9606
3.7394	4.3354
3.8396	4.7103
3.9398	5.0851
4.04	5.46

Figure A.2.2: Agar spot assay for detection of antimicrobial activity (The spot on the top is isolate *L. brevis* 14.8.28 (415) that produced a zone of inhibition against indicator *L. brevis* LA 0036 and the spot on the below is the control with no zone of inhibition)

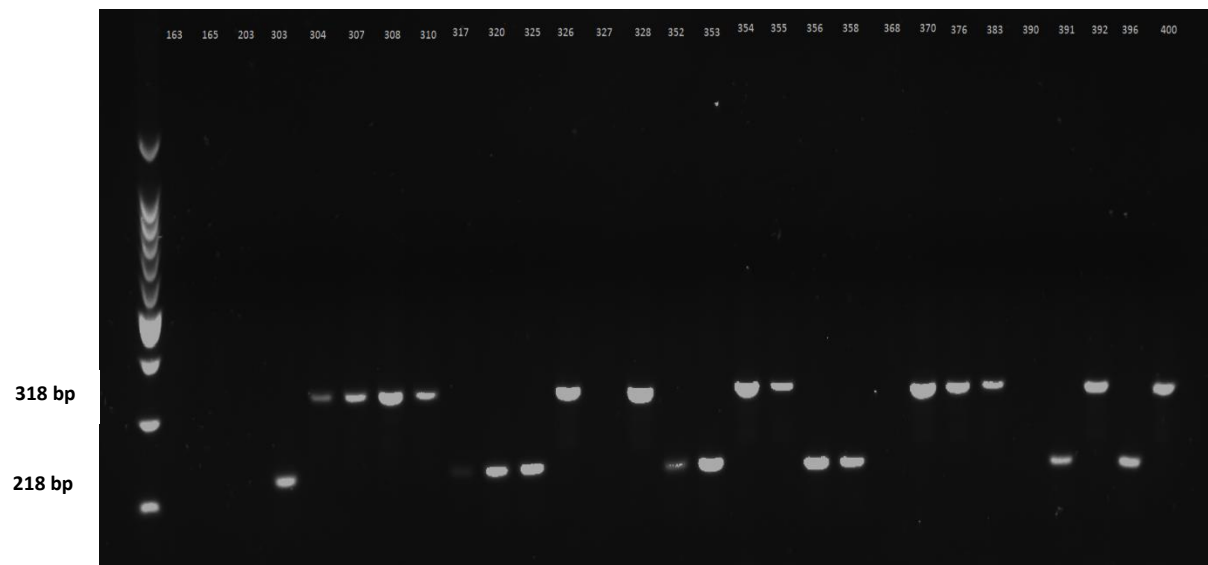


Figure A.2.3: Example of recA based identification of *L. plantarum* and *L. pentosus* isolates from a) NC tank and b) MN tank

(a)



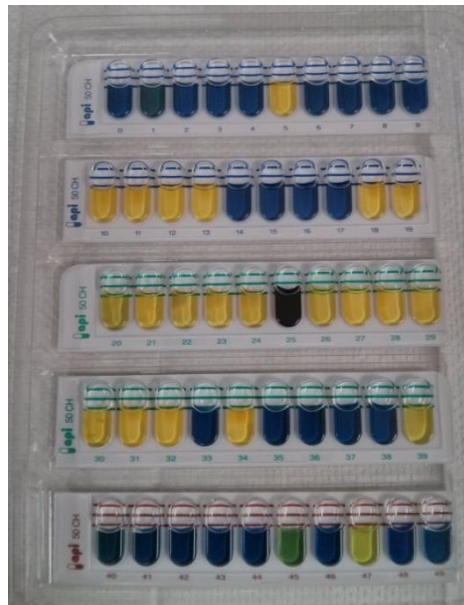
(b)



The *recA* PCR product of *L. pentosus* is 218 bp while *L. plantarum* PCR product size is 318 bp.

*ND- Not Detected

Figure A.3.1: Carbohydrate utilization profile* of *L. plantarum* 3.2.8



A color change from blue to yellow indicates positive and to light green indicates intermediate positive and no color change indicates negative.

*Please refer to (www.fishersci.com/shop/products/biomerieux-api-50-ch-medium/b50300) for the complete list of sugars used in this strip.

