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**Chemical Composition, Probiotic Survivability and Shelf Life Studies of
Symbiotic Buttermilk**

A Thesis Presented

by

Dong Zhang

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
For the Degree of Master of Science
Specializing in Department of Nutrition and Food Sciences

May, 2015

Defense Date: March 24th, 2015
Thesis Examination Committee:

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ABSTRACT

Cultured buttermilk is becoming popular as an ingredient for bakery applications and for direct consumption in the U.S.. The objective of this study was to develop a symbiotic cultured buttermilk, containing inulin as a prebiotic and the probiotics *Lactobacillus acidophilus* and *Bifidobacterium* spp. The cultured buttermilk was prepared using a commercial mesophilic starter CHN22 (*Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*) and the probiotics. The control buttermilk was prepared using CHN22 and the symbiotic buttermilk were analyzed for chemical composition, probiotics survivability, mold, yeast and coliform counts. Changes in pH, titratable acidity and proteolysis were also determined during storage at 4°C for 12 weeks. The chemical composition of the control and symbiotic buttermilk were: protein 3.29±0.05 and 3.30±0.02%; fat 3.28±0.04 and 3.26±0.06%; carbohydrate 4.55±0.05 and 5.16±0.06%; total solids 11.81±0.05 and 12.42±0.03%; ash 0.69±0.03 and 0.70±0.01%, respectively. The populations of both *Lactobacillus acidophilus* and *Bifidobacterium* spp. were initially above 10⁷ cfu/ml and remained 10⁶ cfu/ml during the 12-week study and no mold or yeast were detected. There were significant differences in pH and titratable acidity between the control and symbiotic buttermilk (p<0.05). There was no considerable difference in proteolysis between the two samples. Results indicated that the symbiotic buttermilk might be considered as a functional food as survival of probiotics was significantly higher compared to other fermented foods.

Key words: Buttermilk, Symbiotic, Inulin, Functional foods, *Lactobacillus acidophilus*, *Bifidobacterium* spp.

ACKNOWLEDGMENTS

I would like to extend thanks to the many people, in many countries, who so generously contributed to the work presented in this thesis.

First of all, I would like to show my grateful feeling to Dr. Mingruo Guo, who taught me and was my project supervisor in the 4th semester. Dr. Guo is a warm-hearted and discipline-keeping person, with whose supervision I accomplished my master's study in time. He is always patient to help me out with questions in terms of administration and rules. Thank you very much, Dr. Guo!

Similarly, I express my warm thanks to Dr. Jana Kraft for her support and guidance at my comprehensive exam and defense.

I am also using this opportunity to express my gratitude to Dr. Catherine Donnelly who supported me throughout the course of Food Safety and Public Policy. I am thankful for her guidance, invaluable constructive criticism and friendly advice during the project work. I am sincerely grateful to her for sharing the truthful and illuminating views on a number of issues related to the project.

Finally, but by no means least, thanks go to mum and dad for almost unbelievable support. They are the most important people in my world and I dedicate this thesis to them.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1. Culture Buttermilk

1.1.1. Introduction

Buttermilk, which is the leftover liquid after churning the butter out of sweet cream, is a by-product of buttermaking (Sodini, et al., 2006). However, cultured buttermilk is a dairy product fermented by mesophilic aromatic microorganisms of pasteurized milk (Chandan, 2013). Buttermilk is a low fat product and the fat content is about 0.5% (Bylund & Pak, 2003). Normally, the chemical composition of buttermilk is very close to skim milk (O'Connell & Fox, 2000). However, buttermilk contains a higher amount of milk fat globule membrane material (MFGM) than skim milk (O'Connell & Fox, 2000). The MFGM is composed of proteins and minerals, especially the high proportion of phospholipids and phosphatidylcholine (known as lecithin) (Morin, Jiménez-Flores & Pouliot, 2007). In contrast, the chemical composition of cultured buttermilk could be totally different and it depends on the milk used in fermentation, such as whole milk, skim milk, and low-fat milk (Bylund & Pak, 2003).

Buttermilk is becoming popular as a dairy ingredient for bakeries and for direct consumption in the USA. It is estimated that the annual production of cultured buttermilk in 2010 was 214,090 tons nationwide (Chandan, 2013).

1.1.2. The chemistry of the flavor compounds

Generally, cultured buttermilk is fermented by multiple mixed microbes, containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar

diacetylactis (Bakhshandeh, et al., 2011). The strains can generate butter-tasting flavor and carbon dioxide (CO₂) (Antunes, et al., 2009). Diacetyl, lactic acid, and acetaldehyde are three important chemical compounds that contribute to the unique flavor of cultured buttermilk (Antunes, et al., 2009). Other chemical components such as acetate, ethanol and acetoin also play a role in buttermilk's aroma.

Diacetyl is a natural by-product of fermentation and it is the most important flavor compound in cultured buttermilk (Levata-Jovanovic & Sandine, 1997). It is a volatile yellow liquid organic compound containing a rich buttermilk flavor, also known as butane-2,3-dione (Krogerus & Gibson, 2013). The molecular formula of diacetyl is C₄H₆O₂ and the molecular structure is made of a C-C bond linking two carbonyls (Eriks, et al., 1983).

Acetaldehyde is a volatile colorless liquid organic compound at 22°C and it has a fruity and pleasant aroma. The chemical formula of acetaldehyde is CH₃CHO. It can be commonly found in coffee, bread, beer, and cultured buttermilk (Lachenmeier & Sohnius, 2008).

The chemical formula of lactic acid is C₂H₄OHCOOH. It has a hydroxyl group adjacent to the carboxyl group, resulting in a α -hydroxy acid. Swedish chemist Carl first isolated lactic acid from curdled milk in 1780 (Datta & Henry, 2006). In dairy manufacturing, lactic acid is produced by fermentation of lactose. Lactic acid bacteria can transfer simple sugar to lactic acid during fermentation processing. The lactic acid causes the casein protein coagulation, resulting in a yogurt-like texture. Additionally, lactic acid can cause the low pH of the products, resulting in a prevention of the unwanted growth of bacteria (Tamime & Robinson, 1999).

Ethanol is a volatile, colorless, liquid with a pleasant flavor, also known as alcohol. The chemical formula of ethanol is $\text{CH}_3\text{CH}_2\text{OH}$ containing a hydroxyl group ($-\text{OH}$) and bonding to a carbon atom (Ballinger & Long, 1960).

Acetoin is a volatile, colorless liquid at 22 °C with an agreeable buttery aroma. The chemical formula of acetoin is $\text{C}_4\text{H}_8\text{O}_2$ containing acetyl methyl carbinol. It is a chiral molecule and (R)-acetoin is generated by bacteria. It can be widely found in apple, butter, wheat, and blackberry (Xiao & Xu, 2007).

1.1.3. Flavor compounds formed

Lactococcus lactis biovar *diacetylactis* is a flavor producing strain that is able to degrade citrate. Citrate metabolism plays a vital role in food fermentation processing. Flavor compounds such as diacetyl, acetoin, ethanol, acetate, and acetaldehyde and also CO_2 can be generated during citrate fermentation. Diacetyl is the most important chemical compound that contributes to a butter-like odor. The level of citrate in raw milk is about 0.8% and mesophilic bacterial strains can take advantage of citrate and produce diacetyl (Laëtitia, Pascal & Yann, 2014). In citrate metabolism, lactic acid bacteria can grow via another carbon source and withstand acidic environment. After that, the citrate/glucid co-metabolism leads to the quick release of organic compounds, known as bacteriostatic effects. In this specific conditions, the C4 pathway can produce diacetyl (Quintans, et al., 2008).

