INFLUENCE OF GROWTH MEDIUM AND pH ON VIABILITY AND FATTY ACID COMPOSITION AFTER FREEZE-DRYING AND STORAGE OF LACTOBACILLI

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1991

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2010

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DEDICATION

This thesis is dedicated to my professor and mentor

Dr. Stanley E. Gilliland

He was truly a very wonderful and generous man and I will

be forever grateful to him.

ACKNOWLEDGMENTS

I have been in the Food Science department of OSU for what seems like forever and have met so many great people and watch them leave. They touched my life in so many ways and this thesis would not have been completed without their valuable help, support, and inspiration. I would like to express my gratitude to those who have made this journey productive and enjoyable.

I appreciate the kindness of the members of my committee: my advisor, Dr. William McGlynn, for all the help he provided me especially during the final submission of this thesis. Thanks to Dr. Christina De Witt and Dr.Rodney Holcomb whose suggestions helped guide my research writing. I am also grateful to Dr. Emilia Paloma Cuesta Alonso for all the help and guidance especially in running the Gas Chromatography. I would also like to thank Dr. Carla Goad for helping in the statistical analysis of my data.

I am very thankful to my friends, Rien and Jessica who were always ready to provide much needed help during my experiments. I will treasure our friendship forever. I also want to thank all my fellow graduate students in Dr. Gilliland's lab for their help and support.

Many friends have been wonderful and helpful throughout these years. I would like thank Bads, Jorge, Pauline, Menchu, and Anh for their love and friendship and for being my family away from home. I special thanks to my fiancé, Scott Shannon for his love and support.

My family has been a source of inspiration to me; especially my *Nanay* (mother) and I dedicate this thesis to her. She has always given me unconditional love, prayers and encouragement. My brothers and sisters have also been very supportive throughout my life and I would never have accomplished all of these without them. Although they are thousands of miles away I know that they are proud of my accomplishments.

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CHAPTER I

INTRODUCTION

Lactic acid bacteria (LAB) play important roles in food, animal products and pharmaceutical industries due to their broad range of application. They are often used as starter cultures in dairy products such as yogurts and cheeses and over the last few decades some strains of lactobacilli have been introduced to food products as probiotics to improve human health (Sodini et al., 2002). Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" (FAO/WHO, 2001). Health benefits include immunomodulation (Herich and Levkut, 2002; Perdigon et al., 1995), lowering serum cholesterol (Brashears et al., 1998; Buck and Gilliland, 1994; Gilliland et al., 1985), improving lactose digestion (Gilliland and Kim, 1984; Lin et al., 1991), and anti-tumor effects (Ma et al., 2004; Zhang et al., 2005). In order to provide health benefits, suitable levels of viable cells of probiotic bacteria are recommended in food products during the shelf life for efficacy. The suggested level for probiotic bacteria per gram of product is $\geq 10^6$ colony forming units (CFU) (Cogan et al., 2007) but some clinical studies use daily doses of 10^9 CFU/day (Meng et al., 2008).

Commercial starter and probiotic culture preparations are utilized for ease of application in the production of foods. Freezing, spray-drying or freeze-drying are

some of the preservation methods employed to maintain the viability and other bacterial properties such as, acid production, aroma production, texture formation and probiotic properties (Wang et al, 2005). Frozen or freeze-dried cultures with high population of viable and uninjured cells are needed and such cultures must be stable during storage and shipping (Gilliland and Speck 1974; To and Etzel, 1997); however, some of these preservation processes result in undesirable effects such as decreased viability and denaturation of sensitive proteins (Carvalho et al., 2004; Gomez-Zavaglia et al., 2000).

The cell membrane is one of the primary sites of damage during these processes and studies have shown that sensitivity or resistance to freezing and freeze-drying is related to changes in the fatty acid composition of some lactic acid bacterial cells (Goldberg and Eschar, 1977; Gilliand and Speck 1974; Smittle et al., 1974; Johnsson et al., 1995). Different factors such as growth medium, culture pH, growth temperature, bacterial strain, growth time, and gas atmosphere can potentially affect cell physiology in a way which contributes to the stability of the processed cells (Saarela et al., 2009) and improve viability (Gilliland and Speck 1974; Smittle et al., 1974; Johnsson et al., 1995; Saarela et al., 2009).

The objective of this study was to determine the influence of Tween 80 and pH on viability and fatty acid composition after freeze-drying and storage of different probiotic strains of lactobacilli.

CHAPTER II

REVIEW OF LITERATURE

Probiotics

Probiotic is derived from the Greek language that means “for life” and over the years its definition has evolved (Itsaranuwat et al., 2003). Probiotic bacteria are living microorganisms that can have beneficial health effects to the host when ingested (Fuller, 1989; Sodini et al., 2002). With the first definition proposed in 1965, probiotics have been gaining popularity (Fioramonti et al., 2003) and there is increasing evidence on the health benefits by consumption of foods containing probiotics (Sullivan and Nord, 2005). Such benefits include immune modulation, improvement of the intestinal flora, prevention or shortening of diarrhea, control of serum cholesterol, and reduction of occurrence of inflammatory bowel disease (Kumura et al., 2004). The mechanisms by which probiotics exert their effects may involve modifying gut pH by producing various acids, antagonizing pathogens through production of antimicrobial or antibacterial compounds such as hydrogen peroxide and bacteriocins, competitions with pathogens for nutrients or adhesion receptors, and stimulation of the immune system (Kopp-Hoolihan, 2001; Marteau et al., 2002).

Aside from health benefits, probiotics produce desirable organoleptic properties and improve nutritional values of food (Parvez et al., 2006). Probiotic bacteria must fulfill several criteria before they can benefit human health, i.e., they must be manufactured and incorporated into food products without losing viability and functionality, must survive passage through the upper gastrointestinal (GI) tract, and must function in the gut environment (Mattilla-Sandholm et al., 2002). Probiotics are marketed as concentrated cultures in frozen, powder or capsule forms (De Roos and Katan, 2000).

The best studied probiotics are the lactic acid bacteria, particularly *Lactobacillus* sp., *Streptococcus* sp., and *Enterococcus* sp., but other organisms such as *Bifidobacterium* ., *Bacteroides* sp., *Bacillus* sp., *Propionibacterium* sp., and various fungi are also used as probiotics (Rolfe, 2000). Lactic acid bacteria (LAB) are Gram positive, facultatively anaerobic, non-spore forming, catalase negative, devoid of cytochromes, cocci or rods, which produce lactic acid as their main fermentation product (Axelsson, 1998). They are among the best studied microorganisms and are widely used in the manufacturing of fermented foods as starter cultures and probiotics. The largest genus within the group of LAB is *Lactobacillus*. It contains very large number of species that have been isolated from various sources including humans, animals, plants and foods. (De Angelis and Gobbetti, 2004).

Preservation of bacterial cultures

Concentrated bacterial starter cultures have been used in the manufacture of dairy foods due to their important role in fermentation processes. They are prepared from bacteria that have been grown under closely controlled conditions, concentrated into smaller volumes, and placed in frozen storage (Gilliland and Speck, 1974). The

development of concentrated commercial starter cultures is the result of widespread interest in the direct inoculation of the culture during the manufacturing of cultured foods (Keogh, 1970). In producing culture concentrates it is economically important to obtain the largest yields of cells possible that possess maximum biological activity (Gilliland and Speck, 1974).

Industrial applications and uses of LAB starter and probiotic cultures depend on the concentration and preservation technologies, which would guarantee long-term stability of cultures in terms of viability and functional activity (Carvalho et al., 2004). Different methods have been used for the preservation of microorganisms for decades. Commercial starter cultures were initially supplied in liquid form before the introduction of concentrated starter cultures and advances in biomass production have later brought frozen and freeze-dried forms of starter cultures (Santivaranghkna et al., 2007). Freezing and freeze-drying are the most common processes of preservation of cultures and have become standard methods for long-term maintenance of bacterial cultures. However, they provide varying degree of success with different bacteria since they cause undesirable side effects, such as denaturation of sensitive proteins and decreased viability of many cell types (Carvalho et al., 2004).

Preservation Methods

Freezing

Commercial starter cultures were initially supplied in liquid form. The dairy fermentation industry first used commercially frozen concentrated lactic starter cultures in 1963 (Speckman et al., 1974). Freezing is a method used to produce frozen bacterial

cultures either as an end product or an intermediate product for subsequent freeze-drying process (Volkert et al., 2008). It is a process commonly used to assure long term viability of LAB while maintaining their acidification activity and organoleptic and preservative properties (Fonseca et al., 2001); however, freezing causes different cellular injuries on LAB (Volkert et al., 2008). Freezing causes damage to cells due to formation of ice crystals and high osmotic pressure brought about by high internal solute concentrations (De Angelis and Gobbetti, 2004). Moreover, frozen cultures are heavy, occupy large volumes, and must be stored at sub-zero temperatures, all of which result in high shipping, storage, and energy costs (Johnsson and Etzel, 1994; Santivaranghkna et al., 2007).

Freeze-drying

Freeze drying or lyophilization is a process in which a solvent is removed from a frozen solution by sublimation (Castro et al., 1997). The procedure consists of the following three phases: freezing, primary, and secondary drying (Meng et al., 2008). Cells are typically frozen at -196 ^{o}C and dried by sublimation under high vacuum (Sativarangkna et al., 2007). This is a commonly used stabilization technique for lactic acid bacteria (Schoug et al., 2008) and it offers ease of storage, handling, and transport, as well as long term viability (Miyamoto-Shinohara et al., 2006). This process also minimizes the degradation reactions and maintains adequate physical, chemical, and biological stability of the product during long-term storage at ambient temperature (Fonseca et al., 2004). Distribution of freeze-dried cultures is more convenient and easier

since it does not require freezing conditions during transportation however; the process is lengthy and more expensive than other drying processes (Santivaranghkna et al., 2007).

Freeze-drying results in a variety of survival levels with different methods and species. Miyamoto-Shinora et al., (2006) reported the survival of a variety of microorganisms following storage for up to 20 years. They observed that *L. acidophilus* and *Enterococcus faecium* exhibited survival of 62.5 and 85.2%, respectively, shortly after freeze drying. The survival rate of Gram positive bacteria, like *Brevibacterium flavum, B. lactofermentum, Corynebacterium acetoacidophilum,* and *Streptococcus mutans,* were around 80% and did not decrease greatly during the storage period of 10 years except for *S. mutans* which decreased about 20% (Miyamoto-Shinohara et al., 2000). They also reported that survival of Gram negative bacteria, i.e., *Escherichia coli, Pseudomonas putida,* and *Serratia marcescens,* was around 50% and decreased for the first 5 years and stabilized to around 10% thereafter.

Spray-drying

Spray-drying is an alternative inexpensive method that has higher production yields compared to freeze-drying (Meng et al., 2008; Morgan et al., 2006). It involves the injection at high velocity air at temperatures up to 200 ^{o}C which then blasts the slurry solution through a nozzle to form granules. Aside from heat stress, the bacterial cells are exposed to dehydration, oxygen and osmotic pressure and this can lead to increased cell permeability and cell damage (Brennan et al., 1986; Texeira et al, 1995)

Factors affecting growth and survival after preservation

Several factors are relevant for the preparation of bacterial cultures that will have high number of cells and long term viability during storage. These include intrinsic factors, such as cell shape and size, genetic composition, and differences in cell wall and membrane composition, growth conditions, drying medium and storage conditions (Carvalho et al., 2004). Most studies on freeze-drying of lactic acid bacteria were performed on the effects of freezing media, freezing and drying conditions, modified atmosphere packaging and rehydration and storage conditions but there are few studies on the influence of growth media on subsequent survival of the cells during freeze drying (Champagne et al., 1991).

During freezing, drying and storage, distinct species of one genus or strains within the same species may exhibit different behaviors (Carvalho et al., 2004). Several hypotheses such as variation in genetic constitution and cell wall composition were cited to cause variability (Carvalho et al., 2004). Cell morphology, i.e., size and shape, as well as cell structure, were also reported to be correlated to viability of cells (Gilliland and Speck, 1974; Carvalho et al., 2004; Santivaranghkna et al., 2007). Small spherical cells, like streptococci, are apparently more resistant to freezing and freeze-drying than rod-shaped cells like lactobacilli (Fonseca, et al., 2000). According to this research, during freezing membrane damage is higher with cells with bigger volume due to extracellular ice crystal formation that can eventually cause osmotic stress to cells (Fonseca et al., 2006).

Growth Medium Composition

Lactobacilli are nutritionally fastidious microorganisms that have complex growth requirements such as carbohydrates, vitamins, proteins, amino acids, and nucleic acid derivatives (Partanen, et al., 2001; Ringo and Gatesoupe, 1998). Because of their high demands for nutrients they are abundant in habitats that can provide these requirements such as rotting plants, milk, and mammalian intestinal tracts and mucous membranes (Partanen, et al., 2001).

The growth medium is a critical parameter in the survival of the bacteria subsequent to freeze-drying (Carvalho et al., 2004). It must contain all the nutrients needed for optimum growth of the bacterial cells and produce cells that will retain viability and desirable characteristics during frozen storage (Mitchell and Gilliland, 1983). For starter cultures the resulting cells must also contain the necessary complement of enzymes and biological activity to insure proper performance (Gilliland and Speck, 1974). Several factors have been identified which may explain the protection afforded by each of various growth media, such as type of sugar substrate, osmotic stress, production of exopolysaccharides and altered fatty acid profile of the membrane (Carvalho et al., 2004).

The general medium used for enrichment, cultivation and isolation of majority of the lactobacilli is MRS (de Man, Rogosa and Sharpe) medium (de Man et al., 1960). It contains a rich nutrient base, as well as polysorbate (Tween 80), acetate, magnesium and manganese which are known to act as special growth factors for lactobacilli (de Man et

al., 1960). Tween 80 is a non-ionic detergent and a water soluble ester of oleic acid which is added to the growth medium to enhance growth (Goldberg and Eschar, 1977). Oleic acid is known to be an essential growth factor for several microorganisms (Williams et al., 1947) and it had been reported that non ionic detergents containing oleic acid, free oleic acid and cis-vaccenic acid can replace the requirement for biotin by lactobacilli (Smittle et al., 1974). It can also be used as a carbon source by some bacteria (Li et al., 2001).

In addition to the growth enhancement effect of Tween 80, it was described that cultivation in the presence of Tween 80 changed the fatty acid composition of some LAB and these changes can influence their subsequent resistance to freezing and other environmental stresses (Corcoran et al., 2007; Kimoto et al., 2002; Partanen et al., 2001; Johnsson et al., 1995; Goldberg and Eschar 1977; Smittle et al., 1974). It was shown that cells of lactic streptococci grown in medium supplemented with Tween 80 had improved resistance to freezing (Gilliland and Speck, 1974). Higher survival rate during storage of freeze-dried *Enterococcus durans* was also reported by Carvalho et al. (2003) when the organism was grown in a medium supplemented with Tween 80.

Effect of pH

The population of LAB is also influenced by the pH of the growth medium. In a controlled pH study conducted by Cogan et al. (1970), favorable increase in cell population in lactic streptococci was observed. They reported that growing lactic streptococci at pH 6.0 - 6.5 favored the production of high cell numbers (Cogan et al.,

1970; Peebles et al., 1969). Similar results were also reported by Gilliland and Speck (1974). They found that lactic streptococci population was greatly increased by controlling the pH of the growth medium. The effect of the pH of growth medium on the survival of *L. delbrueckii* subsp. *bulgaricus* during spray drying was studied by Silva et al., (2005). They found that cells grown under uncontrolled pH were found to be more resistant than cells grown under controlled pH (at pH 6.5) but no significant differences were observed during storage.