Figure A.3.2: Example of biogenic amine detection on plates by color change from yellow to purple. Production of putrescine from ornithine by a) *Lactobacillus 30a* (positive control) and no color change by b) *L. plantarum* 3.2.8

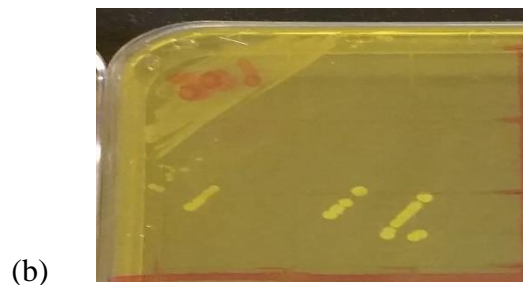


Figure A.3.3: Agar spot assay on isolates *L. pentosus* 1.2.11 (top) and 1.2.13 (bottom) against a) *I. occidentalis* Y0089, b) *Z. globiformis* Y0069, c) *P. manshurica* Y098 and d) *C. etchellsii* Y095. *L. pentosus* isolates in this study did not produce zones of inhibition against the yeasts tested

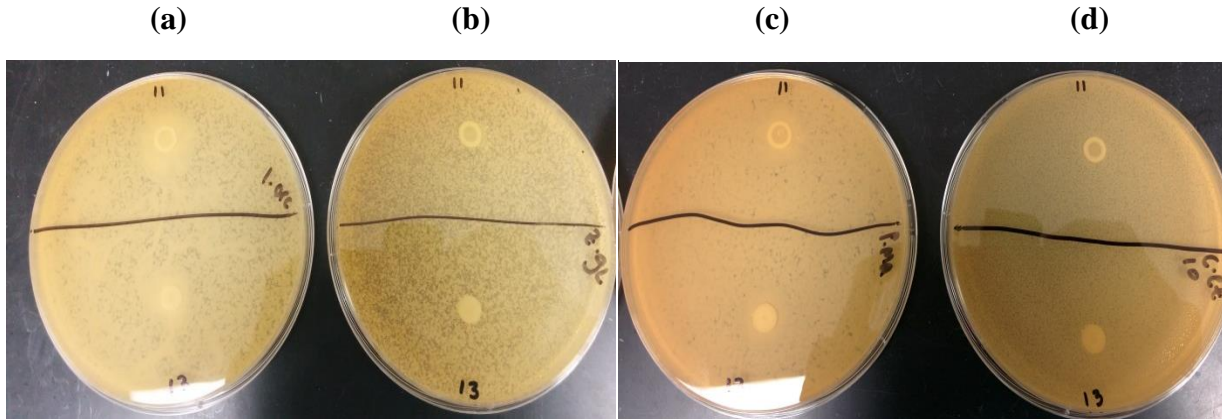


Figure A.3.4: Example of MIC determination using Etest[®] strips. Zones of inhibition formed by *L. plantarum* 3.2.8 against erythromycin (EM; MIC- 1 µg/mL) and chloramphenicol (CL; MIC- 6 µg/mL)

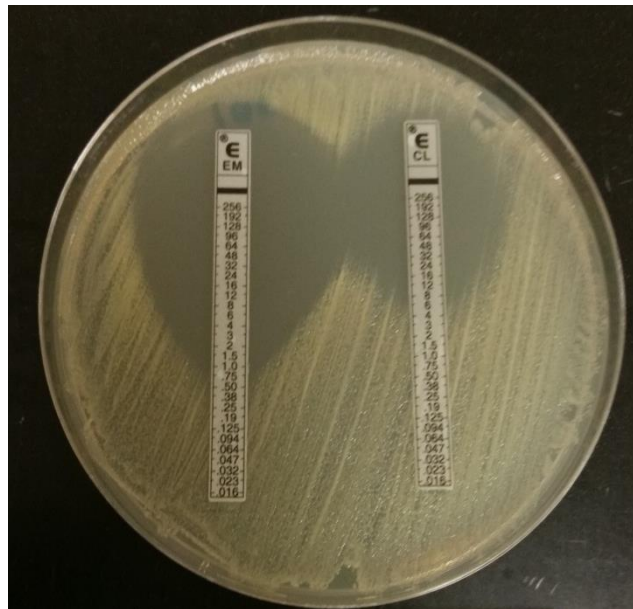


Figure A.4.1: Jar fermentation set up in the incubator



Figure A.4.2: Cucumber pickles after 14 days of fermentation in control jar (left) and pickle from the jar inoculated with *L. plantarum* 3.2.8 with excess white spots (right)