Lactococcus lactis is a Gram-positive, non-spore forming, non-flagellated, rod-shaped bacterium that can ferment lactose to lactic acid by a homofermentative pathway (Madigan, et al., 1997). With enough glucose and limited oxygen (O_2), a mole of glucose can release two moles of lactic acid and ATP. In the Embden-Meyerhof-Parnas (EMP pathway), one mole of glucose is first converted to two mole

of pyruvate by glycolysis. Subsequently, pyruvate is converted to lactic acid due to a terminal electron acceptor (Zuniga, Pardo & Ferrer, 1993). *Lactococcus lactis* has two subspecies, *lactis* and *cremoris*, which have been widely used in fermented food, such as cheese and cultured buttermilk. *Lactococcus lactis* can utilize lactose to produce ATP and lactic acid in fermentation processing. Lactic acid cannot only lower the pH of the fermented food and limit the growth of unwanted bacteria, it also can give the products a pleasant acidic taste (Hofvendahl & Hahn-Hägerdal, 2000).

Leuconostoc is a heterofermentative bacterium that converts lactose to lactic acid, acetate, ethanol and CO₂. In heterofermentation, one mole of glucose-6-phosphate is first converted to 6-phosphogluconate. After that, 6-phosphogluconate is decarboxylated, resulting in one mole of CO₂ and pentose-5-phosphate. Subsequently, the byproduct pentose-5-phosphate is fermented into one mole glyceraldehyde phosphate (GAP) and one mole of acetyl phosphate. GAP can be further fermented to lactate with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. Finally, one mole of glucose can generate one mole of ethanol, lactic acid, ATP and CO₂ (Zuniga, Pardo & Ferrer, 1993). *Leuconostoc* also utilizes citrate releasing flavor compounds (diacetyl, acetoin, ethanol, acetate, CO₂) and is furthermore able to convert acetaldehyde to ethanol.

1.2. The Role of Prebiotics, Probiotics, and Symbiotic in Human Health

1.2.1. Introduction

In recent years, the market for functional foods has grown rapidly worldwide (Barbara, et al., 2013). Functional foods have been simply defined as the foods that may have a positive effect on human health beyond basic nutrition and without changing eating habits (Bech-Larsen & Grunert, 2003). Traditionally, most of the first

generation functional foods on the market are vitamins and mineral supplements (Ziemer & Gibson, 1998). Due to the high metabolic and endocrine activity of the human colon, microorganisms in the gastrointestinal tract (GI tract) play an important role in human health (Ziemer & Gibson, 1998). To benefit microflora in the gastrointestinal tract, probiotics and prebiotics are currently used to enable the symbiotic relationship between microbes and human beings (Walker & Duffy, 1998).

1.2.2. Prebiotics

Prebiotics have been defined as a non-digestible food ingredient that could benefit the growth of microflora in the human GI tract (Manning, et al., 2004). The populations of viable lactic acid bacteria and other microbes in the intestine are pertinent to the host's immune health. These microbes increase mineral absorption, minimize the growth of harmful microbes, and decrease blood cholesterol levels (Manning, et al., 2004). Prebiotics could act as a carbohydrate source for these microbes and increase the survivability. Chicory, garlic, onion, raw oats, acacia gum, and unrefined wheat are very good sources of prebiotics (Ziemer & Gibson, 1998). Inulin, oligofructose, fructooligosaccharide, and lactulose are the non-digestible fiber considered as prebiotics in the diet (Jardine, 2009).

The inadequate intake of calcium could lead to a higher risk of osteoporosis, especially for the elderly (Scholz-Ahrens, et al., 2001). In recent years, it was reported that the intake of prebiotics, such as inulin, lactulose, and oligosaccharides, could increase calcium absorption and prevent osteoporosis. Numerous studies have scientifically proven this in animal trials (Manning, et al., 2004). The mechanisms of this action is the fermentation of prebiotics that can cause an increase of short chain fatty acid and lower the pH in the luminal colon, which finally results in increasing

calcium solubility and levels in the GI tract (Manning, et al., 2004). In one study, 9 and 12 volunteers were fed 40 g/day of inulin (high doses) and 15 g/day (low doses) for 28 days, respectively. The result indicated that the high dose group had a significant increase in calcium absorption and the low dose group had a negative effect (Manning, et al., 2004). Future studies should focus on the appropriate doses for humans and more studies on human trials need to be carried out.

Prebiotics cross from the human mouth and finally get into the large intestine where they are thoroughly broken down by the beneficial microorganisms (Delzenne & Roberfroid, 1994). In the end, some gases and short-chain fatty acid are produced. In the meantime, the mass of bacteria in the large intestine increases, which not only reduces the pH of stools, but also leads to higher stool frequency and stool weight (Delzenne & Roberfroid, 1994). This can result in a regularization of bowel habits (Jardine, 2009). As a dietary fiber, prebiotics are also considered as a low-calorie food (Roberfroid, Gibson & Delzenne, 1993). Due to its non-digestibility, it helps prevent diabetes and helps regulate insulin secretion (Jardine, 2009).

Recently, the prevalence of colon cancer has become very high, especially in the large intestine. The microbes in the large intestine can produce toxins and carcinogens. Therefore, scientists think that microbes in the large intestine are the key to develop colon cancer (Manning, et al., 2004). A number of studies have been reported that prebiotics have an effect in the reduction of colon cancer, especially inulin, lactulose and oligofructose (Tuohy, et al., 2003). The intake of prebiotics can increase the numbers of clostridia and eubacteria, which increase the production of butyrate in the gut. Butyrate has been proven as an energy source for healthy colonocytes and it could increase apoptosis in colonic cancer cell lines (Manning, et al., 2004). Another mechanism of this action is that prebiotics may change bacterial

metabolism where proteolysis to saccharlysis do not occur, which results in a reduction of toxins and carcinogens (Tuohy, et al., 2003). In animal trials, lactulose has been successfully used to protect against DNA damage. However, lactulose showed a negative effect in human trials (Tuohy, et al., 2003). Currently, research on human and animal trials is still limited. Further studies should focus on identification of prebiotics and how they can stimulate the growth of eubacteria.

Pathogens have been defined as biological agents such as viruses, protozoans, and bacteria that can make the host sick. Prebiotics can be used to resist the pathogens by stimulating the survivability of bifidobacteria and lactobacilli (Manning, et al., 2004). The increase of bifidobacteria and lactobacilli can produce more acid and drop the pH in the gut, which limits pathogen growth. Also, bifidobacteria can produce antimicrobial effects that could effectively kill the pathogens in the intestine (Tuohy, et al., 2003). A recent animal trial showed that prebiotics have positive effects on reducing *Escherichia coli* O157: H7 and *Campylobacter* spp. (Manning, et al., 2004).

Prebiotics improve human health and nutrition by modulating the microflora of the GI tract. Prebiotics have also been considered a nutritional supplement for years, but more studies need to be carried out. The safety dosage for infants, elderly, and patients also need to be determined. More fortified functional foods with prebiotics should be developed to meet the needs of the market. Finally, more human trials on prevention of cancer and growth of pathogens are needed.

1.2.3. Probiotics

The International Life Sciences Institute (ILSI) has defined probiotics as live microorganisms that could benefit the host's GI tract (Salminen & Gueimonde, 2004). In addition, the number of live microorganisms in the products should be maintained

at least 10^6 viable cells per ml or g during the shelf life, otherwise it can't be considered a functional food for human beings (Dave & Shah, 1997). As age, consumption of medicine, and stress increases, the balance of essential microflora in the human body could be destroyed, resulting in diarrhea, indigestion, and pronounced illness (Bylund & Pak, 2003). The consumption of probiotics is helpful in not only reducing the symptoms mentioned above, but also it can reduce the risk of stomach cancer and strengthen the immune system (Bylund & Pak, 2003). Production of probiotic products is rising rapidly and such products have dominated the Japanese and European functional food markets (Siro, et al., 2008). In 2004, probiotic products occupied 56% of the functional foods' market worldwide, which is about 31.1 billion US dollars (Siro, et al., 2008). *Lactobacillus acidophilus* (*L. acidophilus*) and *Bifidobacterium* spp. are the two probiotics that have been most widely used in dairy products (Saarela, et al., 2000). *L. acidophilus* is a microaerophilic Gram-positive, non-flagellated, and non-spore forming rod shaped bacterium that exists in the small intestine. *Bifidobacterium* spp. is an obligate anaerobic, Gram-positive, non-flagellated, and non-spore forming V-shaped bacterium that occupies the large intestine (Bylund & Pak, 2003).