Bacterial Fatty Acid

Lipids are the major component of the bacterial cell membrane. In particular, phospholipids play important roles in the maintenance of membrane functions (Tsuchiya et al., 1986). Membrane lipids are one of the most adaptable molecules that respond to perturbations, like temperature, chemicals, ions, pressure, nutrients, and the growth phase of the microbial culture (Denich et al., 2003). The fluidity of a biological membrane may be influenced by the lipid structure and the portion of saturated, unsaturated, branched, or cyclic fatty acids in individual phospholipids (Sajbidor, 1997).

All bacteria contain fatty acids and the most prevalent are usually in the range of C15 to C19 (Tracey and Britz, 1989). The lipids of Gram positive bacteria, like *Lactobacillus*, are located primarily in the membrane and mesosomal lipids of typical lactobacilli and contain even-carbon, straight-chain saturated and monoenoic acids together with cyclopropane acids (Suutari and Laakso, 1992). Optimum membrane fluidity is crucial to the survival of bacteria at low environmental temperature

(Chattopadhyay, 2006; Suutari and Laakso, 1994). It is known that the lipid composition of membranes can be altered when microbial growth temperature changes and exposure to low temperature results in the formation of a close array of acyl chains of fatty acids in the cytoplasmic membrane which results in a gelling effect on the lipid bilayer (Russell, 2002; Chattopadhyay and Jagannadham, 2001). Some strategies are adopted by microorganisms to maintain the fluidity of the cell membrane, such as, conversion of saturated fatty acids into unsaturated fatty acids by enzymes and preferential synthesis of short-chain fatty acids, branched chain fatty acids, straight chain fatty acids and iso fatty acids (Chattopadhayay, 2006; Chattopadhyay and Jagannadham, 2001; Suutari and Lakso, 1994).

Cyclopropane Fatty Acid

Cyclopropane fatty acids (CFAs) have been known for several decades to occur as phospholipids and glycolipid esters in membranes of many different bacteria that include *Enterobacteriacea*, *Pseudomonadaceae*, *Rhizobiaceae,* and *Lactobacillaceae* (Nikkila et al., 1996). The role of CFAs in lactic acid bacteria is poorly understood but they are believed to increase the fluidity of the membrane in a manner similar to that of polyunsaturated fatty acids (Johnsson et al., 1995). The increase in the unsaturated fatty acid content of membrane phospholipids decreases solid- to –fluid transition temperature and thus increase membrane fluidity (Wang et al., 2005). Cyclopropane fatty acids are formed by the addition of a methylene group derived from the methyl group of S-adenosylmethionine, across the carbon-carbon double bond of unsaturated fatty acids (Grogan and Cronan, 1997). The main CFAs in lactobacilli are, lactobacillic acid [11, 12-

methyleneoctadecanoic acid; C19:0cyc11] and dihydrosterculic acid [9,10-methyleneoctadecanoic acid; C19:0cyc9], are formed by methylation of cis-vaccenic and oleic acid, respectively (Nikkila et al., 1996). Lactobacillic acid, named after the organisms from which it was originally isolated, is the first type of CFA to be discovered in bacteria (O'Leary, 1962).

Relationship between viability and cellular fatty acid composition

A close relationship between resistance to freezing in liquid nitrogen and cellular fatty acid composition for *L. bulgaricus* was reported by Smittle et al. (1974). The storage stability and viability of cells after freezing in liquid nitrogen was improved by supplementing the medium in which the cells had been grown with sodium oleate (Tween 80). It was observed that they contained larger amounts of C18:1 and CFA and less saturated fatty acids. In a study conducted by Johnsson et al. (1995), it was reported that when the growth medium of *Lactobacillus* was supplemented with Tween 80, two new fatty acids (oleic and dihydrosterculic acid) appeared in the profile. Tween 80 contained oleic acid which was potentially transported into the bacterial cells (Johnsson et al. 1995).

Goldberg and Eschar (1977) studied the viability of *Streptococcus lactis* and *Lactobacillus* species grown in a medium supplemented with Tween 80 and they reported a pronounced change in the cellular fatty acid composition. An increase in the ratio of unsaturated to saturated fatty acids was observed resulting with an improvement in viability of both bacteria after freezing. The incorporation of Tween 80 in the growth medium for *L. rhamnosus* GG showed significant enhancement of survival of the cells at low pH of gastric juice and a change in the fatty acid composition of the cell membrane (Corcoran et al., 2007). In a study by Nikkila et al. (1995), *L. buchnerii* and *L. brevis*

cultivated in MRS broth with Tween 80 yielded different results. *Lactobacillus buchnerii* had two different CFAs, namely, dihydrosterculic and lactobacillic acid, while *L. brevis* contained dihydrosterculic acid only. This shows that the cells were able to cyclisize oleic acid supplemented by its growth medium. Incorporation of Tween 80 in the phospholipids of a non lactic acid organism was also reported in *Aspergillus niger* (Nemec and Jernejc, 2002). They reported that sterol esters and triacylglycerols were increased with the supplementation of 0.1% Tween 80 in the growth medium. The fatty acyl chains of phospholipids and the ratio of unsaturated fatty acids, as well as sterols are among the most important factors modulating the fluidity and integrity of the membrane (Nemec and Jernejc, 2002).

Membrane fatty acid alterations were reported to occur as means to adapt to different environmental stresses (Guerzoni et al., 2001; Fozo et al., 2004 Suutari and Lakso, 1992). Guerzoni and his colleagues (2001) studied the cellular fatty acid composition of *L. helveticus*, a widely used starter in the dairy industry, in response to environmental stresses such as high salt, acid, as well as, oxidative and thermal stresses. They were able to elucidate that this change in the fatty acid composition was a cellular response that protected the cells from toxic oxygen species and high temperatures. In *L. fermentum*, the interconversion of oleic, vaccenic and dihydrosterulic acids were also observed to be a response to changes in growth temperatures (Suutari and Laakso, 1992). In response to low pH, aciduric oral bacteria like *Streptococcus gordonii* DL1, *S. salivarius* 57.I, and *L.* casei 4646 also were shown to alter their membrane composition to contain increased levels of long-chained, mono-unsaturated fatty acids (Fozo et al., 2004).

Improved cryotolerance in *L. acidophilus* also was reported by Wang et al., (2005). In this study the effect of different fermentation temperature and pH were determined and the results showed a high ratio of unsaturated to saturated fatty acid, low C18:0 content and high C16:0 and CFA concentrations. They concluded that the high resistance during frozen storage was related to high CFA concentrations. Gomez-Zavaglia et al., (2000) also reported that high cycC19:0 concentration favored cryotolerance of *L. helveticus* and *L. acidophilus*. Castro et al., (1996) reported that the lipid composition of the cells of *L. bulgaricus* showed a decrease in the unsaturated and saturated fatty acid content supporting the hypothesis that membrane damage had occurred. The resistance to freezing and frozen storage of *S. thermophilus* was also shown to be related to membrane fatty acid composition (Beal et al., 2001). In this study, the incorporation of oleic acid (Tween 80) in the culture medium and the decrease in fermentation pH enhanced the ratio of unsaturated into saturated fatty acids and improved the acidification activity of the organism.

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CHAPTER III

INFLUENCE OF TWEEN 80 ON VIABILITY AND FATTY ACID COMPOSITION AFTER FREEZE-DRYING AND STORAGE OF LACTOBACILLI

Abstract

The influence of Tween 80 on survival, storage stability, and fatty acid composition during freeze-drying of strains of probiotics species of lactobacilli was studied. *Lactobacillus acidophilus* (L-1, L-23, O-16, and 381-IL-28), *L. casei* (E-5 and E-10), and *L. reuteri* X-18 were grown in MRS broth supplemented with different concentrations of Tween 80, freeze dried, sealed under vacuum, and stored at 5 ^{o}C for 21 days. At regular intervals during storage, viable cells were enumerated and at the end of storage period fatty acid was extracted and analyzed by gas chromatography. Results showed that Tween 80 improved survival of *L. reuteri* X-18 but did not improve survival of the other cultures. Tween 80 was shown to alter the fatty acid composition of the cultures. In general, addition of Tween 80 increased oleic acid concentration in the

cell membranes of most cultures. Significantly higher C18:1 and C19:0cyc11 concentrations in strain X-18 grown with Tween 80 could have contributed to improved survival during freeze-drying and storage. Minimal decline in population during storage was observed in most cultures and this was attributed to the absence of oxygen since the vials were sealed under vacuum. Strains that showed the best resistance to freeze-drying should be studied further and optimization of growth conditions is needed to increase survival during freeze-drying and storage. Further studies should be conducted to determine the optimum concentration of Tween 80 that can produce higher biomass and improve survival among the strains of probiotic lactobacilli used in this study.

Introduction

The dairy foods industry has grown over the years and consumption of cultured products like yogurt, sourdough, and cream cheese, are driving its growth. Lactic acid bacteria are commonly used as starter cultures in a wide range of fermented dairy and meat products, as well as in infant formulas and dietary supplements (Kurtmann et al., 2009). In addition its role in the fermentation process, such as acid production, aroma production, and texture formation, some strains of lactobacilli are introduced to these products as probiotics to improve human health.

Freeze-drying is used in the preservation of LAB because easy of storage, handling, transport and viability for long periods (Miyamoto-Shinohara et al., 2006). Freeze-dried lactic acid cultures are used for direct vat inoculation or added directly to various dairy products. The process of freeze-drying involves many stressful conditions

and brings some undesirable effects such as loss of viability due to damage to the cell membrane lipids and structural proteins (Gomez-Zavaglia et al., 2000; Carvalho et al., 2002).

Lactobacillus is one of the most common probiotic bacteria used in the dairy industry and several studies have been conducted to maximize its survival during the freeze-drying process, as well as improve storage stability. Survival of these bacteria throughout the preservation processes and subsequent storage is dependent on many factors such as initial cell concentration, growth medium, pH, growth temperature, and bacterial strain (Gilliland and Speck, 1974; Smittle et al., 1974; Johnsson et al., 1995). Growth medium has been shown to influence subsequent survival of cells during freeze-drying and storage and Tween 80, a nonionic surfactant, has been reported to have a cryprotective effect on cells (Gilliland and Speck, 1974; Smittle et al., 1974; Goldberg and Eschar, 1977).

The cell membrane is the primary site of damage during freezing or freeze-drying which results in drastic changes in permeability (Gomez-Zavaglia et al., 2000) and sensitivity to damage was found to be related to alterations in fatty acid composition that may protect cells against freezing (Baati et. al, 2000). Tween 80 was also reported to alter fatty acid composition of cells (Gilliland and Speck, 1974; Smittle et al., 1974; Goldberg and Eschar, 1977; Johnsson et al., 1995; Wang et al., 2005).

The objective of this study was to determine the influence of Tween 80 on survival, storage stability and fatty acid composition during freeze-drying and subsequent storage of different strains of lactobacilli.

Materials and Methods

Microorganisms and Maintenance of Cultures

Cultures of *Lactobacillus acidophilus* (strains L-1, O-16, L-23 and 381-IL-28), *L. casei* (strains E-5 and E-10) and *L. reuteri* X-18 were obtained from the culture collection in the Food Microbiology Laboratory (Robert M. Kerr Food and Agricultural Products Center, Oklahoma State University, Stillwater, OK, USA). Identification of each culture was confirmed by Gram stain, catalase test, growth at 15 ^{o}C and 45 ^{o}C, and fermentation patterns determined using API 50 CH kits (BioMérieux, Brusells, Belgium) following the manufacturer's instructions. Identification was based on characteristics presented in Bergey's Manual of Determinative Bacteriology (Sneath, 1986).

The cultures were maintained by subculturing weekly using 1% inoculum into lactobacilli Man Rogosa and Sharpe (MRS) broth (BD, Beckton Dickinson & Co., Sparks, MD, USA) and incubation at 37 ^{o}C for 18 hours. Before each experiment, all cultures were subcultured at least three times in lactobacilli MRS broth and kept at 4^{o}C between each transfers.

Determination of harvest time

Growth curves of the cultures were constructed to determine incubation time required to reach the stationary phase. The cultures were inoculated (1%) into 10 ml of MRS broth and incubated in a 37 ^{o}C water bath. Absorbance value (λ 620 nm) was read every hour for 16 h using a spectrophotometer (Spectronic 21D, Spectronic Instruments,

Rochester, NY). The values were recorded and growth curves for each culture were made by plotting absorbance values against time (Appendix B).

Growth of lactobacilli in culture media supplemented with Tween 80

Cultures were inoculated at 1% into 100 ml MRS broth supplemented with different concentrations of Tween 80 (Sigma, Wisconsin, USA). The concentrations of Tween 80 used were 0.1%, 0.2%, 0.3%, 0.4% and 0.5% (w/v). For the control, the cultures were inoculated into MRS broth without Tween 80. Cultures were incubated at 37 ^{o}C for 15 h and concentrated cultures were prepared.

Preparation of concentrated cultures

Cultures were grown in MRS broth for 15 h as described in the previous section and were concentrated using the procedure of Peebles et al. (1969). Bacterial cells were harvested by centrifugation at 10,000 x g for 10 min at 2^{o}C. Supernatant was discarded and cells were resuspended in 10 ml sterilized 10% skim milk. Aliquots of 0.5 ml were aseptically dispensed into sterile freeze-dryer vials, frozen at -70^{o}C and freeze-dried.

Preparation of cells for fatty acid analysis

Cultures were grown in MRS broth for 15 h with different concentrations of Tween 80 as described in previous section. Cells were harvested by centrifugation at 10,000 x g for 10 min at 2 ^{o}C. The supernatant was decanted and the pellets were washed twice with sterile deionized water. Cells were resuspended in 10 ml sterile deionized

water and dispensed in freeze-dryer vials. The cells were frozen at -70 ^{o}C and freeze-dried.

Freeze drying

Freeze-drying was performed in a Labconco® 6 L Benchtop Freeze Dry System with a Freezone ® Stoppering Tray Dryer (Labconco Corporation, Kansas City, MO, USA). The freeze-dryer was programmed to operate for 1 h initial freezing at -40 ^{o}C followed by primary drying at -25 ^{o}C at 0.10 mbar pressure for 48 h and secondary drying at 5 ^{o}C for 3 h with the same pressure. After the end of the freeze-drying cycle, the vials were sealed under vacuum and stored at 5 ^{o}C until analyzed (Labconco Corporation, 2007).

Determination of cell viability

Number of viable cells before freeze-drying and after storage for 1, 7, 14 and 21 days was determined by pour plate method. Dilutions were prepared from milk suspension before freezing and plated on MRS agar plates (Day 0 count). Freeze-dried samples were resuspended to the original volume (0.5 ml) in 0.1% peptone water and appropriate dilutions were prepared and plated on MRS agar. Plates were incubated at 37 ^{o}C for 48 h. Cell viability for each storage day after freeze-drying is reported as percentage survival and was calculated using the following equation:

$$\% \text{ Survival} = \left(\frac{\frac{\text{CFU}}{\text{ml}} \text{after freeze} - \text{drying}}{\frac{\text{CFU}}{\text{ml}} \text{before freeze} - \text{drying}} \right) \text{x } 100$$

Fatty acid extraction

Fatty acid methyl esters (FAMEs) were prepared according to the method described in Supelco Analytical Manual (2008) with some modifications. Twenty to thirty mg of freeze-dried cells was weighed into 13 x 90 mm screw capped test tube. Two ml BCl_3-methanol 12% w/w and 100 µl internal standard (C17:0) (Fluka, Sigma Aldrich, Switzerland) were added. After mixing, samples were heated at 60 ^{o}C for 10 min on a water bath and immediately cooled. One ml hexane and 1 ml deionized water were added to the test tube. After mixing, samples were centrifuged at 3,500 x g for 2 min. Upper (organic) layer was transferred into a clean tube with ~300 mg of sodium sulfate (Na_2SO_4) to absorb residual moisture. Hexane layer was transferred into clean test tube and dried under a stream of nitrogen using Turbo Vap® LV Evaporator (Zymark Corp. Hopkinton, MA, USA). Dried FAMEs were re-suspended in 200 µl hexane and analyzed by gas chromatography (GC).

Gas Chromatography

Fatty acid composition of the FAMEs were determined by gas chromatography using Agilent 6890 Gas Chromatograph (Agilent Technologies, Inc., Wilmington, DE, USA) equipped with a flame ionization detector and split injection. A wall coated open tubular (WCOT) fused silica capillary column (50 m x 0.25 mm x 0.2 µm) (Varian CP6173, Lake Forest, CA, USA) was used. Injection volume was 2 µl and was done at a split mode (40:1 split). Hydrogen served as the carrier gas with a flow rate of 1.7 ml/min. The oven temperature was held at 40^{o}C for 3 min after sample injection and subsequently

increased at 5 ^{o}C per min until the final temperature of 240 ^{o}C was reached. Fatty acid methyl esters were identified by comparing their retention times with known standards (C12:0, C14:0, C16:0, C18:0, C16:1, C18:1, C19:0, and C19:0cyc11). Relative percentage of each fatty acid was calculated as the ratio of the surface area of the peak to the total area of all peaks.

Statistical Analysis

Experiments were conducted in triplicate. Effect of Tween 80 was analyzed using a completely randomized design for repeated measures with Tween 80 as main effect and trials as random effects with storage day as repeated measure using GLIMMIX procedure of SAS software (SAS Institute, 2006). All two and three-way interactions and main effects were included in the model. The main effects were considered only when two or three-way interactions were not significant. Due to the departure from normality, a beta distribution was used to model the response variable, and a covariance structure for the repeated measures was also modeled. Degrees of freedom were adjusted using the Kenward-Roger method. Least Square Means were presented and compared using protected Fischer methods for comparison. Results were presented as least square means (LSMeans) ± SEM (standard error of mean)

Data for fatty acid were analyzed using PROC GLM of SAS and least square means were determined and separated by Duncan's t-test. Differences were considered significant at the $P < 0.05$ level.

Results

Confirmation of Identity of cultures of Lactobacillus

The identity of the cultures of *Lactobacillus* used in this study was confirmed when characteristics and fermentation patterns were compared to those listed in Bergey's Manual of Determinative Bacteriology (Sneath, et al., 1986). The complete culture characteristics of these organisms are listed in Appendix A.

Determination of harvest time

The stationary phase of growth for each of the cultures was determined by constructing growth curves (Appendix A). Constant growth was observed from 12 h until 16 h, thus harvest time used in the experiments was 15 h.

Effect of Tween 80 on total numbers of lactobacilli

The initial populations of the cultures harvested at 15 h in growth media supplemented with different concentrations of Tween 80 were determined. Depending on the strain and concentration, the general trend was higher population when grown in MRS broth supplemented with Tween 80 compared to those grown without Tween 80. Strains O-16, 381-IL-28 and X-18 showed significantly ($P < 0.05$) higher populations than the control when grown in MRS with 0.1% to 0.5% Tween 80 (Table 1). Higher cell population was observed only at 0.5% Tween 80 in strains E-5 and E-10 while L-23 exhibited better growth in medium with 0.1 and 0.5% Tween 80.

Effect of Tween 80 on viability of cultures of lactobacilli after freeze-drying and storage

Three factors were considered with regards to viability of the cultures of lactobacilli during freeze-drying and storage: concentration of Tween 80, storage time (day), and strain. The main effects of these factors, as well as their interactions were determined for each of the cultures. Main effects of Tween 80 and storage were statistically significant while effect of strain was not significant and there were no significant two-way or three-way interactions among these factors.

In general, viability (% survival) of the strains of lactobacilli after freeze-drying and subsequent storage did not improve when cultivated in medium with Tween 80 (Table 2). Higher percent survival was observed only in *L. reuteri* X-18 grown in medium with 0.1% to 0.5% Tween 80. In *L. casei* E-5, addition of Tween 80 did not increase viability of cells; however, cells grown with 0.1% Tween 80 exhibited significantly higher percentage survival than those grown in broth with 0.3%, 0.4%, and 0.5% Tween 80. For *L. casei* E-10, viability was lower when cells were grown in MRS with 0.5% Tween 80 while other concentrations (0.1% to 0.4%) did not show any significant effect on survival compared to the control. Strains L-1, L-23, O-16, and 381-IL-28 exhibited no increase in viability when grown with Tween 80.

The stability of the different cultures during storage for 21 days was determined (Table 3). Statistical analyses showed that there was no Tween 80 x day interaction. Strains E-10, L-1, 381-IL-28, and X-18 did not exhibit significant decrease in viability during storage from day 1 to day 21. Viability of strain E-5 decreased during the first week of storage but remained the same from day 7 to day 21. Strain L-23 did not show

decrease in viability from day 1 to day 14 but declined from day 14 to day 21. In strain O-16, the decrease in percentage survival was significant only between day 1 and day 21.

Data did not reveal any strain effect or any two- or three-way interactions; however, numerically, the cultures exhibited differences in percentages of survival during freeze-drying and storage. This could be due to high variability in the data (Table 3).

Effect of Tween 80 on fatty acid composition

Membrane fatty acid composition of the strains of lactobacilli was characterized to understand the physiological modifications induced by the previously observed responses. The main fatty acid of the cultures grown in the presence and absence of Tween 80 are shown in Table 4. Main fatty acid peaks identified were myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), and lactobacillic acid (C19cyc11). Small amounts of lauric (C12:0) and nonadecanoic (C19:0) were also detected in some cultures (data not shown).

Fatty acid analysis showed that cultivation in the presence of Tween 80 affected the cellular fatty acid profile of the different strains of lactobacilli. In general, addition of Tween 80 tend to increase the concentrations of unsaturated fatty acids like oleic, palmitoleic, and lactobacillic acid in some of the cultures. A trend of significantly lower concentrations of the saturated fatty acid, palmitic acid also was also observed in most cultures grown with Tween 80.

Discussion

Freeze-drying is a method preferred in the preservation of microorganisms for long term storage. Producing cultures with high population of viable and stable cells depend on several factors like growth phase, growth medium composition, pH and temperature, among others. In this study, cells were harvested during stationary phase of growth because they are reported to be less sensitive to environmental stresses than exponential phase cells (Davis, 1996). Stationary phase induces various physiological changes within the cells like formation of capsular material (Brashears and Gilliland, 1995), condensed cytoplasm, and less fluid cell membrane (Ishihana, 1997). They are also able to survive under stress conditions due to systems they express for DNA repair and protection of membranes and proteins (Hengge-Aronis, 1993). This changes would trigger stress responses to allow higher survival and viability of the cells as under several environmental stresses, like freezing, as reported in *L. bulgaricus* (Texeira et. al., 1995; Carvalho et. al., 2003), *L. rhamnosus* (Corcoran et. al., 2004), and *L. acidophilus* (Brashears and Gilliland, 1995; Kim et. al., 2001).

In the present study, effect of Tween 80 on initial population of cultures was determined. Higher initial population was generally obtained from cultures grown in MRS broth supplemented with Tween 80 compared to those without Tween 80; however the maximum concentration of Tween 80 varied for each strain. Some strains, like L-1, E-5 and E-10 had higher cell populations when grown with 0.5% Tween 80 while strain L-23 at 0.4% and 0.5% Tween 80**.** The increase in cell number can be attributed to the growth enhancement effect of Tween 80. Previous studies have shown that Tween 80 enhanced growth of bacteria since it contains essential growth factors like oleate

(William et al., 1947; de Mann et al., 1960), biotin (William et al., 1947; Waller and Lichstein, 1967) and some fatty acids (Partanen et al., 2001). The production of higher number of cells would be economically important in the commercial preparation of concentrated cultures since higher yields will be obtained from the same volume of growth medium (Gilliland and Rich, 1990).

Growth medium is one of the important factors in the preparation of concentrated cultures to maximize initial cell population and survival following freeze-drying and storage (Fonseca et al., 2001; Carvalho et al., 2003). Substances, such as Tween 80, sugars, glycerol, adonitol, sorbitol, glutamate, and aspartate are added as protectants to improve viability and stability of cells (Carvalho et al., 2002; Tymczyszyn, et al., 2007). In the present study, the influence of Tween 80 on survival during freeze-drying and storage was determined. The cryoprotective effect of Tween 80 was not observed since in general most of the cultures did not exhibit improvement in survival during freeze-drying and storage. Improved percentage survival was observed only in *L. reuteri* X-18, which showed higher viability when grown in medium with Tween 80. This result is in agreement with previous studies that reported improved survival in frozen and freeze-dried cultures of lactic acid bacteria associated with addition of Tween 80 in the growth medium (Smittle et al., 1972; Gilliland and Speck, 1974; Smittle et al., 1974; Carvalho et al., 2003; Corcoran et al., 2007).

In strains O-16 and E-10 higher concentration of Tween 80 (0.5%) resulted in lower survival. The same result was also observed by Smittle et al. (1972) in *L. bulgaricus*; however, there are limited or no studies that could explain this observation. Our results showed that different species of one genus can exhibit different behaviors

during freeze-drying and storage. The optimum concentration of Tween 80 that might be required by some cultures to improve survival and confer the best resistance to freeze-drying should be studied further.

Cell membrane is the primary site of damage during freezing and freeze-drying (Brennan, et al., 1986) and changes in fatty acid composition enable microorganisms to maintain membrane functions during environmental stresses like freezing and desiccation (Baati et al, 2000; Guerzoni et al., 2001). Variations in membrane lipid structure such as increase in proportions of unsaturated fatty acids and cyclopropane fatty acids affect membrane fluidity and thus preventing death of some bacterial cells (Goldberg and Eschar, 1977). In the present study, alteration in the cell membrane was apparent upon examination of cultures grown in medium with Tween 80. Cells grown with Tween 80 had increased amount of oleic acid (C18:1) in their cell membranes. This shows that the cells were able to incorporate the oleic acid in Tween 80 in their cell membranes. This is in agreement with several studies on the lactic acid bacteria (Smittle et al., 1974; Jacques, et al., 1995; Johnsson et al., 1995). Jacques, et al (1985) hypothesized that the fatty acid component of the detergent was released by hydrolysis and incorporated directly into the membrane lipids of the cell. The ability of LAB in incorporating exogenous fatty acids from the growth medium was further studied by Kankaapaa, et al. (2004). They reported that polyunsaturated fatty acids (PUFA) added to the growth medium were incorporated to the cell lipids of *L. rhamnosus, L. casei,* and *L. delbrueckii.*

Several studies have shown the relationship between survival during freezing and increase in C18:1 and other unsaturated fatty acids in the cell membrane of lactic acid bacteria (Smittle et al., 1972; Smittle et al., 1974; Goldberg and Eschar, 1977; Schoug et.

al., 2008; Zhao et. al., 2009). They reported that increase in degree of unsaturation of the membrane helped regulate membrane fluidity, thus increasing the survival of cells during freeze-drying. In the present study, increase in C18:1 was observed in strain X-18 and this may have contributed to improvement in survival; however, this observation was not consistent with other strains which also had higher C18:1 in their membranes. Aside from the concentration of C18:1, several studies also reported the influence of cyclopropane fatty acid (CFA) in conferring resistance to freezing (Smittle et al., 1972; Smittle et al., 1974; Goldberg and Eschar, 1977). In the present study *L. reuteri* X-18 had significantly higher C19:0cyc11 when cultivated in medium with Tween 80. It is probable that in addition to C18:1, increase in amount of C19:0cyc11 could have contributed to the improvement in survival of this strain since this fatty acid can be considered as unsaturated fatty acid (Goldberg and Eschar, 1977). Moreover, lower amount of saturated fatty acids (C18:0 and C16:0) were also observed in strain X-18 cultivated with Tween 80. These results suggest that more than one fatty acid is responsible for cell membrane fluidity. This is in agreement with study by Gilliland and Speck (1974) which reported that stability of the membrane probably involves more than just the oleic acid content but an overall favorable balance of fatty acids and this was probably not observed in other cultures.

It is also important for concentrated cultures to maintain their viability during storage. In the present study, stability of the cultures during storage was determined by calculating percentage survival weekly up to 21 days. Addition of Tween 80 did not have significant effect in maintaining stability of cultures during storage. In general, all cultures exhibited similar trends of viability during the storage period regardless of

Tween 80 concentration. The decline in survival observed during the first week of storage might be likely due to damage to cells during the freeze-drying process. According to Reilly and Gilliland (1990), the greatest injury of cells during freezing occurs during the early stages and cells became stable during storage. In a study conducted by Mitchell and Gilliland (1983), they observed the greatest decline in survival of *L. acidophilus* during the first 24 hours of storage in liquid nitrogen and no additional decrease was observed during subsequent storage for 28 days. Other factors might have also contributed to the results. These factors include temperature, light, water activity, and oxygen level (Wang, et. al., 2004; Otero, et al., 2007; Kurtmann et. al., 2009). In the present study, effect of oxygen level could have contributed to the stability of cells. The vials of freeze-dried cells sealed under vacuum and according to Bosgra (1947), storing under vacuum can help preserve viable organisms. Sealing can prevent oxidation of cells as shown in other studies (Castro et al., 1996; Miyamoto-Shinohara et al., 2006; Kurtmann et al., 2009). Another advantage of vacuum sealing is the high level of desiccation that ensures low moisture and relative humidity (Miyamoto-Shinohara, 2006). In their study, excellent survival of some freeze-dried microbial species after 20 years of storage was attributed to high level of desiccation and sealing under vacuum. Decrease in water activity and reduced oxygen level also increased the storage stability of freeze-dried and vacuum sealed cultures of *L. acidophilus* (Kurtmann et al., 2009).

Conclusion

The results showed that in general Tween 80 did not significantly improve survival of most of the cultures of lactobacilli except for *L. reuteri* X-18. Statistical

analyses also showed that Tween 80 did not influence viability during storage since the cultures exhibited the same trend of survival regardless of Tween 80 concentration. Fatty acid evaluation showed significant increase in C18:0 in cells grown with Tween 80. This showed that the oleic acid present in Tween 80 was incorporated in the cell membrane of the organisms. Relationship between fatty acid and viability was observed in *L. reuteri* X-18 which had significantly higher C18:1 and C19:0cyc11 concentrations when grown with Tween 80. This could have contributed to increase in degree of unsaturation in the cell membrane making the cell more resistance to freeze-drying. Further studies should be conducted to determine the optimum concentration of Tween 80 that can produce higher biomass and improved survival among the strains of probiotic lactobacilli used in this study.