Although lactose intolerance is not very common in America, around 75% of people worldwide have reported lactose intolerance, particularly individuals in Asian countries (Marteau & Boutron-Ruault, 2002). Lactose intolerant individuals are unable to digest lactose due to the lack of sufficient β -galactosidase (Rolfe, 2000). The presence of lactose in the large intestine can break the osmotic balance and produce gas, which could result in diarrhea and nausea (Scheinbach, 1998). Several studies showed that the consumption of fermented dairy products with probiotics could efficiently relieve the symptoms of lactose intolerance (Salminen & Gueimonde,

2004). Probiotics can convert the lactose into simple sugar during fermentation and increase the levels of β -galactosidase after consumption (Salminen & Gueimonde, 2004). However, not all probiotics are capable of releasing active β -galactosidase in the gut or fermenting lactose (Rolfe, 2000). Usually, *L. acidophilus* and *Bifidobacterium* spp. are added into dairy products to increase the digestibility of lactose (Rolfe, 2000).

Individuals with hypercholesterolemia may have a high risk of cardiovascular diseases because of high levels of serum cholesterol (Lourens-Hattingh & Viljoen, 2001). Research suggests that the consumption of probiotics such as *Bifidobacterium* spp. can lower the cholesterol level in the human body. Probiotics could assimilate the cholesterol and break down the bile acid *in vitro*, which could inhibit the absorption of the bile acid into the body again (Tahri, et al., 1995). In a rat-feeding experiment, three trials of rats were fed probiotic yogurt, yogurt (control), and unfermented soymilk (control), respectively. The results suggested that there were significant increases in the liver lipids and bile salt concentration in control groups, while cholesterol level in the plasma of the probiotic group were reduced (El-Gawad, et al., 2005). Unfortunately, since there are no successful trials in humans, reduction of cholesterol by probiotics cannot be scientifically applied to humans and further research is recommended.

Numerous studies reported that the use of probiotics could help prevent several different kinds of diarrheas, such as antibiotic-associated diarrhea, traveller's diarrhea, and rotavirus diarrhea.

About 25% of patients who consume antibiotics suffer from diarrhea, resulting from the disturbance of microflora (Toure, et al., 2003). The toxin-producing *Clostridium difficile* exists in the human gut, normally in low numbers. However, the

intake of antibiotics will lead to a dramatic growth of *Clostridium difficile* due to lack of competition of other microorganisms in the gut, which could result in mild diarrhea (Andersson, et al., 2001). A few randomized double-blind trials suggested that probiotics (*Saccharmyces boulardii*, *Lactobacillus rhamnnsus* GG, and *Enterococcus faecium* SF68) could more efficiently prevent diarrhea when compared to a control group (Marteau & Boutron-Ruault, 2002). The mechanism of this action has not been thoroughly understood. In addition, only a small number of probiotics have been scientifically proven for use in treating antibiotic-associated diarrhea. Future studies should analyze probiotics for treatment of antibiotic-associated diarrhea.

The risk of traveller's diarrhea does not only occur in developing countries, but also in developed countries. Some studies have shown that the consumption of probiotics could reduce the risk of traveller's diarrhea, although some other studies reported that there were no significant effects (Salminen & Gueimonde, 2004). A group of Danish tourists in Egypt participated in a double-blind placebo-controlled study. The results indicated that the intake of probiotics had positive outcomes on prevention of traveller's diarrhea, reducing 43% of frequency of occurrence in the probiotics group (Ericsson, 2003). However, the current data on human studies is still limited. Hence, more convincing trials and data on prevention of diarrhea by probiotics need to be verified in the future.

Diarrhea in children is predominantly caused by rotavirus and symptoms include vomiting, acute diarrhea, and dehydration, resulting in a high infant morbidity and mortality (Roos & Katan, 2000). Oral rehydration and vaccine have been commonly used to treat it (Rolfe, 2000). Of 74 children who had diarrhea by rotavirus who participated in a trial, the duration of diarrhea was dramatically shorter in

children who took probiotics *Lactobacillus GG* (Rolfe, 2000). In conclusion, probiotics are an efficient way for children to be treated for rotavirus related diarrhea.

Probiotics will eventually play a bigger role in human nutrition due to the advanced nutrition and therapeutic effects. Research has shown that the consumption of probiotics also has positive effects on cancer, irritable bowel syndrome, inflammatory bowel disease, and other health related complications. However, the mechanism of actions is poorly understood and problems including probiotics safety, dosage, and efficiency need to be scientifically proven by human trials. Additionally, future studies should focus on prolonging the shelf life, developing new strains and probiotic-containing functional foods.

1.2.4. Symbiotics

In functional foods, it has been defined that probiotics and prebiotics work together and benefit the gut microflora of the host by increasing the survivability of microbes in the gastrointestinal tract (Pharmaceutiques, 1995). The functions of prebiotics and probiotics have been individually reviewed as beneficial to human health. However, only a few papers have been published on the symbiotic relationship. The advantages of symbiotics greatly outweigh the advantages of pre- or probiotics alone (Jardine, 2009).

The benefits of probiotics and prebiotics on human digestive health have been previously discussed separately where they are also has numerous positive effects on human digestive health, such as balance of colonic microflora, bowel habits, and treatment of diarrhea (Aline, 2014).

A recent study has shown that symbiotic formula can decrease the rate of infant diarrhea. A group of infants were fed infant formula containing probiotics,

prebiotics, and symbiotics. The result showed that the consumption of symbiotic formula could lead to a lower rate of diarrhea (Chouraqui, et al., 2008).

Symbiotic infant food might play a role in potential functional food market, although they are not currently popular food products. The balance of human gut microflora is the key to health and disease, especially for infants (Bakker-Zierikzee, 2005). Due the advantages of probiotics and prebiotics, they have been separately approved for infant health. Probiotics such as bifidobacteria and lactobacilli have a positive effect on gut microflora. Prebiotics can increase the number of viable resident bacteria to benefit infant health (Jardine, 2009). Symbiotic infant formula should be developed for these reasons.

Constipation has been commonly found in the elderly. Microflora decreases with age, resulting in a decrease of bifidobacteria and an increase of putrefactive (Jardine, 2009). Recent research found that the consumption of symbiotic yogurt drink containing probiotics and prebiotics can improve gut health and prevent constipation by increasing the numbers of viable bifidobacteria (Aline, 2014).

More studies on the benefits of symbiotics needs to be carried out to provide scientific evidence to support the benefits of development of functional foods containing symbiotics.

1.2.5. Summary

Probiotics, prebiotics, and symbiotics play a large role in human nutrition. However, the microflora in the gut is a very complicated and diverse ecosystem. Many mechanisms of probiotics, prebiotics, and symbiotics haven't been well understood and more human studies need to be completed.

1.3. *Lactobacillus acidophilus*

1.3.1. Introduction

L. acidophilus is a microaerophilic, Gram-positive, non-flagellated, and non-spore forming rod bacterium (Gomes & Malcata, 1999). The optimum growth temperature of *L. acidophilus* is around 37°C (Baati, et al., 2000). *L. acidophilus* can utilize glucose to produce lactic acid in the homofermentation and lactic acid, CO₂, and ethanol in heterofermentation (Jardine, 2009). It has been naturally found in human and animal GI tract.