Table 1. Growth of cultures of lactobacilli in MRS broth at 37 ^{o}C for 15 h with and without supplementation of Tween 80

Tween 80 (%)	$\log_{10}$ CFU/ml[1]						
	L. casei		*L. acidophilus*				*L. reuteri*
	E-5	E-10	L-1	L-23	O-16	381-IL-28	X-18
0	10.22 ^{b}	9.97 ^{b}	8.79 ^{b}	8.53 ^{b}	8.54 ^{b}	9.96 ^{b}	9.21 ^{b}
0.1	10.28^{ab}	10.18^{ab}	9.96^{ab}	10.05^{ab}	10.40^{a}	10.15^{a}	10.14^{a}
0.2	10.29^{ab}	10.16^{ab}	10.18^{a}	10.05^{ab}	10.38^{a}	10.20^{a}	10.17^{a}
0.3	10.32^{ab}	10.23^{ab}	10.14^{ab}	10.07^{ab}	10.52^{a}	10.21^{a}	10.25^{a}
0.4	10.32^{ab}	10.24^{ab}	10.14^{ab}	10.21 ^{a}	10.51^{a}	10.22^{a}	10.22^{a}
0.5	10.38^{a}	10.39 ^{a}	10.23^{a}	10.24 ^{a}	10.32^{a}	10.27^{a}	10.35^{a}

[1]Values are means from three trials

^{ab} Values in the same column with different superscript are significantly different ($P > 0.05$)

Table 2. Effect of Tween 80 on survival of freeze-dried cultures of lactobacilli during storage

	Survival (%) (LS Means ±SEM)[1]						
	L. casei		*L. acidophilus*				*L. reuteri*
Tween 80 (%)[2]	E-5	E-10	L-1	L-23	O-16	381-IL-28	X-18
0	65.64 ± 5.87^{abc}	73.96 ± 8.42^{a}	57.35 ± 11.53^{a}	35.82 ± 16.64^{a}	67.60 ± 10.27^{a}	58.90 ± 13.65^{a}	43.56 ± 10.35^{b}
0.1	78.46 ± 5.16^{a}	78.23 ± 7.71^{a}	56.82 ± 11.52^{a}	52.27 ± 17.88^{a}	40.88 ± 10.12^{a}	68.98 ± 12.5^{a}	76.16 ± 9.19^{a}
0.2	72.00 ± 5.65^{ab}	82.45 ± 6.62^{a}	33.05 ± 10.75^{b}	59.80 ± 17.17^{a}	35.31 ± 10.58^{a}	67.57 ± 12.7^{a}	82.79 ± 7.70^{a}
0.3	58.64 ± 6.24^{bc}	70.37 ± 9.12^{ab}	44.20 ± 11.56^{ab}	60.1 ± 17.16^{a}	39.06 ± 10.90^{a}	73.27± 11.64^{a}	70.90 ± 9.65^{a}
0.4	60.44 ± 6.18^{bc}	82.64 ± 6.59^{a}	57.94 ± 11.44^{a}	48.06 ± 17.70^{a}	37.58 ± 10.80^{a}	60.4 ± 13.50^{a}	78.71 ± 8.42^{a}
0.5	50.89 ± 6.31^{c}	56.42 ± 10.43^{b}	39.43 ± 11.25^{ab}	43.61 ± 17.64^{a}	54.90 ± 11.39^{a}	61.40 ±13.41^{a}	57.96 ± 10.35^{a}

[1] Values are least square means from three trials, LS Means= Least Square Means; SEM = Standard Error Mean

[a,b,c] Values in the same column without a common superscript are significantly different ($P < 0.05$)

[2] Cultures were grown for 15 h in MRS broth supplemented with or without Tween 80, freeze-dried and stored at 5^{o}C for 21days

Table 3. Survival during storage at 5 °C of freeze-dried cultures of lactobacilli[1]

Day[3]	Survival (%) (LS Means ± SEM)[2]						
	L. casei		*L. acidophilus*				*L. reuteri*
	E-5	E-10	L-1	L-23	O-16	381-IL-28	X-18
1	69.76 ± 2.47[a]	81.37 ± 6.76[a]	58.55 ± 10.53[a]	62.21± 14.79[a]	59.51 ± 10.50[a]	70.75 ± 7.99[a]	79.35 ± 4.25[a]
7	66.21 ± 2.37[b]	76.97 ± 7.11[a]	47.43 ± 10.79[a]	53.43 ± 14.58[a]	51.89 ± 10.78[ab]	67.14 ± 8.66[a]	71.01 ± 4.26[a]
14	62.56 ± 3.08[b]	72.64 ± 7.88[a]	41.15 ± 11.17[a]	49.76 ± 15.10[a]	38.54 ± 10.30[bc]	59.63 ± 10.92[a]	65.17 ± 5.63[a]
21	60.85 ± 3.69[b]	67.09 ± 9.38[a]	44.95 ± 11.29[a]	34.51 ± 13.10[b]	34.14 ± 9.79[c]	63.12 ± 9.82[a]	61.28 ± 5.57[a]

[1] Cultures were grown for 15 h in MRS broth supplemented with and without Tween 80, freeze-dried and stored at 5°C for 21days

[2] Values are least square means from all Tween 80 concentrations for three trials, SEM = Standard Error Mean

[a,b,c] Means within columns without a common superscript are significantly different (P>0.05)

Table 4. Fatty acid composition of lactobacilli cultivated in MRS broth with or without Tween 80.

Culture	Tween 80 (%)	Fatty Acid composition (%)[1] C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc 11
L. casei	0	1.20^{d}	59.21^{a}	1.55^{b}	7.91^{a}	1.52^{b}	27.05^{b}
E-5	0.1	3.22^{bc}	47.79^{a}	3.36^{a}	3.53^{a}	23.12^{a}	18.98^{c}
	0.2	3.05^{c}	25.18^{b}	1.54^{b}	3.71^{a}	19.14^{a}	47.54^{a}
	0.3	3.97^{abc}	15.80^{b}	1.14^{b}	5.69^{a}	25.75^{a}	41.80^{a}
	0.4	4.34^{ab}	14.82^{b}	1.84^{b}	6.18^{a}	25.82^{a}	46.81^{a}
	0.5	5.00^{a}	15.26^{b}	3.05^{a}	11.90^{a}	19.34^{a}	47.41^{a}
L. casei	0	1.51^{b}	47.68^{a}	6.54^{a}	1.41^{b}	23.66^{a}	22.62^{a}
E-10	0.1	2.31^{ab}	43.32^{ab}	13.77^{a}	2.45^{b}	14.08^{a}	18.59^{a}
	0.2	3.50^{a}	17.57^{bc}	3.02^{a}	5.21^{ab}	40.97^{a}	29.74^{a}
	0.3	2.50^{ab}	13.16^{c}	14.34^{a}	3.65^{b}	39.30^{a}	42.01^{a}
	0.4	2.88^{ab}	20.63^{bc}	2.88^{a}	4.09^{b}	34.00^{a}	35.52^{a}
	0.5	4.30^{a}	15.04^{bc}	5.38^{a}	10.29^{a}	10.84^{a}	41.78^{a}
L. acidophilus	0	1.07^{a}	43.11^{a}	24.83^{a}	0.73^{b}	1.78^{b}	28.33^{a}
L-1	0.1	1.32^{a}	6.33^{b}	1.55^{b}	2.55^{ab}	74.27^{a}	12.34^{a}
	0.2	1.69^{a}	16.15^{b}	2.60^{b}	4.49^{ab}	46.23^{a}	28.62^{a}
	0.3	2.03^{a}	6.94^{b}	2.40^{b}	5.54^{ab}	67.36^{a}	15.42^{a}
	0.4	2.67^{a}	8.18^{b}	4.20^{b}	8.61^{ab}	58.52^{a}	17.59^{a}
	0.5	1.65^{a}	9.43^{b}	6.4^{b}	11.45^{a}	48.26^{a}	22.01^{a}
L. acidophilus	0	0.83^{a}	23.82^{a}	13.11^{a}	23.19^{a}	13.10^{a}	26.09^{a}
L-23	0.1	0.71^{a}	6.44^{a}	42.76^{a}	2.69^{a}	17.86^{a}	16.77^{a}
	0.2	3.38^{a}	10.61^{a}	4.38^{a}	7.46^{a}	37.30^{a}	19.71^{a}
	0.3	4.21^{a}	12.26^{a}	4.79^{a}	9.21^{a}	29.22^{a}	18.81^{a}
	0.4	6.91^{a}	10.56^{a}	19.93^{a}	4.32^{a}	30.03^{a}	18.95^{a}
	0.5	8.70a	11.01^{a}	18.55^{a}	4.32^{a}	43.12^{a}	19.49^{a}
L. acidophilus	0	0.85^{a}	44.09^{a}	23.24^{a}	0.81^{a}	0.92^{b}	29.62^{a}
O-16	0.1	1.29^{a}	3.20^{b}	2.48^{b}	ND	70.86^{a}	21.67^{a}
	0.2	1.95^{a}	20.24^{b}	3.26^{b}	3.47^{a}	49.38^{a}	20.73^{a}
	0.3	2.28^{a}	9.37^{b}	2.84^{b}	5.21^{a}	52.83^{a}	25.58^{a}
	0.4	2.39^{a}	10.46^{b}	1.64^{b}	7.63^{a}	47.70^{a}	27.10^{a}
	0.5	2.73^{a}	12.10^{b}	1.91^{b}	8.27^{a}	37.87^{a}	27.07^{a}

Culture	Tween 80 (%)	Fatty Acid composition (%)[1] C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc 11
L. acidophilus 381-IL-28	0	1.22[a]	33.62[a]	1.05[b]	33.36[a]	2.03[b]	28.73[a]
	0.1	1.43[a]	28.69[a]	48.27[a]	4.62[b]	16.48[a]	24.32[b]
	0.2	2.77[a]	14.97[b]	3.20[b]	1.84[b]	36.88[a]	40.53[a]
	0.3	3.15[a]	13.86[b]	3.54[b]	1.60[b]	37.23[a]	40.61[a]
	0.4	3.11[a]	13.75[b]	4.40[b]	1.73[b]	39.45[a]	27.04[a]
	0.5	3.93[a]	15.26[b]	4.41[b]	1.33[b]	37.84[a]	38.71[a]
L. reuteri X-18	0	2.33[a]	51.05[a]	0.92[b]	10.76[a]	14.36[b]	21.61[b]
	0.1	1.77[a]	28.41[b]	0.65[b]	4.41[b]	40.72[a]	23.89[a]
	0.2	2.59[a]	10.37[b]	3.71[a]	2.67[b]	40.77[a]	39.80[a]
	0.3	2.76[a]	11.20[b]	4.09[a]	1.81[b]	38.91[a]	41.27[a]
	0.4	3.28[a]	13.34[b]	4.32[a]	1.91[b]	37.30[a]	40.85[a]
	0.5	3.71[a]	15.00[b]	4.30[a]	1.69[b]	30.47[a]	41.40[a]

[1] Each value is the mean from three trials expressed as relative concentration; Fatty acids are: Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11); ND = Not detected

[a,b,c] Means within strain in the same column with common superscript are not significantly different ($P > 0.05$)

CHAPTER IV

INFLUENCE OF GROWTH pH ON VIABILITY AND FATTY ACID COMPOSITION OF LACTOBACILLI AFTER FREEZE-DRYING AND STORAGE

Abstract

The influence of pH on viability, storage stability, and fatty acid composition during freeze-drying of strains of lactobacilli was studied. Seven cultures of lactobacilli were evaluated for viability and stability during freeze-drying and subsequent refrigerated storage following growth in medium maintained at four different pH levels. The growth pH influenced the resistance to freeze-drying of the strains of lactobacilli. Generally, cultures grew well in all pH levels except for *L. casei* E-5 and *L. reuteri* E-10 which did not grow well at pH 4.5. after freeze-drying and storage, most cultures grown in broth with more acidic pH (4.5 and 5.0) generally exhibited higher viability except for *L. acidophilus* O-16 which had higher survival at pH 5.5 and 6.0. During subsequent storage at 5 ^{o}C significant decrease in viability was observed only in some strains (L-1 and O-16). Viability declined during the first two weeks but remained stable until the end of the storage period. This could be attributed to minimal exposure to oxygen, low temperature,

and absence of light during storage. Relationship between pH, survival and fatty acid composition was not clearly shown due to high variability in the data. Other factors could have possibly contributed to the improved survival of cells grown at lower pH; however, they were not investigated in this study.

Results also suggested that strains exhibited different responses to changes in growth pH so further studies can be conducted to determine the best pH for each strain to survive better during freeze-drying and remain stable throughout storage, as well as investigate other possible stress responses that these strains of probiotic lactobacilli exhibit when grown in a pH-controlled environment.

Introduction

Fermented food products with probiotics are a major part of people's diet all over the world and several health benefits associated with consumption have been reported. Concentrated cultures of lactic acid bacteria are produced, stored and added to foods during manufacturing. They are frozen or freeze-dried for ease of storage and distribution.

Growth conditions to produce cells for concentrated cultures greatly influence survival during freeze-drying and storage. Optimum growth of microorganisms occurs within a specific pH range which influences cell growth and metabolism (Nannen and Hutkins, 1991), as well as cell physiology and membrane lipid composition (Saarela et al., 2009). Survival of lactic acid bacteria during freezing or freeze-drying were reported to be affected by growth pH (Gilliland and Rich, 1990; Palmfeldt and Hahn-Hagerdal, 2000; Silva et al., 2005) and pH also was reported to change fatty acid composition of

different bacteria (Russell et al., 1995; Wang et al., 2005; Hua et al., 2009; Saarela et al., 2009).

The objective of this study was to determine the influence of growth pH on fatty acid composition, survival, and storage stability of different strains of lactobacilli after freeze-drying and subsequent refrigerated storage at 5 ^{o}C.

Materials and Methods

Microorganisms and Maintenance of Cultures

The same strains of lactobacilli were used in this experiment and the cultures were maintained using the same methods and conditions described in the previous chapter.

Growth in culture medium with controlled pH

Production of cell crops for each of the cultures was similar to the method used by Gilliland and Rich (1990). Cultures were grown using 1% inocula into 500 ml MRS broth for 15 h at 37 ^{o}C in a 1 L bench top scale fermentor system (Biostat® Q- 1000, Sartorius BBI Systems GmbH, Melsugen, Germany) equipped with a digital instrumentation system, combination pH electrode connected to an automatic pH controlled unit, inoculation and sampling ports and acid and base ports. The temperature was controlled via the jacket of the culture vessel by a thermostat unit with a 1 kW heater and circulation pump which supplied heated water in conjunction with a cooling water supply.

The growth medium was maintained at four different pH values: 4.5, 5.0, 5.5, and 6.0. Prior to inoculation, the acidity of the broth was adjusted to the desired pH by the addition of sterile 20% lactic acid solution (Fischer Scientific, Fischer Scientific Chemicals, Fair Lawn, NJ, USA). Temperature was maintained at 37 ^{o}C throughout the fermentation process with agitation at 299 rpm and pH was maintained by the automatic addition of sterile neutralizer (20% sodium carbonate in 20% ammonium hydroxide). After 15 h incubation, 100 ml of broth was aseptically drawn from the sampling port using a sterile syringe and concentrated cultures were prepared using the same procedures described in the previous chapter.

Preparation of cells for fatty acid analysis

Cultures were grown in MRS broth maintained at different pH for 15 h as described in previous section. Cells for fatty acid analysis were prepared using the same procedures described in the previous chapter.

Freeze drying

Cells were freeze-dried using the same procedures as described in the previous chapter.

Enumeration of lactobacilli and storage stability

Cells were enumerated using the same procedure described in the previous chapter and the percentage storage was calculated using the same formula.

Fatty acid extraction and Gas Chromatography

Fatty acid methyl esters (FAMEs) were prepared and ran in the gas chromatographer using the same procedures described in the previous chapter. Fatty acids were identified by comparing them with known standards. Results were reported as relative percentage of fatty acid calculated as the ratio of the peak over the total area of all peaks.

Statistical Analysis

Experiments were conducted in triplicate. Effect of pH was analyzed using a completely randomized design for repeated measures with pH as main effects and trials as random effects with storage day as repeated measure using GLIMMIX procedure of SAS software (SAS Institute, 2006). All two and three-way interactions and main effects were included in the model. The main effects were considered only when two or three-way interactions were not significant. Due to the departure from normality, a beta distribution was used to model the response variable, and a covariance structure for the repeated measures was also modeled. Degrees of freedom were adjusted using the Kenward-Roger method. Least Square Means were presented and compared using protected Fischer methods for comparison. Results were presented as least square means (LSMeans) ± SEM (standard error of mean)

Data for fatty acid were analyzed using PROC GLM of SAS and least square means were determined and separated by Duncan's t-test. Differences were considered significant at the $P < 0.05$ level.

Results

Effect of pH on initial population of lactobacilli

Most cultures grew well at each of the four pH levels. The highest population in most cultures was obtained at pH 5.5 although the average $\log_{10}$ counts for each culture obtained at each pH were not significantly different ($P > 0.05$) except for strains E-10 and X-18 which did not grow well at pH 4.5 (Table 3).

Effect of pH on survival of cultures of lactobacilli after freeze-drying and storage

Within strain, the main effects on survival of pH levels and storage time (day), as well as the two-way interactions were determined. There was no significant pH x day interaction observed in most strains except for strain E-10. Figure 2 shows that cells of strain E-10 grown at pH 4.5 had the highest survival ($P < 0.05$) compared with all other pH levels. Within days of storage, percentage survival of strain E10 also differed. At day 1 survival of cells grown at pH 4.5 was significantly higher that all other pH levels. On day 7 and 14 and 21 cells had higher survival at pH 4.5 and 5.0 than at pH 5.5 and 6.0.

Table 4 shows strain E-5 exhibited higher survival at pH 4.5, 5.0, and 5.5 compared to pH 6.0. In strain L-1, cells grown at pH 4.5 and 5.0 exhibited higher percentage survival than cells grown at pH 5.5 and 6.0. Cells of strains L-23 and X-18 survived better when grown at pH 4.5 than at all other pH levels. In strain O-16, cells grown at pH 6.0 had higher survival than at other pH levels.

Effect of storage on viability

For storage test, there was no pH x day interactions among the other six strains of lactobacilli. Results showed that regardless of growth pH, the cultures exhibited similar trends of viability during storage. Strains E-5, L-23, 381-IL-28, and X-18 showed no decrease in viability during storage from day 1 to day 21 while strain L-1 showed decrease in survival from day 1 to 7 but remained stable until day 21 (Table 6). In strain O-16, viability of freeze-dried cells did not decrease from day 1 to day 14 but decreased from day 14 to day 21.

For strain comparison, significant strain x pH interaction ($P < 0.05$) at each of the four days of storage was observed. Among species, *L. reuteri* had the highest percentage survival at pH 4.5. Within strains of *L. acidophilus*, L-1, L-23 and 381-IL-28 showed higher survival than strain O-16 at pH 4.5, 5.0, and 5.5. Strain O-16 and L-23 exhibited higher survival at pH 6.0 compared to other strains of *L. acidophilus*.

Effect of pH on fatty acid composition

The fatty acid composition of the cultures of lactobacilli was determined after growing the cultures at different pH values. The lipid membrane of the lactobacilli contained the following fatty acids: C14:0, C16:0, C16:1, C18:0, C18:1 and C19cyc11 acid (Table 9). In general, most cultures did not exhibit significant differences in the concentrations of cellular fatty acids.

Discussion

Effect of pH during growth and survival during freeze-drying of the cultures was determined. All the cultures grew well at each of the four pH levels but showed better growth at pH 5.0, 5.5, and 6.0. Most lactic acid bacteria, except for some genera of *Lactobacillus*, *Leuconostoc*, and *Oenococcus* have optimal pH for growth between 5 and 9 (van de Guchte et. al., 2002). Some cultures also grew well at pH 4.5 and the ability of some of the cultures to grow in pH below their optimum pH can be explained by their acidophilic characteristics (Gilliland and Rich, 1990). *Lactobacillus reuteri* X-18 did not grow well at pH 4.5 since its optimum growth pH is 5.0 to 6.0 (Ragout et al., 1994; Palmfeldt and Hahn-Hagerdahl, 2000). Our results suggested that growing the cultures in their optimum pH has the advantage of obtaining higher yields for concentrated culture preparation. Moreover, cultivation at a lower pH can prevent growth of contaminants since growth at pH 5.0 or 5.5 would be less favorable for other microorganisms (Gilliland and Rich, 1990).

Generally, most cultures grown at lower pH showed higher survival after freeze-drying and storage than those grown at pH 6.0. These results were in agreement with previous studies wherein lower pH during growth improved viability during freezing or freeze-drying (Gilliland and Rich, 1990; Palmfeldt and Hahn-Hagerdahl, 2000; Wang et al., 2005). Higher survival during freezing was observed with cells grown at pH 5.0 in *L. acidophilus* (Gilliland and Rich, 1990), pH 5.5 in freeze-dried *L. coryneformis* (Schoug et. al., 2008), and pH 3.2 - 4.8 in freeze-dried *Oenococcus oeni* (Hua et. al., 2009; Zhao et. al., 2009). A 25 to 45 fold higher survival after freeze-drying was also observed in cells of *L. delbrueckii* spp. *lactis* when grown at pH 5.0 than at pH 6.0. Palmfeldt and Hanh-Hagerdahl (2000) also reported that *L. reuteri* culture grown at pH 5.0 had a 30%

increase in survival after freeze-drying compared to pH 6.0. Also, cells of *L. delbrueckii* subsp. *bulgaricus* were more resistant to spray drying when grown at pH 6.0 than at pH 6.5 (Silva et al., 2005). A study by Lorca and de Valdez also showed that *L. acidophilus* grown at pH 6.0 was more sensitive to freeze-drying than those grown at lower pH.

Among the cultures, only strain O-16 showed significantly ($P < 0.05$) higher survival at pH 6.0 than at lower pH values. This result was similar to previous study by Reilly and Gilliland (1999) wherein they observed that *Bifidobacterium longum* was more stable and higher survival when grown at pH 6.0 than lower pH. These results indicated that the survival was also dependent on strain used. The variation in the response of the different strains of lactobacilli was clearly shown in this study and this observation is in agreement with previous studies that resistance to freeze-drying was strain dependent (Koch et al., 2008).

Changes in growth conditions were reported to affect cell physiology and membrane lipid composition which contribute to stability of the cells (Saarela et al., 2009). In the present study, improved viability after freeze-drying and storage of cultures grown in more acidic pH was not found to be related to fatty acid composition since significant difference among the concentrations of fatty acids was not observed. This result is not in agreement with previous studies which showed that changes in fatty acid composition were influenced by culture pH (Russell et al, 1995; Wang et al., 2005; Saarela et al., 2008; Hua et al., 2009). These studies showed that growing the cultures at low pH values caused increase in unsaturated fatty acids (C18:1, 16:1 and C19:0cyc11) that consequently increased membrane fluidity, hence more stable. Beal et al., 2001 also reported that decreasing the pH from 6.5 to 5.5 led to lower C16:0 and higher C18:1 and

C19:0cyc 11 concentrations. Improved tolerance to freezing was also attributed to high concentration of C16:0 in *L. acidophilus* (Wang et al., 2005).

Based from the results of the present study, higher percentage survival of some strains at lower pH might have been caused by other factors aside from cellular fatty acids. Kim et al. (2001) reported that better tolerance against environmental stresses such as low freezing and desiccation of low pH-grown cells can be a result of specific or general stress response of the cells. In a study by Palmfedt and Hanh-Hagerdal (2000) the higher survival observed for cells of *L. reuteri* grown at pH 5.0 than pH 6.0 was explained as a stress response triggered by growing the cells in more acidic pH. In the present study, the higher survival observed in cultures grown at low pH can be attributed to the response mechanism brought about by acid stress during growth called acid tolerance response; however, most of the studies on the effect of pH was observed when cells were subjected to acid shock and not by growing under controlled pH (Silva et al., 2005). Adaptive treatments of *Oenococcus oeni* at pH 3.2 and 3.5 increased its survival during freeze-drying which was due to acid-induced cross protective response in this organism.

Conclusion

Most cultures exhibited higher percentage survival during storage when cultivated at lower pH levels. In general, trend of decreasing percentage survival during storage was observed among the strains; however, in most of the strains the values were not statistically significant from day 1 to day 21. This shows that cells remained viable and stable during refrigerated storage. Relationship between pH, survival and fatty acid

composition was not clearly shown since significant differences in fatty acid percentages were not observed. Other factors could have possibly contributed to the improved survival of cells grown at lower pH; however, they were not investigated in this study.

The results suggested that strains exhibited different responses so further studies can be conducted to determine the best pH for each strain to survive better during freeze-drying and remain stable throughout storage, as well as investigate other possible stress responses that these strains of probiotic lactobacilli when grown in a pH-controlled environment.

Table 5. Growth of cultures of lactobacilli in MRS broth at 37 ^{o}C for 15 h at various pH

Growth pH	$\log_{10}$ CFU/ml[1]						
	L. casei		*L. acidophilus*				*L. reuteri*
	E-5	E-10	L-1	L-23	O-16	381-IL-28	X-18
4.5	10.37[a]	9.89[b]	10.35[a]	10.51[a]	10.47[a]	10.23[a]	8.15[b]
5.0	10.59[a]	10.63[a]	10.58[a]	10.64[a]	10.82[a]	10.43[a]	10.40[a]
5.5	10.50[a]	10.69[a]	10.60[a]	10.65[a]	10.48[a]	10.74[a]	10.76[a]
6.0	10.59[a]	10.43[a]	10.66[a]	10.33[a]	10.39[a]	10.67[a]	10.58[a]

[1] Values are means from three trials

[a,b] Means within columns without a common superscript are significantly different (P>0.05)

Table 6. Effect of growth pH on survival of freeze-dried cultures of lactobacilli during storage at 5 °C for 21 days

	Survival (%) (LS Means ±SEM)[1]					
	L. casei	*L. acidophilus*				*L. reuteri*
pH[2]	E-5	L-1	L-23	O-16	381-IL-28	X-18
4.5	54.70 ± 5.98[a]	58.62 ± 7.49[a]	53.89 ± 6.99[a]	12.90 ± 6.24[b]	69.91 ± 10.11[a]	82.09 ± 6.32[a]
5.0	58.42 ± 5.22[a]	51.65 ± 7.59[a]	30.03 ± 6.06[b]	12.07 ± 5.97[b]	56.26 ± 11.67[a]	60.36 ± 9.79[b]
5.5	49.47 ± 5.16[a]	24.97 ± 6.43[b]	22.74 ± 5.25[b]	20.30 ± 8.84[a]	29.22 ± 10.09[b]	20.85 ± 6.98[c]
6.0	31.89 ± 5.76[b]	17.33 ± 5.43[b]	27.86 ± 5.83[b]	37.29 ± 11.88[a]	24.99 ± 9.40[b]	22.09 ± 7.25[c]

[1] Values are least square means from three trials. SEM = Standard Error Mean

[a,b,c] Means within columns without a common superscript are significantly different (P>0.05)

[2] Cultures were grown for 15 h in MRS broth at different pH, freeze-dried and stored at 5°C for 21days

Table 7. Viability of freeze-dried cultures of lactobacilli grown at different pH during storage

Day	Survival (%) (LS Means ±SEM)[1]					
	L. casei	*L. acidophilus*				*L. reuteri*
	E-5	L-1	L-23	O-16	381-IL-28	X-18
1	50.53 ± 2.92[a]	46.03 ± 5.21[a]	34.89 ± 5.55[a]	25.53 ± 9.00[a]	54.53± 10.49[a]	49.71 ± 9.88[a]
7	50.49 ± 4.88[a]	35.25 ± 5.19[b]	35.27 ± 5.79[a]	22.28 ± 8.20[ab]	47.89 ± 10.74[a]	47.74 ± 10.02[a]
14	46.24 ± 3.17[a]	35.17 ± 4.77[b]	31.89 ± 5.40[a]	19.82 ± 7.69[b]	40.89 ± 10.36[a]	43.48 ± 9.34[a]
21	46.47 ± 1.99[a]	29.53 ± 5.17[b]	29.44 ± 5.61[a]	11.25 ± 5.40[c]	35.02 ± 9.30[a]	42.86 ± 9.28[a]

[1] Values are least square means from three trials. SEM = Standard Error Mean
[a,b,c] Means within columns without a common superscript are significantly different (P>0.05)
[2] Cultures were grown for 15 h in MRS broth with different pH, freeze-dried and stored at 5^{o}C for 21days

Table 8. Fatty acid composition of lactobacilli cultivated in MRS broth at various pH

Culture	pH[1]	Fatty Acid composition (%)[2]					
		C14:0	C16:0	C16:1	C18:0	C18:1	C19cyc 11
L. casei	4.5	2.42[a]	29.70[a]	1.84[a]	3.12[a]	23.60[a]	39.20[a]
E-5	5.0	3.55[a]	40.65[a]	2.31[a]	3.7[a]	23.32[a]	26.46[a]
	5.5	3.37[a]	45.13[a]	3.50[a]	4.74[a]	21.09[a]	22.17[a]
	6.0	3.17[a]	28.37[a]	2.02[a]	4.30[a]	35.51[a]	19.44[a]
L. casei	4.5	10.08[a]	27.67[a]	0.28[a]	3.45[a]	23.72[a]	32.00[a]
E-10	5.0	3.30[a]	53.94[a]	2.21[a]	4.69[a]	14.21[a]	21.65[a]
	5.5	3.15[a]	51.74[a]	2.50[a]	5.06[a]	19.19[a]	18.26[a]
	6.0	2.69[a]	37.23[a]	2.15[a]	5.16[a]	29.12[a]	20.40[a]
L. acidophilus	4.5	3.26[a]	36.94[a]	0.45[a]	14.02[a]	5.90[b]	22.30[ab]
L-1	5.0	3.74[a]	49.95[a]	0.73[a]	4.91[a]	16.30[b]	23.49[a]
	5.5	9.68[a]	34.18[a]	4.71[a]	24.46[a]	6.26[b]	21.00[ab]
	6.0	12.26[a]	15.84[a]	8.73[a]	10.80[a]	43.82[a]	3.15[b]
L. acidophilus	4.5	2.67[a]	48.60[a]	0.82[a]	6.10[a]	18.55[a]	22.66[a]
L-23	5.0	3.71[a]	48.33[a]	0.74[a]	11.64[a]	12.64[a]	14.40[a]
	5.5	3.70[a]	48.55[a]	1.83[a]	5.44[a]	21.48[a]	18.49[a]
	6.0	3.70[a]	25.56[a]	7.21[a]	3.97[a]	30.50[a]	14.21[a]
L. acidophilus	4.5	4.52[a]	2.22[a]	0.56[a]	2.49[a]	61.82[a]	27.86[a]
O-16	5.0	0.56[a]	4.87[a]	ND	0.93[a]	63.67[a]	25.77[a]
	5.5	1.76[a]	2.91[a]	0.12[a]	3.07[a]	68.41[a]	23.17[a]
	6.0	0.58[a]	3.52[a]	2.05[a]	1.09[a]	28.81[a]	17.55[a]
L. acidophilus	4.5	2.90[a]	52.83[a]	0.28[a]	4.77[a]	6.97[a]	32.25[a]
381-IL-28	5.0	3.03[a]	43.87[a]	0.88[a]	4.85[a]	21.00[a]	29.09[a]
	5.5	3.13[a]	49.58[a]	1.52[a]	5.37[a]	20.36[a]	20.66[a]
	6.0	2.00[a]	28.90[a]	11.44[a]	5.14[a]	27.67[a]	13.77[a]
L. reuteri	4.5	4.52[a]	2.22[a]	0.56[a]	2.49[ab]	61.82[a]	27.86[a]
X-18	5.0	0.56[a]	1.30[a]	ND	3.19[a]	63.67[a]	30.68[a]
	5.5	1.76[a]	2.91[a]	0.12[a]	3.07[ab]	68.35[a]	23.08[a]
	6.0	3.03[a]	1.85[a]	1.71[a]	1.89[b]	41.52[a]	18.09[a]

[1] Cultures were cultivated in MRS broth under controlled pH for 15 h

[2] Each value is the mean from three trials expressed as relative percentage. Fatty acids are: Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND = Not detected

[a,b] Means within strain in the same column with common superscript are not significantly different (P > 0.05)