1.3.2. The factors of survivability of *L. acidophilus*

The population of viable *L. acidophilus* in food products should be maintained at least 10⁶ viable cells per ml or g during shelf life, otherwise probiotics lose its functions (Dave & Shah, 1997). Although the survivability of *L. acidophilus* during storage plays an important role in a successful product, previous research reported that the number viable *L. acidophilus* could decrease rapidly, which could be affected by differences strains, environment acidity, oxygen content, incorporation of micronutrients, and competition with other strains, etc. (Ng, Yeung & Tong, 2011; Dave & Shah, 1997; Talwalkar, et al., 2004).

The strain variation in fermented products is the key factor to the survival of *L. acidophilus*. There are more than 20 strains and most of them are considered as probiotics, such as commercial strains A3, A9, 08, 53, and LA-5 (Vinderola, Mocchiutti & Reinheimer, 2002). Different strains may have different survivability under the same conditions. Additionally, modification of strains might result in raising a higher population of viable bacteria. In order to avoid disadvantage properties of strains, genes can be deleted or replaced with the favorable genes from

the other strains (Tamime & Robinson, 1999). In conclusion, careful strain selection and monitoring are very important, which could lead to a high quality commercial strain.

The viability of *L. acidophilus* can be affected by the co-culture involved in the fermentation (Vinderola, Mocchiutti, & Reinheimer, 2002). For example, the presence of *L. bulgaricus* can result in a loss of viable *L. acidophilus* because of post-acidification during fermentation and storage. *B. bifidum* cannot grow in pure milk by itself due to the lack of proteolytic ability. Because of proteolytic ability *L. acidophilus* can work with *B. bifidum* as a symbiotic culture (Lourens-Hattingh & Viljoen, 2001).

L. acidophilus is a microaerophilic organism, therefore, it cannot completely reduce oxygen to hydrogen peroxide because it lacks an electron-transport chain. Furthermore, this organism is unable to decompose the hydrogen peroxide due to absence of catalase (Talwalkar & Kailasapathy, 2004). The accumulation of O₂ can lead probiotics cell death. This process is called “oxygen toxicity” (Talwalkar & Kailasapathy, 2004). Usually, yogurt products are considered as high oxygen content foods due to the incorporation of oxygen during processing and storage. Homogenization, mixing, and agitation are the three main processing steps that could increase the levels of oxygen in the yogurt and lead to a low survival of *L. acidophilus* (Talwalkar & Kailasapathy, 2004). Numerous methods have been scientifically demonstrated to change the levels of oxygen and increase the number of viable *L. acidophilus*. These methods include use of ascorbate, L-cysteine, special high-oxygen consuming strains, microencapsulation, and changing the packing material (Talwalkar & Kailasapathy, 2004).

L. acidophilus is very sensitive to low pH environment and it stops growing below pH 4.0 (Shah, et al., 2000). Since *L. acidophilus* grows very slowly, *L. delbrueckii ssp. Bulgaricus* is commonly inoculated as a starter culture along with *L. acidophilus* in yogurt manufacture. During fermentation and storage, *L. delbrueckii ssp. Bulgaricus* continues to produce lactic acid, known as post-acidification, and the eventual result is a loss of viable *L. acidophilus* (Shah, 2000). A recent study by (Kailasapathy, et al., 2008) showed that the survival of *L. acidophilus* was affected by different fruit mixtures. Plain-yogurt had a higher population during storage of viable *L. acidophilus* than the yogurt containing passion fruits and mixed berries. However, the yogurt containing mango and strawberry had a better survival of *L. acidophilus* than the plain-yogurt (Kailasapathy, et al., 2008). In conclusion, this study showed that any food ingredients that could decrease the environmental pH could lower the survival of *L. acidophilus*.

Bile is also an important factor that can decrease the survival of *L. acidophilus* (Tuomola, et al., 2001). This is because bile salts can damage bacterial cell membranes. In order to make a successful commercial strain that can survive and pass through the stomach and small intestine, the ability to tolerate bile is also important (Tuomola, et al., 2001).

The presence of hydrogen peroxide can lead to *L. acidophilus* cell death because of the toxic oxygen metabolism. Numerous studies have shown that the probiotic yogurt containing *L. delbrueckii ssp. Bulgaricus* has a poor survival of *L. acidophilus* during storage due to the production of hydrogen peroxide.

The temperature during storage can affect the viability of *L. acidophilus*. The optimum growth temperature of *L. acidophilus* is around 37°C (Baati, et al., 2000). Normally, yogurt is fermented at 43°C. Manipulation of the incubation temperature to

37°C, and increased incubation time can increase the population of viable *L. acidophilus*. The mechanism of this action is that low temperature restricts the growth of *L. bulgaricus* and therefore avoids the resulting over-acidification (Lourens-Hattingh & Viljoen, 2001). Research has shown that *L. acidophilus* is tolerant to low temperatures (Lourens-Hattingh & Viljoen, 2001).

The survival of *L. acidophilus* could also be affected by inoculum size, fermentation medium, and micronutrients (Shah, et al., 1995).

1.3.2. Applications

L. acidophilus has been widely utilized in fermented dairy products, especially in yogurt, cultured buttermilk and cheese. *L. acidophilus* has many advantageous health benefits. Considerable research has shown that *L. acidophilus* has a positive effect on the relief of lactose intolerance symptoms, prevention of different types of diarrheas, and alleviation of irritable bowel syndrome. For elderly, *L. acidophilus* can be consumed to prevent cancer, diabetes, and to boost the immune system (Andersson, et al., 2001). Therefore, the demand for *L. acidophilus* is rapidly growing in the function food market worldwide.

1.4. *Bifidobacterium*

1.4.1. Introduction

Bifidobacterium can be isolated from the feces of human and animals. In 1974, bifidobacteria were first isolated from a healthy child and then it was named by modern taxonomic tools in 1990 (Jardine, 2009). Because of its health benefits, it is reported that more than 70 dairy products containing *Bifidobacteria* spp. can be found in the functional markets (Antunes, et al., 2009).

1.4.2. Physiology of *bifidobacterium*

Bifidobacteria are an obligate anaerobe, Gram-positive, non-flagellated, and non-spore forming V-shaped bacteria (Jardine, 2009). Although they are classified as obligate anaerobes, they can survive in low levels of oxygen (<10%). The optimum growth temperature of bifidobacteria is between 37 and 43°C (Jardine, 2009). Bifidobacteria can ferment carbohydrate to 2:3 ratio of lactate and acetate by hexose metabolic pathway. The main enzyme in this pathway is glucose-6-phosphate (Gomes & Malcata, 1999).

In addition, bifidobacteria are able to produce different types of water-soluble vitamins in dairy fermentation, such as nicotinic acid, folate and thiamine (Tahri, et al., 1995).

1.4.3. Applications

Bifidobacteria have been mainly reported to benefit digestive health. Many research studies have shown that the consumption of bifidobacteria has significant effects on not only traveller's diarrhea, antibiotic-associated diarrhea, and childhood diarrhea, but also prevention of cancer, reduction of blood cholesterol level, and relief of lactose intolerance symptom (Tahri, et al., 1995).

1.5. Inulin

1.5.1. Introduction

Inulin is a white odorless non-digestible carbohydrate that has been found in many types of natural plants, and commercially is most often extracted from chicory roots (Roberfroid, 1993). Leek, onion, banana, and rye are also good sources of inulin and these plants use the inulin as a carbohydrate reserve to survive under cold condition (Jardine, 2009). Inulin was first found by a German scientist in 1804

(Boeckner, Schnepf& Tungland, 2001). The production process of inulin is very similar to making sucrose from sugar beets. The chicory root was initially extracted in hot water, followed by purification technologies, and finally evaporation and spray-drying (Jardine, 2009). Inulin has been considered as part of a normal human diet for nearly 100 years and the estimated daily intake is around 3 to 11 g in Europe and 1 to 4 g in the USA (Roberfroid, 2005).

1.5.2. Chemical properties

Inulin mainly consisted of a polydispersed carbohydrate with the β (2,1) glucosyl-fructosyl structure (Jardine, 2009). The numbers of fructose units bonded together varies from 2 to 70 and the degree of polymerization (DP) is between 4 and 25 (Roberfroid, 2000). Oligofructose is a short-chain with about 2-10 DP. Industrially, the long-chain inulin (DP 25), produced by physical separation technology, is used as a fat replacement and for texture improvement (Roberfroid, 2000). Due to its β (2,1) structure, inulin cannot be broken down by human enzymes, and so has functions of dietary fiber, prebiotics, and reduced calorie value (Franck, 2002).

1.5.3. Physical properties

Standard inulin (DP 12, $n=2-60$) as well as high performance inulin, are a white powders that have a neutral taste. Comparably, the standard inulin is 10% as sweet as sugar. In contrast, the oligofructose (DP 4, $n=2-10$) has a sweeter taste, which is about 35% greater than sugar (Jardine, 2009). Compared with oligofructose, inulin has a moderate solubility in water. Both inulin and oligofructose have a very low viscosity in water. Inulin is also considered a perfect fat replacement because inulin can form a stable tri-dimensional gel when dissolved in water, which can also improve the stability of foams and emulsions (Jardine, 2009). Because of high

solubility, heat lability and sweetness, inulin has been used as a sugar replacement and the production process is very similar to manufacture of sugar and glucose syrup (Roberfroid, 2000).

1.5.4. Applications

Inulin has been widely used as functional food ingredient because of its prebiotics properties, especially in dairy and baked products (Franck, 2002). In the bakery products, a 2-15% dosage level of inulin is used to improve the taste and texture, resulting in a crispy texture. Similarly, a 2-5% level of inulin is added to low-fat dairy products to impart a creamy texture (Al-Sheraji, et al., 2013)

In addition, inulin can be digested by human beings due to the β (2,1) glucosyl-fructosyl structure, and it can pass through from the mouth, stomach, and small intestine without hydrolysis. In the large intestine, inulin can be slowly broken down by bacteria and turned into bacterial mass, short-chain fatty acid, and some gases. Due to advantageous nutrition properties, inulin can be added into food as dietary fiber, resulting in improved digestive health, reduced stool pH, reduced constipation and increased stool weight (Roberfroid, 1993).

CHAPTER 2: MANUSCRIPT

Chemical Composition, Probiotic Survivability and Shelf Life Studies of Symbiotic Buttermilk

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2.1. Abstract

Cultured buttermilk is becoming popular as an ingredient for bakery applications and for direct consumption in the U.S.. The objective of this study was to develop a symbiotic cultured buttermilk, containing inulin as a prebiotic, and the probiotics *Lactobacillus acidophilus* and *Bifidobacterium* spp. The cultured buttermilk was prepared using a commercial mesophilic starter CHN22 (*Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteorides* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*) and the probiotics. The control buttermilk was prepared using CHN22, and along with the symbiotic buttermilk, was analyzed for chemical composition, probiotics survivability, mold, yeast and coliform counts. Changes in pH, titratable acidity and proteolysis were also determined during storage at 4°C for 12 weeks. The chemical composition of the control and symbiotic buttermilks were: protein 3.29±0.05 and 3.30±0.02%; fat 3.28±0.04 and 3.26±0.06%; carbohydrate 4.55±0.05 and 5.16±0.06%; total solids 11.81±0.05 and 12.42±0.03%; ash 0.69±0.03 and 0.70±0.01%, respectively. The populations of both *Lactobacillus acidophilus* and *Bifidobacterium* spp. were initially above 10⁷ cfu/ml and remained at 10⁶ cfu/ml during the 12-week storage period with no mold and yeast growth. There were significant differences in pH and titratable acidity between the control and symbiotic buttermilk (p<0.05). There was no considerable difference in proteolysis between the two samples. Results indicated the symbiotic buttermilk might be considered as a functional food as survival of the probiotic cultures was significantly higher compared to other fermented foods.

Key words: Buttermilk, Symbiotic, Inulin, Functional foods, *Lactobacillus acidophilus*, *Bifidobacterium* spp.

2.2. Introduction

Buttermilk is a by-product in buttermaking manufacture (Antunes, et al., 2009). Cultured buttermilk is a fermented dairy product that made by mesophilic aromatic strains. Diacetyl is the most important aroma component that contributes to buttermilk's unique flavor (Sodini, et al., 2006). In recent years, cultured buttermilk is popular in cooking, especially in baking. Due to its advantageous nutritional value and special flavor, it is also consumed as a beverage (Chandan, 2013). However, only a few studies have been carried out using cultured buttermilk as a carrier of probiotics and prebiotics.

Probiotics are live beneficial microbes that can improve digestive health. The consumption of probiotics can not only reduce the cholesterol level in the blood and relieve lactose intolerance, but can also boost the immune response and reduce the risk of getting some cancers. *L. acidophilus* and *Bifidobacterium* spp. are the two well-known probiotics that have been used in the functional food market, especially in fermented products. *L. acidophilus* is a microaerophilic, Gram-positive, non-flagellated, and non-spore forming rod-shaped bacterium that has been found in the small intestine. *Bifidobacterium* spp. is an obligate anaerobic, Gram-positive, non-flagellated, and non-spore forming V-shaped bacterium which resides in the large intestine (Gomes & Malcata, 1999).

The viability of probiotics plays an important role in qualifying buttermilk as a functional food. It is recommended that only 10^6 cfu/ml or more numbers of viable probiotics are useful for human health benefits (Shah, et al., 1995). However, most viable probiotics die off after a few weeks of storage, especially *L. acidophilus*. There are a number of contributing factors, including excess oxygen, pH, and temperature (Talwalkar, et al., 2004).

Inulin is a natural polysaccharide that exists in the roots of many plants such as leeks, onion, and banana. Chicory root is the best sources of inulin. It can increase calcium and magnesium absorption. It is also a very suitable food for diabetics because it can control blood sugar regulation. Beyond its nutritional value, inulin can be used as prebiotic in symbiotic dairy products to promote the growth of probiotic cultures (Coussement, 1997).

The objective of this research was to develop a symbiotic buttermilk product containing both prebiotics and probiotic cultures and to evaluate the chemical composition, physiochemical properties, probiotic survivability and microbiological properties of the buttermilk.

2.3. Materials and Methods

2.3.1. Materials

A freezer-dried mesophilic aromatic starter culture F-DVS CHN22 containing multiple mixed strains of *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* was obtained from Chr-Hansen. The probiotics, *L. acidophilus* (LA-5) and *Bifidobacterium* spp. (BB-12) were also from Chr-Hansen. Inulin was obtained from Oraftic®GR. The pasteurized whole milk was purchased from a local market.

2.3.2. Preparation of symbiotic cultured buttermilk and the control

The symbiotic cultured buttermilk was made by combining CHN22 (0.015%, w/w), *L. acidophilus* (LA-5) and *Bifidobacterium* spp. (BB-12) (0.1%, w/w), and inulin (0.8%, w/w). Buttermilk with only starter culture CHN22 (0.015%, w/w) was also prepared as a control. The pasteurized whole milk and inulin were heated up to

85°C in a water bath and held for 5 minutes until the inulin was totally dissolved. The milk was cooled to 22°C using ice bath and inoculated with the starter culture CHN22 and probiotics (*L. acidophilus* and *Bifidobacterium* spp). Finally, the sample was incubated at 22.5°C for 20 hours. The samples were then stored at 4°C before testing. Three batches of samples were prepared on three different days for chemical composition, microbiology analyses and shelf life testing.

2.3.3. Chemical composition

Protein content of the symbiotic cultured buttermilk and the control were analyzed by the Kjeldahl method and fat content was determined by the Babcock method (Wehr & Frank, 2004). The quantity of total solids was determined by drying samples in a forced-drafted oven at 105°C for 3 hours (Wehr & Frank, 2004). The ash content was determined by ignition in a muffle furnace at 550°C for 6 hours (Wehr & Frank, 2004). The content of carbohydrate was calculated by the difference of total solids minus protein, fat, and ash as described by Guzman-Gonzalez (Guzmán-González, et al., 1999). All analyses were measured in triplicate.