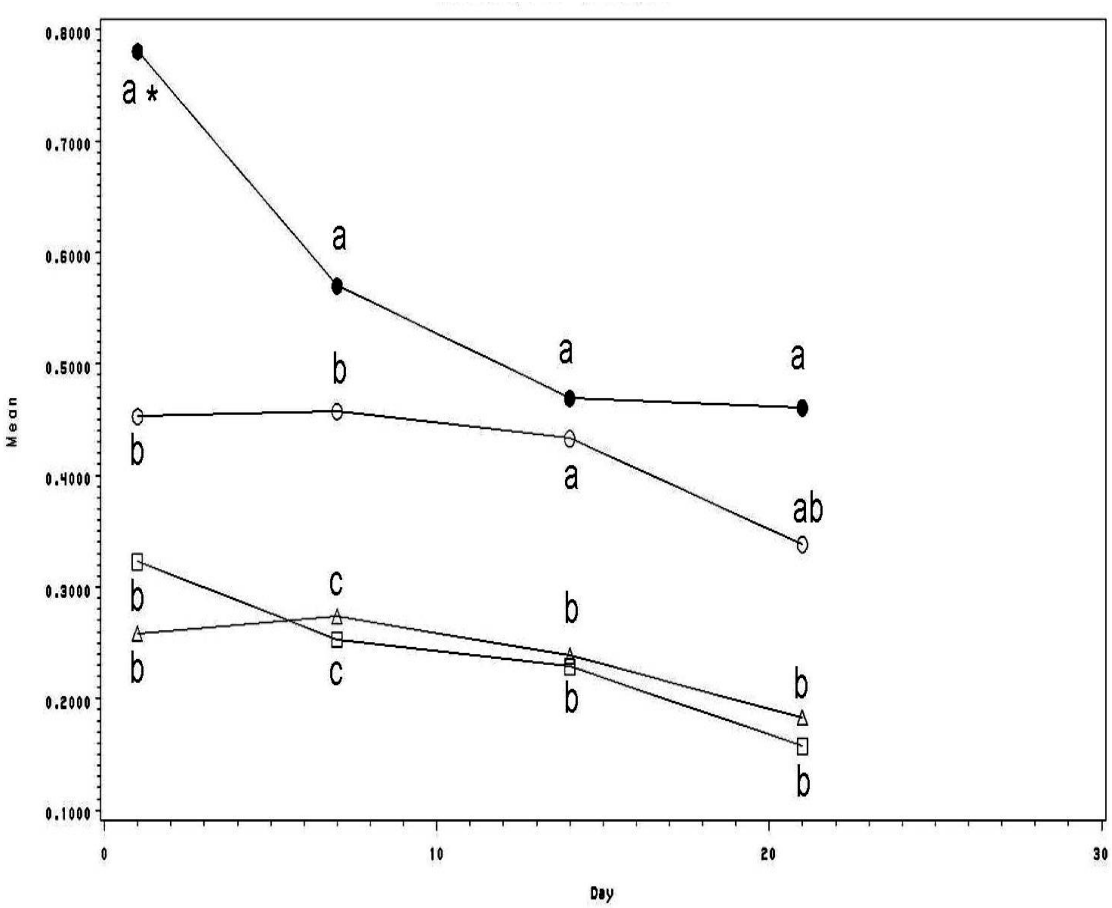

Figure 1. Survival during storage of freeze-dried cells of *Lactobacillus casei* E-10 grown in MRS broth with different pH values; ● pH 4.5, ○ pH 5.0, Δ 5.5, □ 6.0.
[a,b,c] Means within day with different letters differ significantly ($P<0.05$);
* Within pH, mean differ from all other

CHAPTER V

OVERALL CONCLUSION

The present study provided evidence that different cultures and strains of lactobacilli exhibited different responses to changes in growth conditions. In general Tween 80 did not have a significant effect on viability of most cultures except *L. reuteri* X-18. Analysis of the fatty acid composition showed higher C18:1 and C19:0cyc11 concentrations in this strain and this could have contributed to better resistance during freeze-drying. In the study on growth pH, most of the cultures generally exhibited higher survival when grown in more acidic pH; however, results were not consistent in all strains. Our study also showed the physiological effect of pH on fatty acid composition; however, due to high variability in the data the relationship between these changes in membrane composition and survival was not clearly elucidated.

In the storage stability test, slight decline in viability for both Tween 80 and pH studies were not significant. Stability of the cultures during storage was attributed to storage conditions like low temperature, absence or minimal exposure to oxygen and light since the vials were sealed under vacuum and stored at 5 ^{o}C. The present study was limited to only 21 days and it is interesting to investigate how the cultures will hold up during prolonged storage.

Further research need to be done to improve viability of lactobacilli during freeze-drying like investigation of other factors and/or combined effect of pH and Tween 80. Knowledge of the optimum growth conditions would be beneficial in the selection of appropriate additives and growth pH since different organisms often exhibit different behaviors in the production of concentrated cultures.

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APPENDIX A

Table A1. Confirmation of identity of *Lactobacillus acidophilus* using API 50 CH

Test	*L. acidophilus*				Bergey's
	L-1	L-23	O-16	381-IL-28	
D-Arabinose	-	-	-	-	-
D-Ribose	-	-	-	-	-
D-Xylose	-	-	-	-	-
D-Galacatose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	-
L-Rhanmnose	-	-	-	-	-
D-Mannitol	-	-	-	-	-
D-Sorbitol	+	-	-	-	
Amygladine	+	+	+	+	+
Esculine	+	+	+	+	+
Salicine	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
D-Malstose	+	+	+	+	+
D-Lactose	+	+	+	+	+
D-Melibiose	+	+	+	+	+
D-Saccharose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Melezitose	-	-	-	-	-
D-Raffinose	-	-	-	-	±
Amidon	-	-	-	-	+
Growth at 45 ^{o}C	+	+	+	+	+
Growth at 15 ^{o}C	-	-	-	-	-

[1]All cultures were Gram positive rods, catalase negative
[2] *Lactobacillus acidophilus* reactions listed in the 9th edition of Bergey's Manual of Systematic Bacteriology, 1986

Table A2. Confirmation of identity of *Lactobacillus casei* using API 50 CH

Test	*L. casei*		Bergey's
	E-5	E-10	
D-Arabinose	-	-	-
D-Ribose	+	+	+
D-Xylose	-	-	-
D-Galacatose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Rhamnose	-	-	-
D-Mannitol	+	+	+
D-Sorbitol	+	+	+
Amygladine	+	+	+
Esculine	+	+	+
Salicine	+	+	+
D-Cellobiose	+	+	+
D-Maltose	+	+	+
D-Lactose	+	+	±
D-Melibiose	+	+	-
D-Saccharose	+	+	+
D-Trehalose	+	+	+
D-Melezitose	+	+	+
D-Raffinose	+	+	-
Growth at 45 ^{o}C	+		+
Growth at 15 ^{o}C	-	-	-

[1]All cultures were Gram positive rods, catalase negative

[2] *Lactobacillus acidophilus* reactions listed in the 9th edition of Bergey's Manual of Systematic Bacteriology, 1986

Table 9. Confirmation of identity of *Lactobacillus reuteri* using API 50 CH

Test	*L. reuteri* X-18	Bergey's
D-Arabinose	-	-
D-Ribose	-	-
D-Xylose	-	-
L-Xylose	-	-
D-Galacatose	+	+
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	+	-
L-Rhamnose	-	-
D-Mannitol	-	-
D-Sorbitol	-	-
Amygladine	-	-
Esculine	-	-
Salicine	+	-
D-Maltose	+	+
D-Lactose	+	+
D-Melibiose	+	+
D-Saccharose	+	+
D-Trehalose	+	-
D-Melezitose	-	-
D-Raffinose	+	+

[1]All cultures were Gram positive rods, catalase negative

[2] *Lactobacillus reuteri* reactions listed in the 9th edition of Bergey's Manual of Systematic Bacteriology, 1986

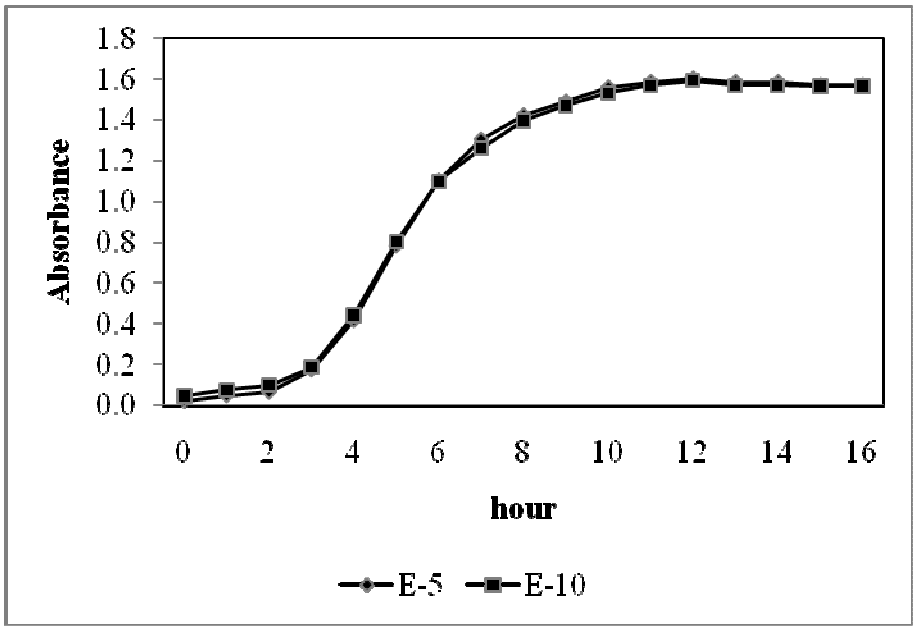

Figure A1. Growth curve of *Lactobacillus casei*; Values were means of absorbance values at 620 nm from 3 replications; Cultures were grown in MRS broth at 37 ^{o}C

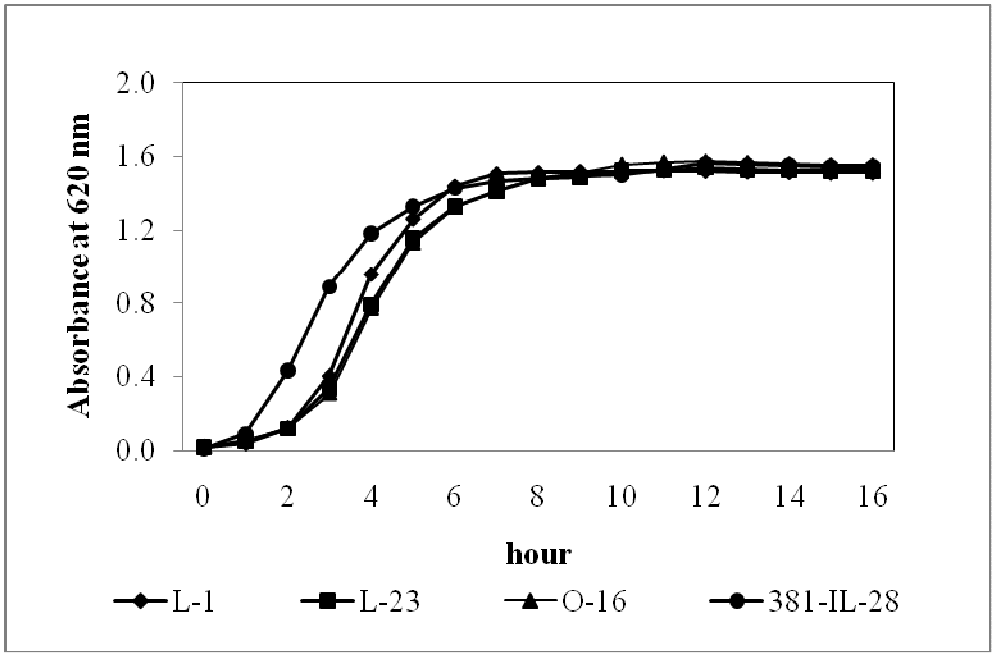

Figure A2. Growth curve of *Lactobacillus acidophilus*;
Values were means of absorbance values at 620 nm from three replications;
Cultures were grown in MRS broth at 37 ^{o}C

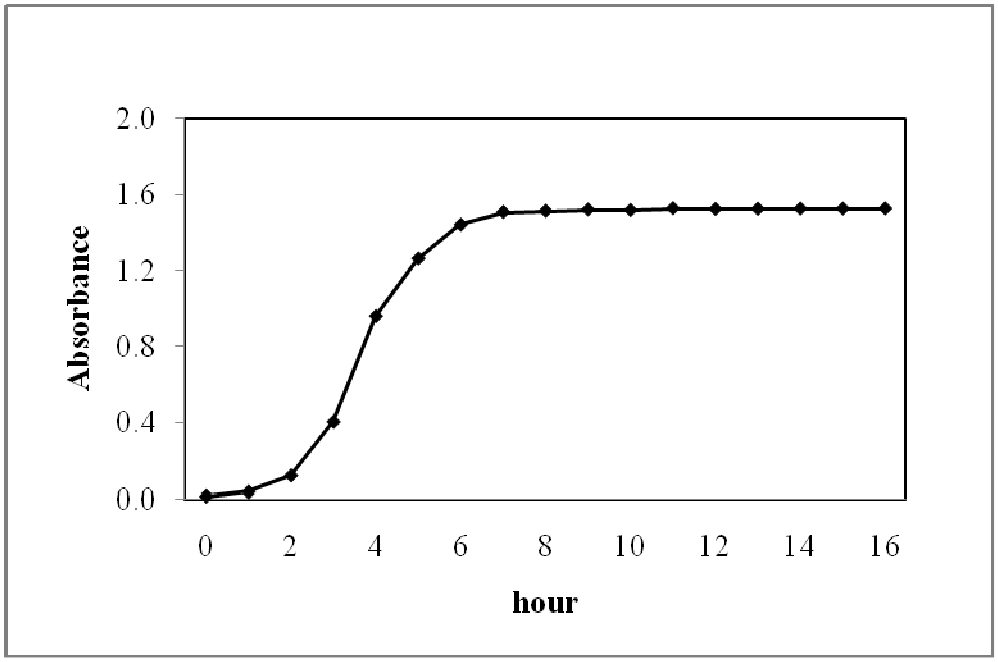

Figure B3. Growth curve of *Lactobacillus reuteri* X-18; Values were means from three replications; Culture was grown in MRS broth at 37 ^{o}C

APPENDIX B
FATTY ACID RAW DATA

Table B1. Fatty acid composition of *Lactobacillus* casei E-5 grown in MRS[1] at different pH

	Relative concentration (%)[2]					
pH	C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc9
4.5						
rep 1	1.33	24.27	2.44	3.07	32.14	36.40
rep 2	3.51	14.15	2.80	1.70	34.23	43.61
rep 3	2.43	50.68	0.30	4.58	4.42	37.58
Mean	**2.42**	**29.70**	**1.85**	**3.12**	**23.60**	**39.20**
Std Dev	**1.09**	**18.86**	**1.35**	**1.44**	**16.64**	**3.87**
5.0						
rep 1	3.27	43.19	3.69	3.29	31.75	14.83
rep 2	2.52	22.92	2.24	1.82	22.11	48.39
rep 3	4.86	55.85	1.02	5.99	16.12	16.17
Mean	**3.55**	**40.65**	**2.32**	**3.70**	**23.33**	**26.46**
Std Dev.	**1.19**	**16.61**	**1.34**	**2.12**	**7.89**	**19.00**
5.5						
rep 1	2.47	41.13	4.16	2.91	11.94	37.39
rep 2	2.86	42.51	5.06	3.62	28.88	17.13
rep 3	4.78	51.74	1.28	7.69	22.51	12.00
Mean	**3.37**	**45.13**	**3.50**	**4.74**	**21.11**	**22.17**
Std Dev	**1.24**	**5.77**	**1.97**	**2.58**	**8.56**	**13.43**
6.0						
rep 1	2.70	12.16	2.53	1.86	28.64	52.10
rep 2	3.00	32.40	1.51	5.04	34.85	22.88
rep 3	3.81	40.54	2.01	5.99	43.05	4.20
Mean	**3.17**	**28.37**	**2.02**	**4.30**	**35.51**	**26.39**
Std Dev	**0.57**	**14.61**	**0.51**	**2.16**	**7.23**	**24.14**