2.3.4. Physicochemical analyses

The pH was measured weekly in triplicate using a pH meter (model 240, IQ Scientific Instrument, Inc., San Diego, CA) over 12 weeks.

The apparent of viscosity (mPa.s) was measured weekly in triplicate by a Brookfield Viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) at room temperature (21±2°C) over 12 weeks. All samples were analyzed using spindle 3 at 100 rpm for 30 seconds.

Titrateable acidity (TA) was used to determine the percentage of lactic acid. 9 grams samples were dissolved into 25 ml water and titrateable acidity (TA) was

measured weekly in triplicate by titrating with 0.1 N NaOH using 0.5 N phenolphthalein as an indicator for 12 weeks (Wehr & Frank, 2004).

2.3.5. Microbiological analyses

Mold and yeast Film (3M, Petrifilm™) was counted once every two weeks by incubation at 21 °C for 72 hours. Coliform film was counted once every two weeks (3M, Petrifilm™) incubation at 35 °C for 48 hours.

2.3.6. Survivability of probiotics

The pour plate method was used to determine the survivability of *L. acidophilus* and *Bifidobacterium* spp. The procedure followed the Chr-Hansen standard methods (Chr-Hansen, 2007). Samples were diluted to 10^{-5} , 10^{-6} , and 10^{-7} using sterile peptone water. The enumeration of *L. acidophilus* was done using MRS agar (Difco 288210) with the addition of clindamycin stock solution (Sigma C5269) and ciprofloxacin stock solution (BAYER 02838560). The enumeration of *Bifidobacterium* spp. was done using MRS agar (Difco 288210) containing dicloxacillin stock solution (Sigma D-9016), LiCl stock solution (Merck No 5679), and CyHCl stock solution (Merck No 2839). Both *L. acidophilus* and *Bifidobacterium* spp. were anaerobically incubated at 43°C for three days. The colonies of *L. acidophilus* were small, irregular, and star shaped. The colonies of *Bifidobacterium* spp. were large, white, and circled shaped. Each sample was counted weekly in duplicate for 12 weeks and the results expressed as log cfu/ml.

2.3.7. Proteolysis (SDS-PAGE)

Standard yogurt fermented by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Chr-Hansen F-DVS YF-L901) was prepared. Symbiotic buttermilk, buttermilk (control), standard yogurt, and whole milk were

frozen for 1 hour at -85°C and freeze-dried for 48 hours in a freeze-drier (LABCONCO, Models 7751020) after 1-week storage and 8-week storage, respectively. 90% whey protein isolate and whole milk (Hannaford) were also prepared.

The SDS-PAGE procedure was adopted from Guo (1999) and Laemmli (1970). All samples were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing 10% sodium dodecyl sulfate, 3% 2-mercaptoethanol, 10% glycerol, 1%(w/v) bromophenol blue and 50-mM Tris-HCl, pH 6.8. Electrophoresis was conducted with 7.5% separating gels and 4% stacking gel at a constant current of 60 mA for 45 minutes by Bio-Rad mini gel device. The gel was fixed with 10% (v/v) glacial acetic acid and 25% (v/v) propan-2-ol overnight. The gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad) for 4 hours followed by a destaining in a 25% (v/v) methanol and 10%(v/v) acetic acid solution.

2.3.8. Statistical analysis

The data on chemical composition of symbiotic buttermilk and control was analyzed by one-way ANOVA. The pH, TA, and viscosity of symbiotic buttermilk and control trials were statistically analyzed and compared using a 2-way repeated measure ANOVA and Bonferroni post-test by SPSS statistical software version 21(SPP Inc., Chicago, IL, USA). A p-value <0.05 was considered significant differences for all analyses.

2.4. Results and Discussions

2.4.1. Chemical composition

The chemical composition of the symbiotic buttermilk and control (%) is presented in Table 1. Since both symbiotic buttermilk and control were made with

whole milk, there were no significant differences between the symbiotic buttermilk and control in protein, ash, and fat ($p>0.1$). However, the contents of total solids and carbohydrates in symbiotic buttermilk are higher than the control, because inulin was added into symbiotic buttermilk as a carbohydrate source, which also increased the level of total solids. According to Bylund (2003) the content of fat, protein, carbohydrate, ash, and total solids in commercial cow's milk is 3.7%, 3.5%, 4.8%, 0.7%, and 12.7%, respectively. The chemical composition of symbiotic buttermilk in this research is close to commercial cow's milk, except for carbohydrate. In conclusion, the symbiotic buttermilk had not a significantly difference in nutritional value when compared to commercial cow's milk.

2.4.2. Changes in pH, titratable acidity and viscosity during storage

The pH was significantly impacted between symbiotic buttermilk and control ($p<0.05$). Figure 1 shows that the pH of symbiotic buttermilk is constantly lower than the pH of control during 12-weeks storage. This result indicates that probiotics and inulin may interact with starter culture, resulting in an increase of lactic acid production. The increase of lactic acid production cause of is the lower pH. A similar study has reported that inulin and probiotics may have positive effects of development of acid (Akın, Akın, & Kırmacı, 2007).

In Figure 2, the titratable acidity was significantly impacted between the control and symbiotic buttermilk ($p<0.05$) and there was no change by 12 weeks storage for both groups ($p>0.1$). This result shows that the additional probiotics and inulin may interact with starter culture, resulting in an increase in lactic acid production. The TA of both groups changes slightly during storage, and we concluded

that there is no post-acidification during storage. Post-acidification could have a negative effect on survivability of probiotics.

There was no significant change in viscosity between control and symbiotic buttermilk over the 12 week storage ($p>0.1$), and there were no changes between weeks for both groups ($p>0.1$). Figure 3 shows that the initial viscosity of symbiotics and control are 70.1 mPa.s and 74.3 mPa.s , respectively. During 12-week storage, the viscosity of symbiotic and control are slightly changed and finally 76.0 mPa.s and 72.8 mPa.s, respectively. Although EI-Nagar (2002) has report that inulin can slightly increase the viscosity of dairy products due to its dietary fiber effect and ability of water binding ability, we concluded that 0.8 % inulin has no effect on viscosity in cultured buttermilk. In future studies, a higher dosage of inulin would be investigated to see the impact on viscosity of cultured buttermilk.

2.4.3. Mold and yeast

No growth of total coliform and yeast/mold in the symbiotic buttermilk was seen at any point during storage.

2.4.4. Survivability of probiotics

Figures 4 and 5 show that the populations of both *L. acidophilus* and *Bifidobacterium* spp. were initially above 10^7 cfu/ml and remained at 10^6 cfu/ml over 12-week storage period. Usually, the number of viable probiotics in yogurt products declines rapidly within a few days during storage (Ng, et al., 2011). However, in this research, probiotics had a very good survival rate and the populations of probiotics remained 10^6 cfu/ml during the 12-week study.

First of all, inulin could play a role in increasing the survivability of probiotics due to the prebiotic effects. Inulin could act as a carbohydrate source for probiotics

and therefore increase the survivability (Ziemer & Gibson, 1998). Similar studies have been reported by Gibson (2003) and Akin (2005).

The high level of oxygen is fatal to probiotics. Numerous studies have reported that plastic packaging compared to the use of glass bottles might play a role in survival of probiotics (Ranadheera, et al., 2012). In this study, the plastic cups were used and sealed during storage. Hence, oxygen cannot get into the products, which might result a desired survivability of probiotics during storage.

The probiotic strains used in this study might be modified, which could lead to a better survivability (Pennacchia, et al., 2004). The modified probiotics strains may have a better ability of oxygen tolerance and low pH tolerance. The probiotics strains used in this study are BB-12 and LA-5 from a commercial supplier. Additionally, further studies need to be determined if LA-5 and BB-12 have a better survivability compared to other probiotic strains.

A commercial starter culture from Chr-Hansen, containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconstoc mesenteorides* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, was used as a co-culture with probiotics in this research. The starter culture can produce CO₂, which can lower the oxygen levels of sealed product. The low oxygen content can give probiotics a better environment to survive. Additionally, the starter culture CHN22 may interact with the probiotics, resulting in a desirable survival of probiotics. According to Antunes (2009), LA-5 and BB-12 had a better survivability during 28-days of storage along with CHN22. However, the interaction between probiotics and starter cultures are not well understood.

In conclusion, these might be the main reasons that resulted in a good survival of probiotics. However, the mechanisms of these actions need to be determined by future studies.

2.4.5. Proteolysis

The proteolysis ability of different starter cultures and probiotics during the 8-week storage period was shown by SDS-PAGE in Figure 6. Symbiotic buttermilk, buttermilk (control), standard yogurt, whole milk, and whey protein isolate lanes were compared with casein and whey. About 80% of milk proteins are casein mainly containing α -casein, β -casein, and κ -casein. Only 20% proteins are found in whey, mainly α -LA and β -LG. In our study, there was no considerable difference in proteolysis among the control, symbiotic buttermilk, whole milk and standard yogurt during storage.

We concluded that CHN22, L901, or probiotics could not hydrolyze milk protein and that symbiotic cultured buttermilk is very stable during storage.

2.5. Conclusions

The results indicated that symbiotic cultured buttermilk could be used as a stable and safe functional food over a 12-week storage period. The survivability of probiotics remained above 10^6 cfu/ml during the 12-week storage and there was no mold, yeast, or coliform detected. There is no significant difference in pH, TA, viscosity and proteolysis during the storage.

2.6. Acknowledgements

Financial support for this project was provided by USDA-NIFA Hatch (VT-H01924S).

We thank Dr. Alan Howard for assistance in the statistical analysis of the results.

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Table 1. Chemical composition of symbiotic buttermilk and control buttermilk (%)

	Symbiotic Buttermilk	Control
Protein	3.30±0.02	3.29±0.05
Fat	3.26±0.06	3.28±0.04
Ash	0.70±0.01	0.69±0.03
Total Solids	12.42±0.03 ※	11.81±0.05 ※
Carbohydrates	5.16±0.06 ※	4.55±0.05 ※

※P<0.05

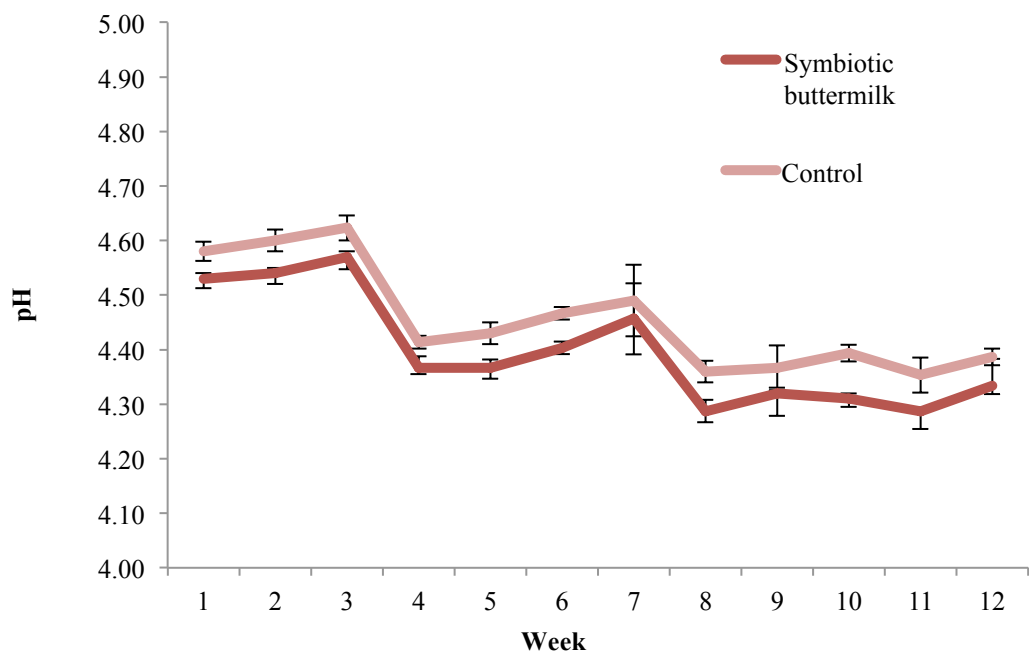


Figure 1. Changes in pH of symbiotic buttermilk and control during storage

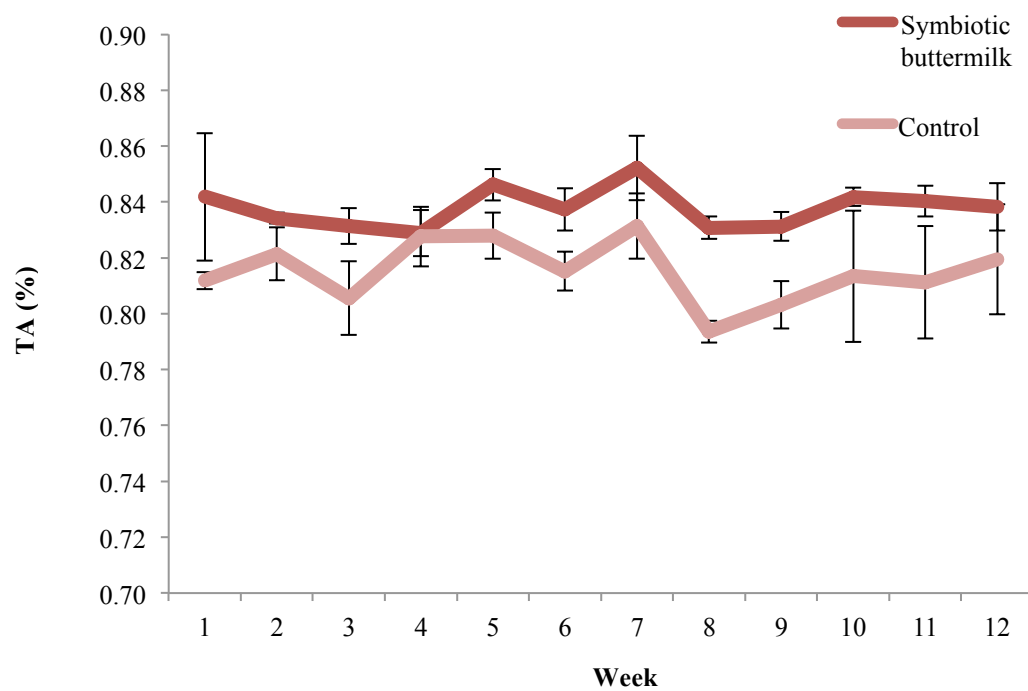


Figure 2. Changes in titratable acidity of symbiotic buttermilk and control during storage