[1] MRS – Man Rogosa and Sharpe broth

[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not detected

Table B2. Fatty acid composition of *Lactobacillus casei* E-10 grown in MRS[1] at different pH

	Relative concentration (%)[2]					
pH	C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc9
4.5						
rep 1	25.37	8.65	0.01	2.41	44.79	10.36
rep 2	3.24	50.97	0.84	5.02	6.53	33.39
rep 3	1.62	23.38	0.00	2.90	19.85	52.24
Mean	**10.08**	**27.67**	**0.28**	**3.44**	**23.72**	**32.00**
Std Dev	**13.27**	**21.48**	**0.48**	**1.39**	**19.42**	**20.97**
5.0						
rep 1	2.6	46.42	4.71	3.51	20.5	22.26
rep 2	4.3	55.26	1.33	5.13	14.56	19.42
rep 3	2.99	60.13	0.6	5.44	7.56	23.28
Mean	**3.30**	**53.94**	**2.21**	**4.69**	**14.21**	**21.65**
Std Dev	**0.89**	**6.95**	**2.19**	**1.04**	**6.48**	**2.00**
5.5						
rep 1	2.75	50.88	4.54	2.84	13.33	25.65
rep 2	4.48	54.65	1.44	4.51	14.93	20.00
rep 3	2.23	49.68	1.53	7.82	29.32	9.13
Mean	**3.15**	**51.74**	**2.50**	**5.06**	**19.19**	**18.26**
Std Dev	**1.18**	**2.59**	**1.76**	**2.53**	**8.81**	**8.40**
6.0						
rep 1	2.56	17.15	2.71	1.98	21.80	53.80
rep 2	3.14	45.36	2.17	5.88	35.99	7.11
rep 3	2.38	49.18	1.57	7.60	29.58	0.28
Mean	**2.69**	**37.23**	**2.15**	**5.15**	**29.12**	**20.40**
Std Dev	**0.40**	**17.49**	**0.57**	**2.88**	**7.11**	**29.13**

[1] MRS – Man Rogosa and Sharpe broth
[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B3. Fatty acid composition of *Lactobacillus acidophilus* L-1 grown in MRS[1] at different pH

	Relative concentration (%)[2]					
pH	C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc9
4.5						
rep1	3.49	51.16	0.74	4.94	8.90	30.77
rep 2	2.51	1.55	0.00	31.86	0.32	12.41
rep 3	3.80	58.11	0.60	5.27	8.50	23.72
Mean	**3.27**	**36.94**	**0.45**	**14.02**	**5.91**	**22.30**
Std Dev	**0.67**	**30.85**	**0.39**	**15.45**	**4.84**	**9.26**
5.0						
rep1	3.59	47.92	0.00	4.89	11.42	30.15
rep 2	3.31	43.40	2.19	5.04	25.80	20.25
rep 3	4.31	58.54	0.00	4.81	11.67	20.08
Mean	**3.74**	**49.95**	**0.73**	**4.91**	**16.30**	**23.49**
Std Dev	**0.52**	**7.77**	**1.26**	**0.12**	**8.23**	**5.77**
5.5						
rep1	3.48	46.23	1.29	4.54	16.39	27.85
rep 2	19.96	19.80	1.17	23.71	2.03	32.89
rep 3	5.59	36.52	11.67	45.13	0.35	2.28
Mean	**9.68**	**34.18**	**4.71**	**24.46**	**6.26**	**21.01**
Std Dev	**8.97**	**13.37**	**6.03**	**20.31**	**8.82**	**16.41**
6.0						
rep1	31.70	0.00	24.41	24.41	0.00	4.68
rep 2	0.00	6.35	0.00	2.02	90.59	1.04
rep 3	5.08	41.16	1.79	5.98	40.87	3.73
Mean	**12.26**	**15.84**	**8.73**	**10.80**	**43.82**	**3.15**
Std Dev	**17.03**	**22.16**	**13.61**	**11.95**	**45.37**	**1.89**

[1] MRS – Man Rogosa and Sharpe broth
[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B4. Fatty acid composition of *Lactobacillus acidophilus* L-23 grown in MRS[1] at different pH

	Relative concentration (%)[2]					
pH	C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc9
4.5						
rep 1	2.4	39.53	1.25	7.91	39.67	7.45
rep 2	2.4	50.81	0.31	4.41	5.85	36.23
rep 3	3.21	55.45	0.92	5.98	10.13	24.31
Mean	**2.67**	**48.60**	**0.83**	**6.10**	**18.55**	**22.66**
Std Dev	**0.47**	**8.19**	**0.48**	**1.75**	**18.42**	**14.46**
5.0						
rep 1	1.61	41.06	1.3	25.6	6.79	0.26
rep 2	6.08	56.07	0.93	4.34	18.49	13.08
rep 3	3.44	47.86	0	4.97	12.63	29.87
Mean	**3.71**	**48.33**	**0.74**	**11.64**	**12.64**	**14.40**
Std Dev	**2.25**	**7.52**	**0.67**	**12.10**	**5.85**	**14.85**
5.5						
rep 1	2.17	42.77	1.95	4.1	17.67	30.57
rep 2	5.62	51.32	1.46	5.35	24.72	11.22
rep 3	3.31	51.56	2.09	6.87	22.05	13.68
Mean	**3.70**	**48.55**	**1.83**	**5.44**	**21.48**	**18.49**
Std Dev	**1.76**	**5.01**	**0.33**	**1.39**	**3.56**	**10.53**
6.0						
rep 1	0	0	16.82	0	4.4	34.78
rep 2	6.35	38.68	3.15	6.09	41.94	4.37
rep 3	4.76	37.99	1.66	5.81	45.11	3.47
Mean	**3.70**	**25.56**	**7.21**	**3.97**	**30.48**	**14.21**
Std Dev	**3.30**	**22.14**	**8.36**	**3.44**	**22.64**	**17.82**

[1] MRS – Man Rogosa and Sharpe broth
[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B5. Fatty acid composition of *Lactobacillus acidophilus* O-16 grown in MRS[1] at different pH

	Relative concentration (%)[2]					
pH	C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc9
4.5						
rep1	13.3	3.86	1.41	2.31	66.04	12.62
rep 2	0.26	1.45	0.27	2.06	61.19	34.15
rep 3	0	1.34	0	3.09	58.24	36.8
Mean	**4.52**	**2.22**	**0.56**	**2.49**	**61.82**	**27.86**
Std Dev	**7.60**	**1.42**	**0.75**	**0.54**	**3.94**	**13.26**
5.0						
rep1	1.68	1.06	0	2.98	66.15	12.62
rep 2	0	11.91	0	3.17	66.69	28.41
rep 3	0	1.65	0	3.41	58.17	36.29
Mean	**0.56**	**4.87**	**0.00**	**3.19**	**63.67**	**25.77**
Std Dev	**0.97**	**6.10**	**0.00**	**0.22**	**4.77**	**12.05**
5.5						
rep1	4.14	3.29	0	2.71	52.04	36.97
rep 2	0.2	1.01	0	2.85	85.26	10.01
rep 3	0.95	4.43	0.35	3.63	67.94	22.53
Mean	**1.76**	**2.91**	**0.12**	**3.06**	**68.41**	**23.17**
Std Dev	**2.09**	**1.74**	**0.20**	**0.50**	**16.62**	**13.49**
6.0						
rep1	0.55	6.06	3.63	0	2.44	36.65
rep 2	0	0	2.18	0	8.58	1.48
rep 3	1.19	4.5	0.33	3.28	75.42	14.52
Mean	**0.58**	**3.52**	**2.05**	**1.09**	**28.81**	**17.55**
Std Dev	**0.60**	**3.15**	**1.65**	**1.89**	**40.48**	**17.78**

[1] MRS – Mann Rogosa and Sharpe broth

[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B6. Fatty acid composition of *Lactobacillus acidophilus* 381-IL-28 grown in MRS[1] at different pH

	Relative concentration (%)[2]					
pH	C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc9
4.5						
rep1	2.87	48.71	0.41	4.16	7.94	35.92
rep 2	2.87	48.54	0.43	4.22	7.9	36.04
rep 3	2.97	61.24	0	5.92	5.07	24.81
Mean	**2.90**	**52.83**	**0.28**	**4.77**	**6.97**	**32.26**
Std Dev	**0.06**	**7.28**	**0.24**	**1.00**	**1.65**	**6.45**
5.0						
rep1	1.71	25.12	0.74	2.67	30.55	49.21
rep 2	3.52	55.16	0.55	4.86	11.58	22.22
rep 3	3.85	51.32	1.34	7.04	20.86	15.85
Mean	**3.03**	**43.87**	**0.88**	**4.86**	**21.00**	**29.09**
Std Dev	**1.15**	**16.35**	**0.41**	**2.19**	**9.49**	**17.71**
5.5						
rep1	2.05	45.15	1.71	4.47	16.77	32.00
rep 2	3.74	50.44	1.46	5.9	24.87	13.3
rep 3	3.59	53.15	1.38	5.74	19.44	16.69
Mean	**3.13**	**49.58**	**1.52**	**5.37**	**20.36**	**20.66**
Std Dev	**0.94**	**4.07**	**0.17**	**0.78**	**4.13**	**9.96**
6.0						
rep1	0	0.79	31.16	0.75	6.48	28.34
rep 2	3.11	42.63	1.67	6.51	38.57	7.15
rep 3	2.88	43.36	1.49	8.16	37.97	5.83
Mean	**2.00**	**28.93**	**11.44**	**5.14**	**27.67**	**13.77**
Std Dev	**1.73**	**24.37**	**17.08**	**3.89**	**18.36**	**12.63**

[1] MRS – Man Rogosa and Sharpe broth
[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B7. Fatty acid composition of *Lactobacillus reuteri* X-18 grown in MRS[1] at different pH

	Relative concentration (%)[2]					
pH	C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc9
4.5						
rep1	13.31	3.86	1.41	2.31	66.04	12.62
rep 2	0.26	1.45	0.27	2.06	61.19	34.15
rep 3	0.00	1.34	0.00	3.09	58.24	36.80
Mean	**4.52**	**2.22**	**0.56**	**2.49**	**61.82**	**27.86**
Std Dev	**7.61**	**1.42**	**0.75**	**0.54**	**3.94**	**13.26**
5.0						
rep1	1.68	1.06	ND	2.98	66.15	27.33
rep 2	0.00	1.19	ND	3.17	66.69	28.41
rep 3	0.00	1.65	ND	3.41	58.17	36.29
Mean	**0.56**	**1.30**	**ND**	**3.19**	**63.67**	**30.68**
Std Dev	**0.97**	**0.31**	**ND**	**0.22**	**4.77**	**4.89**
5.5						
rep1	4.14	3.29	0.00	2.71	52.04	36.97
rep 2	0.20	1.01	0.00	2.85	85.36	10.01
rep 3	0.95	4.44	0.35	3.63	67.64	22.25
Mean	**1.76**	**2.91**	**0.12**	**3.06**	**68.35**	**23.08**
Std Dev	**2.09**	**1.75**	**0.20**	**0.50**	**16.67**	**13.50**
6.0						
rep1	0.55	6.06	3.63	0.00	2.44	36.65
rep 2	0.00	0.00	1.18	0.00	4.63	0.80
rep 3	1.18	4.50	0.33	3.28	75.42	14.52
Mean	**3.03**	**1.85**	**0.45**	**0.52**	**3.41**	**4.56**
Std Dev	**0.59**	**3.15**	**1.71**	**1.89**	**41.52**	**18.09**

[1] MRS – Man Rogosa and Sharpe broth
[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B8. Fatty acid composition of *Lactobacillus casei* E-5 grown in MRS[1] with different concentrations of Tween 80

Rep	Tween 80 (%)	Relative concentration (%)[2] C14:0	C16:0	C18:0	C16:1	C18:1	C19:0cyc11
1	0	1.27	66.00	1.46	8.78	1.43	21.5
2		1.12	52.41	1.64	7.03	1.6	32.6
3		0.94	52.15	1.72	5.41	2.72	37.06
Mean		**1.20**	**59.21**	**1.55**	**7.91**	**1.52**	**27.05**
Std. Dev.		0.17	7.92	0.13	1.69	0.70	8.01
1	0.1	3.44	45.13	3.88	2.32	26.89	18.34
2		3.00	50.45	3.18	4.4	19.35	19.62
3							
Mean		**3.22**	**47.79**	**3.53**	**3.36**	**23.12**	**18.98**
Std. Dev.		0.31	3.76	0.49	1.47	5.33	0.91
1	0.2	3.46	14.35	4.39	2.35	27.76	48.19
2		2.59	37.33	2.65	0.39	12.03	45.00
3		3.10	23.87	4.09	1.87	17.63	49.44
Mean		**3.05**	**25.18**	**3.71**	**1.54**	**19.14**	**47.54**
Std. Dev.		0.44	11.55	0.93	1.02	7.97	2.29
1	0.3	3.39	13.28	4.56	2.11	29.28	46.84
2		4.29	10.16	7.35	0.89	29.16	46.15
3		4.23	23.95	5.16	0.42	18.81	47.44
Mean		**3.97**	**15.80**	**5.69**	**1.14**	**25.75**	**46.81**
Std. Dev.		0.50	7.23	1.47	0.87	6.01	0.65
1	0.4	3.54	12.84	5.03	2.36	31.69	44.55
2		4.11	10.35	7.01	1.93	29.31	47.28
3		5.38	21.28	6.50	1.22	16.47	49.14
Mean		**4.34**	**14.82**	**6.18**	**1.84**	**25.82**	**46.99**
Std. Dev.		0.94	5.73	1.03	0.58	8.19	2.31
1	0.5	4.07	12.01	6.13	2.11	29.60	46.07
2		5.17	11.85	7.66	0.80	26.89	47.91
3		5.76	21.92	21.92	6.23	1.53	48.23
Mean		**5.00**	**15.26**	**11.90**	**3.05**	**19.34**	**47.40**
Std. Dev.		0.86	5.77	8.71	2.83	15.48	1.17

[1] MRS – Man Rogosa and Sharpe broth

[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B9. Fatty acid composition of *Lactobacillus casei* E-10 grown in MRS[1] with different concentrations of Tween 80

	Tween 80	Relative concentration (%)[2]					
Rep	(%)	C14:0	C16:0	C18:0	C16:1	C18:1	C19:0cyc11
1	0	2.04	44.08	1.24	6.88	45.22	7.34
2		0.97	51.28	1.58	6.2	2.09	37.89
3		1.03	52.11	0.88	16.6	10.25	28.14
Mean		**1.51**	**47.68**	**1.41**	**6.54**	**23.66**	**22.62**
Std. Dev.		0.60	4.42	0.35	5.82	22.91	15.60
1	0.1	0.93	40.88	0.89	24.63	1.78	20.9
2		3.68	45.76	4	2.9	26.38	16.27
3		3.18	18.39	3.28	3.03	30.26	41.86
Mean		**2.31**	**43.32**	**2.45**	**13.77**	**14.08**	**18.59**
Std. Dev.		1.46	14.60	1.63	12.51	15.45	13.64
1	0.2	2.50	12.47	4.02	2.34	42.41	36.26
2		3.92	9.21	7.01	1.44	31.98	46.45
3		4.07	31.02	4.61	5.28	48.52	6.50
Mean		**3.50**	**17.57**	**5.21**	**3.02**	**40.97**	**29.74**
Std. Dev.		0.87	11.76	1.58	2.01	8.36	20.76
1	0.3	2.40	11.17	3.89	1.10	39.64	41.80
2		2.40	11.17	3.89	1.10	39.64	41.80
3		2.70	17.14	3.18	2.29	36.46	42.22
Mean		**2.50**	**13.16**	**3.65**	**14.34**	**39.30**	**42.01**
Std. Dev.		0.17	3.45	0.41	21.92	2.69	0.24
1	0.4	2.47	10.84	3.86	1.22	35.24	46.37
2		4.41	9.87	7.52	1.20	29.44	47.55
3		1.76	41.19	0.88	6.22	37.31	12.63
Mean		**2.88**	**20.63**	**4.09**	**2.88**	**34.00**	**35.52**
Std. Dev.		1.37	17.81	3.33	2.89	4.08	19.83
1	0.5	3.76	15.27	15.27	4.39	1.31	38.03
2		5.55	11.81	11.81	8.05	1.51	46.10
3		3.59	18.04	3.78	3.69	29.70	41.21
Mean		**4.30**	**15.04**	**10.29**	**5.38**	**10.84**	**41.78**
Std. Dev.		1.09	3.12	5.89	2.34	16.33	4.07