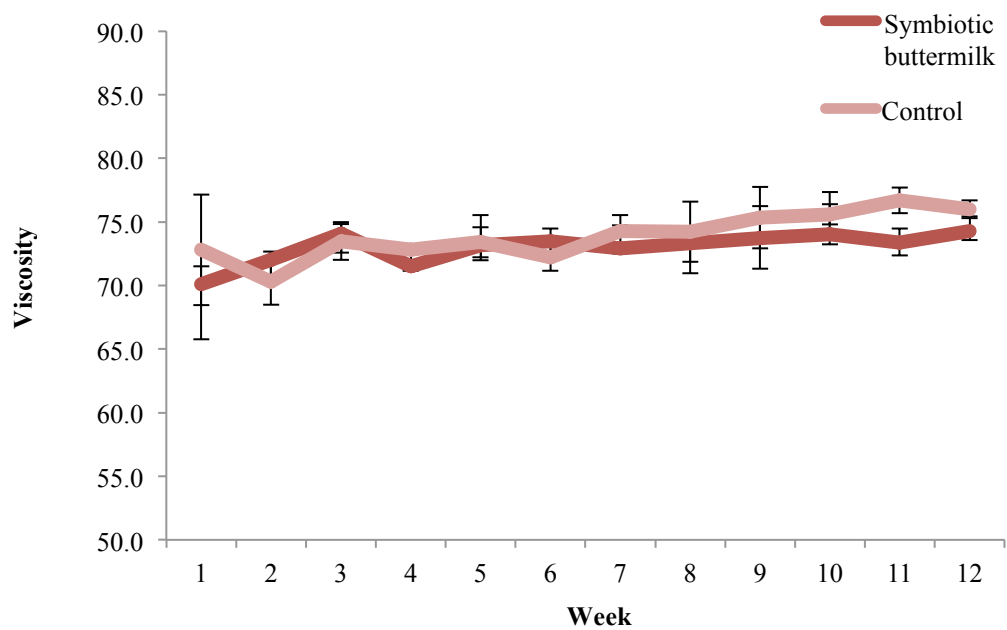


Figure 3. Changes in viscosity of symbiotic buttermilk and control during storage

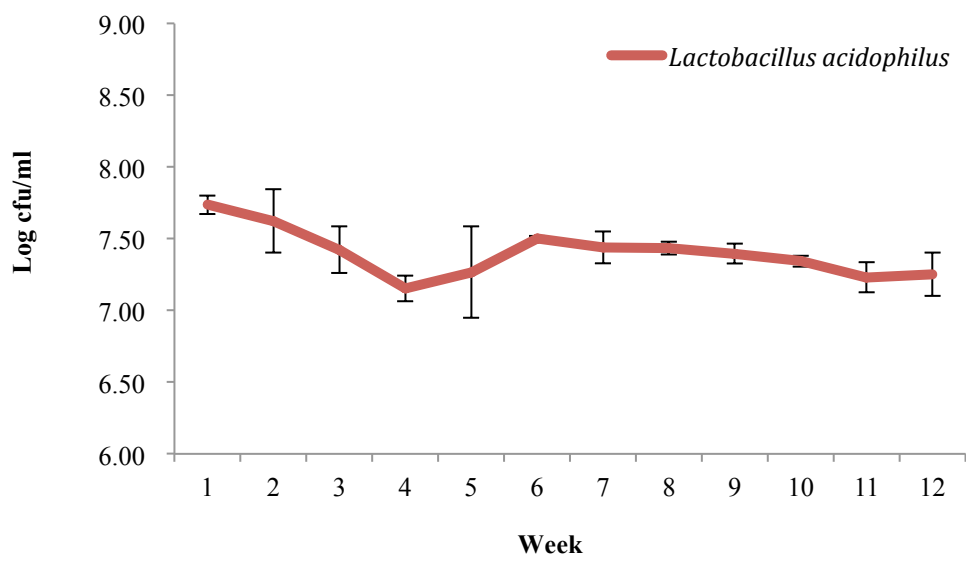


Figure 4. Survivability of *Lactobacillus acidophilus* during storage

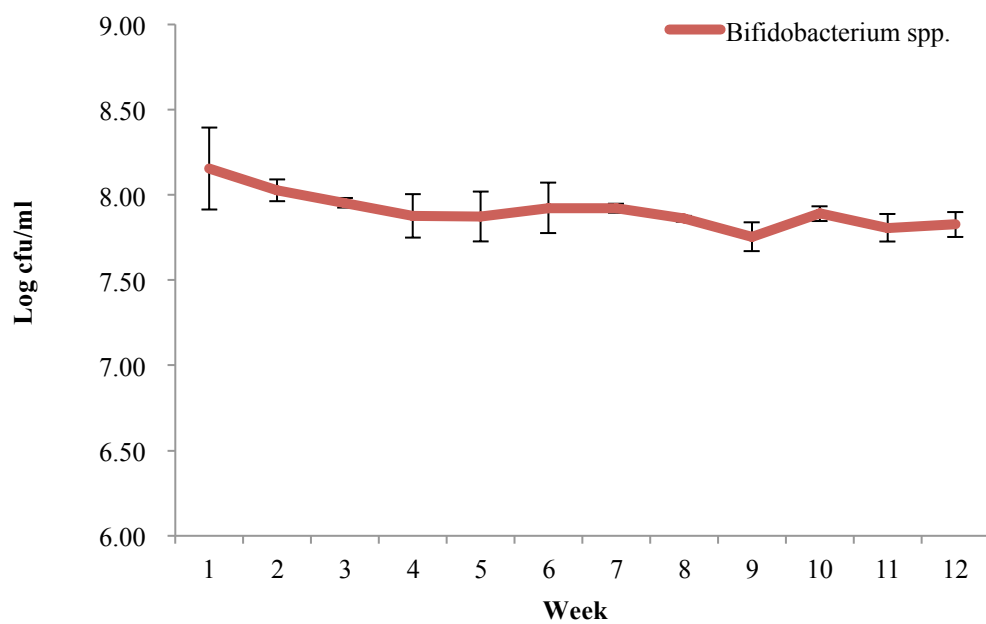


Figure 5. Survivability of *Bifidobacterium* spp. during storage

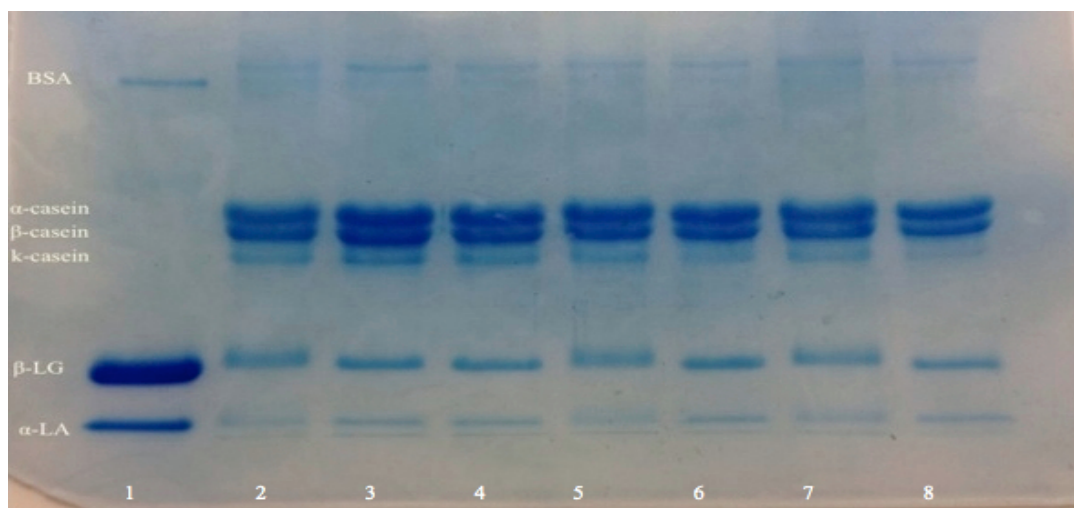


Figure 6. SDS-PAGE photograph of protein profile of symbiotic buttermilk, control, standard yogurt, whole milk, and whey protein isolate. Lane 1, WPI; lane 2, Whole milk; lane 3, standard yogurt fermented by starter culture L901 after 1-week storage; lane 4, standard yogurt fermented by starter culture L901 after 8-week storage; lane 5, culture buttermilk fermented by start culture CHN22 after 1-week storage; lane 6, culture buttermilk fermented by start culture CHN22 after 8-week storage; lane 7, symbiotic culture buttermilk fermented by starter culture CHN22 and *L. acidophilus* and *Bifidobacterium* spp. after 1-week storage; lane 8, symbiotic culture buttermilk fermented by start culture CHN22 and *L. acidophilus* and *Bifidobacterium* spp. after 8-week storage.

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