[1] MRS – Man Rogosa and Sharpe broth

[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B10. Fatty acid composition of *Lactobacillus acidophilus* L-1 grown in MRS[1] with different concentrations of Tween 80

	Tween 80	Relative concentration (%)[2]					
Rep	(%)	C14:0	C16:0	C18:0	C16:1	C18:1	C19:0cyc11
1							
2	0	0.93	41.6	0.65	23.91	1.26	31.35
3		1.21	44.62	0.81	25.75	2.31	25.3
Mean		**1.07**	**43.11**	**0.73**	**24.83**	**1.79**	**28.33**
Std. Dev.		0.20	2.14	0.11	1.30	0.74	4.28
1		2.55	0.93	7.01	0.33	85.09	1.35
2	0.1	0.92	12.29	0.64	2.9	49.77	33.14
3		0.51	5.78	0.01	1.44	87.95	2.55
Mean		**1.33**	**6.33**	**2.55**	**1.56**	**74.27**	**12.35**
Std. Dev.		1.08	5.70	3.87	1.29	21.27	18.02
1		0.86	3.76	7.17	4.8	79.4	3.37
2	0.2	1.8	8.75	3.78	1.76	47.51	36.39
3		2.41	35.95	2.52	1.23	11.79	46.1
Mean		**1.69**	**16.15**	**4.49**	**2.60**	**46.23**	**28.62**
Std. Dev.		0.78	17.33	2.40	1.93	33.82	22.40
1		0.74	4.33	6.91	4.72	78.9	4.4
2	0.3	1.95	9.07	3.97	1.45	46.12	37.44
3		3.42	7.43	5.74	1	77.07	4.43
Mean		**2.04**	**6.94**	**5.54**	**2.39**	**67.36**	**15.42**
Std. Dev.		1.34	2.41	1.48	2.03	18.42	19.07
1		0.71	5.64	14.71	10.12	60.41	6.87
2	0.4	2.38	10.43	4.3	1.63	43.43	37.92
3		4.94	8.46	6.83	0.88	70.9	7.99
Mean		**2.68**	**8.18**	**8.61**	**4.21**	**58.25**	**17.59**
Std. Dev.		2.13	2.41	5.43	5.13	13.86	17.61
1							
2	0.5	0.48	7.26	18.43	11.43	54.76	6.23
3		2.82	11.61	4.48	1.53	41.77	37.79
Mean		**1.65**	**9.44**	**11.46**	**6.48**	**48.27**	**22.01**
Std. Dev.		1.65	3.08	9.86	7.00	9.19	22.32

[1] MRS – Man Rogosa and Sharpe broth

[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B11. Fatty acid composition of *Lactobacillus acidophilus* L-23 grown in MRS[1] with different concentrations of Tween 80

	Tween 80	Relative concentration (%)[2]					
Rep	(%)	C14:0	C16:0	C18:0	C16:1	C18:1	C19:0cyc11
1							
2	0	1.07	46.45	0.93	25.39	1.63	25.54
3		0.59	1.18	45.44	0.83	24.57	26.64
Mean		**0.83**	**23.82**	**23.19**	**13.11**	**13.10**	**26.09**
Std. Dev.		0.34	32.01	31.47	17.37	16.22	0.78
1		0.42	5.26	1.8	81.32	0.29	8.49
2	0.1	2.89	0.67	5.66	ND	1.78	7.61
3		1.05	8.41	0.62	4.2	51.51	34.21
Mean		**1.45**	**4.78**	**2.69**	**42.76**	**17.86**	**16.77**
Std. Dev.		1.28	3.89	2.64	54.53	29.15	15.11
1		3.36	9.05	5.19	1.86	69.12	10.24
2	0.2	0.79	5.07	13.54	9.44	1.58	6.54
3		1.7	9.25	3.66	1.83	41.2	42.35
Mean		**1.95**	**7.79**	**7.46**	**4.38**	**37.30**	**19.71**
Std. Dev.		1.30	2.36	5.32	4.39	33.94	19.69
1		4.85	11.12	7.5	2.32	62.86	10.37
2	0.3	0.65	6.01	16.43	10.27	2.76	6.60
3		1.77	9.25	3.69	1.78	22.05	39.46
Mean		**2.42**	**8.79**	**9.21**	**4.79**	**29.22**	**18.81**
Std. Dev.		2.17	2.59	6.54	4.75	30.69	17.98
1		14.29	12.15	1.65	52.21	52.21	11.99
2	0.4	0.8	4.07	7.21	5.15	0.32	3.62
3		2.38	12.87	4.09	2.43	37.57	41.22
Mean		**5.82**	**9.70**	**4.32**	**19.93**	**30.03**	**18.94**
Std. Dev.		7.37	4.89	2.79	27.99	26.75	19.74
1		16.35	12.97	1.52	48.41	48.51	11.68
2	0.5	0.28	7.21	7.19	5.87	ND	5.54
3		2.55	12.87	4.25	1.37	37.72	41.24
Mean		**6.39**	**11.02**	**4.32**	**18.55**	**43.12**	**19.49**
Std. Dev.		8.70	3.30	2.84	25.96	7.63	19.09

[1] MRS – Man Rogosa and Sharpe broth

[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND = Not Detected

Table B12. Fatty acid composition of *Lactobacillus acidophilus* O-16 grown in MRS[1] with different concentrations of Tween 80

	Tween 80	Relative concentration (%)[2]					
Rep	(%)	C14:0	C16:0	C18:0	C16:1	C18:1	C19cyc11
1	0	0.85	43.78	0.78	23.23	0.92	29.59
2		0.85	44.4	0.84	23.25	0.92	29.64
3							
Mean		**0.85**	**44.09**	**0.81**	**23.24**	**0.92**	**29.62**
Std. Dev.		0.00	0.44	0.04	0.01	0.00	0.04
1	0.1	2.57	5.38	0.01	2.41	66.87	22.32
2		0.01	1.02	0.01	2.56	74.85	21.02
3							
Mean		**1.29**	**3.20**	**0.01**	**2.49**	**70.86**	**21.67**
Std. Dev.		1.81	3.08	0.00	0.11	5.64	0.92
1	0.2	0.48	43.22	1.18	6.24	40.38	6.68
2		2.93	5.72	5.1	2.22	62.25	20.69
3		2.44	11.78	4.13	1.31	45.51	34.83
Mean		**1.95**	**20.24**	**3.47**	**3.26**	**49.38**	**20.73**
Std. Dev.		1.30	20.13	2.04	2.62	11.44	14.08
1	0.3	1.31	9.71	6.17	3.41	52.84	22.01
2		3.13	6.03	5.4	2.26	61.42	20.65
3		2.41	12.37	4.07	2.84	44.23	34.08
Mean		**2.28**	**9.37**	**5.21**	**2.84**	**52.83**	**25.58**
Std. Dev.		0.92	3.18	1.06	0.58	8.60	7.39
1	0.4	0.37	11.4	11.68	1.7	40.99	25.81
2		3.69	6.18	6.77	1.67	60.21	20.3
3		3.1	13.8	4.45	1.54	41.91	35.2
Mean		**2.39**	**10.46**	**7.63**	**1.64**	**47.70**	**27.10**
Std. Dev.		1.77	3.90	3.69	0.09	10.84	7.53
1	0.5	0.31	14.12	12.47	2.74	35.97	24.96
2		4.36	7.03	7.91	1.36	57.82	20.76
3		3.53	15.15	4.43	1.62	39.76	35.5
Mean		**2.73**	**12.10**	**8.27**	**1.91**	**37.87**	**27.07**
Std. Dev.		2.14	4.42	4.03	0.73	11.68	7.59

[1] MRS – Man Rogosa and Sharpe broth

[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B13. Fatty acid composition of *Lactobacillus acidophilus* 381-IL-28 grown in MRS[1] with different concentrations of Tween 80

	Tween 80	Relative concentration (%)[2]					
Rep	(%)	C14:0	C16:0	C16:1	C18:0	C18:1	C19:0cyc11
1	0	1.59	30.65	1.14	38.94	2.46	25.22
2		0.85	36.59	0.95	27.78	1.59	32.23
3		0.93	41.37	0.86	26.03	1.38	29.4
Mean		**1.22**	**33.62**	**1.05**	**33.36**	**2.03**	**28.73**
Std. Dev.		0.41	5.37	0.14	7.00	0.57	3.53
1	0.1	0.76	1.92	48.27	1.25	8.28	39.52
2		2.09	55.45	ND	7.98	24.68	9.11
3		1.67	44.2	1.14	7.58	3.62	8.52
Mean		**1.43**	**28.69**	**48.27**	**4.62**	**16.48**	**24.32**
Std. Dev.		0.68	28.22	33.33	3.78	11.06	17.73
1	0.2	3.85	19.98	2.24	2.1	25.85	46.54
2		2.12	13.11	3.4	1.87	41.84	37.67
3		2.33	11.82	3.95	1.56	42.94	37.38
Mean		**2.77**	**14.97**	**3.20**	**1.84**	**36.88**	**40.53**
Std. Dev.		0.94	4.39	0.87	0.27	9.57	5.21
1	0.3	4.28	18.27	2.53	1.92	26.55	46.43
2		2.66	11.54	4.07	1.49	42.18	38.06
3		2.50	11.76	4.02	1.39	42.97	37.33
Mean		**3.15**	**13.86**	**3.54**	**1.60**	**37.23**	**40.61**
Std. Dev.		0.98	3.82	0.88	0.28	9.26	5.06
1	0.4	2.82	13.82	4.22	1.28	40.31	37.55
2		3.59	13.79	4.7	1.14	36.69	40.07
3		2.93	13.63	4.29	2.76	41.34	3.5
Mean		**3.11**	**13.75**	**4.40**	**1.73**	**39.45**	**27.04**
Std. Dev.		0.42	0.10	0.26	0.90	2.44	20.43
1	0.5						
2		4.1	15.02	4.58	1.36	34.52	40.04
3		3.76	15.49	4.24	1.29	37.84	37.37
Mean		**3.93**	**15.26**	**4.41**	**1.33**	**37.84**	**38.71**
Std. Dev.		0.24	0.33	0.24	0.05	2.35	1.89

[1] MRS – Man Rogosa and Sharpe broth

[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

Table B14. Fatty acid composition of *Lactobacillus reuteri* X-18 grown in MRS[1] with different concentrations of Tween 80.

	Tween 80	Relative concentration (%)[2]					
Rep	(%)	C14:0	C16:0	C16:1	C18:0	C18:1	C19cyc11
1	0	1.57	51.67	0.89	16.48	4.01	28.03
2		3.08	50.42	0.94	5.04	24.71	15.18
3		0.96	42.56	0.58	24.37	1.21	30.33
Mean		**2.33**	**51.05**	**0.92**	**10.76**	**14.36**	**21.61**
Std. Dev.		1.09	4.94	0.20	9.72	12.84	8.16
1	0.1	2.95	50.11	0.88	5.88	23.75	16.42
2		0.58	6.71	0.41	2.93	57.68	31.36
3		0.96	12.34	0.56	3.53	46.31	36.31
Mean		**1.77**	**28.41**	**0.65**	**4.41**	**40.72**	**23.89**
Std. Dev.		1.27	23.60	0.24	1.56	17.27	10.35
1	0.2	4.53	12.53	4.14	1.82	32.47	44.51
2		1.38	8.76	3.38	1.85	49.14	35.25
3		1.87	9.83	3.62	4.34	40.69	39.65
Mean		**2.59**	**10.37**	**3.71**	**2.67**	**40.77**	**39.80**
Std. Dev.		1.70	1.94	0.39	1.45	8.34	4.63
1	0.3	4.55	12.75	4.65	1.94	30.5	45.51
2		1.72	11.03	3.75	1.88	44.65	36.98
3		2.00	9.81	3.87	1.61	41.58	41.31
Mean		**2.76**	**11.20**	**4.09**	**1.81**	**38.91**	**41.27**
Std. Dev.		1.56	1.48	0.49	0.18	7.44	4.27
1	0.4	5.25	17.36	4.47	2.79	27.54	45.6
2		2.26	11.97	4.24	1.49	43.35	36.69
3		2.32	10.7	4.25	1.44	41.02	40.26
Mean		**3.28**	**13.34**	**4.32**	**1.91**	**37.30**	**40.85**
Std. Dev.		1.71	3.54	0.13	0.77	8.54	4.48
1	0.5	5.64	18.81	4.26	2	24.88	44.44
2		2.59	13.38	4.26	1.28	41.27	37.72
3		2.91	12.82	4.39	1.79	36.05	42.05
Mean		**3.71**	**15.00**	**4.30**	**1.69**	**30.47**	**41.40**
Std. Dev.		1.68	3.31	0.08	0.37	8.37	3.41

[1] MRS – Man Rogosa and Sharpe broth

[2] Fatty acids are : Myristic (14:0), Palmitic (C16:0), Palmitoleic (C16:1), Stearic (C18:0), Oleic (C18:1), Lactobacillic (C19:0cyc11), ND=Not Detected

VITA

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Candidate for the Degree of

Master of Science

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Major Field: Food Science

Scope and Method of Study: The influence of growth medium and pH on survival, storage stability, and fatty acid composition after freeze-drying and storage of strains of lactobacilli was studied. *Lactobacillus acidophilus* (L-1, L-23, O-16, and 381-IL-28), *L. casei* (E-5 and E-10), and *L. reuteri* X-18 were grown in MRS broth supplemented with different concentrations of Tween 80 or cultivated in broth at different pH levels. The cultures were freeze dried, sealed under vacuum, and stored at 5 ^{o}C for 21 days. At regular intervals during storage, viable cells were enumerated and viability was determined. Fatty acid was extracted and analyzed by gas chromatography.

Findings and Conclusions: Tween 80 improved growth of most of the cultures but did not improve viability after freeze-drying and storage except in *L. reuteri* X-18. Significantly higher C18:1 and C19:0cyc11 concentrations in cell membrane of X-18 grown with Tween 80 could have contributed to better resistance during freeze-drying and storage. In general, cells grown in more acidic pH (pH 4.5, 5.0, and 5.5) had higher survival after freeze-drying and subsequent storage; however, relationship between viability and fatty acid was not clearly elucidated due to high variability in the data. Stability of cultures during subsequent storage was not affected by pH or Tween 80 since they exhibited similar trends in viability throughout the storage period regardless of Tween 80 concentration or growth pH. It was attributed to minimal exposure to oxygen since the vials were sealed under vacuum, as well as low temperature and absence of light during refrigerated storage. The present study provided evidence that different cultures and strains of lactobacilli exhibited different responses to changes in growth conditions. It will be interesting to investigate the combined effect of Tween 80 and pH on growth and survival to determine optimum conditions that would be beneficial in the selection of appropriate additives and growth pH during the production and preservation of concentrated cultures.

ADVISER'S APPROVAL: Dr. William G. McGlynn