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Use Of Mushroom Extract To Enhance Quality And Health Benefits Of Dairy Products

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Use of Mushroom Extract to Enhance Quality and Health Benefits of Dairy Products

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Dedication

This dissertation is dedicated to my wife Zeinab, my son Mustafa and to my parents, my brothers and sisters, friends, mentors, and advisors who believed in my ability and helped me to realize that all of my dreams can become realities.

Biographical Sketch

Osman A. Hassan was born in Umdawanban, Sudan, where he attended elementary and secondary schools. Osman received his B.S. degree in Dairy Science from Utah State University in 1991. Then, Osman worked for Global Tel in New York. In 2003 Osman earned his M.S. degree in Food and Nutritional Sciences from North Carolina A&T State University. After finishing his master's, Osman worked as Laboratory Manager and Research Associate of Mushroom Biotechnology & Fungus Biological Laboratory at North Carolina A&T State University, Greensboro, NC. Osman annually helped the North Carolina farmer workshop for how to grow mushroom as a cash product. Also Osman helped in mushroom research. In 2008, Osman was joined North Carolina A&T State University to pursue his Ph.D. degree in Energy and Environmental Systems. At North Carolina A&T State University, Osman has been awarded the Waste Management Scholarship for the year 2009 and 2010.

Osman is a member of several honor societies, such as Gamma Sigma Delta of the Agricultural Society and ADSA. He is the first and corresponding author of several peer-reviewed articles and has co-authored others.

After finishing his Ph.D. degree, Osman intends to continue his career in research to investigate the probiotic effects of different strains of bacteria as antimicrobial agents. Osman is very interested in investigating gastrointestinal dysfunctions in Sudanese people, and to study the effect of probiotics on other product beside dairy products.

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Nomenclature

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
°C	Celsius
ml	Milliliters
CFU	Colony forming units
CFU/ml	Colony forming units/ milliliters
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FOS	Fructooligosacchride
g	Grams
Gal U/ml	Galactose unit per milliliter
GI	Gastrointestinal tract
GRAS	Generally Recognized As Safe
h	Hours
IBS	Possible treatment of inflammatory bowel disease
IgA	Immunglobutin A
LA	Lactic Acid
LAB	Lactic Acid Bacteria
mg	Milligram
mins.	Minutes
µl	Microliters
mM	Millimolar

mol%	Molecular percentage
MRS	DeMann Rogosa Sharpe media
N	Normality
nm	Nanometers
rpm	Revolution per minute
Spp.	Species
WHO	World Health Organization
w/v	Weight/ volume

Abstract

Bifidobacteria and *Lactobacilli* are purportedly beneficial to human health and are called probiotics which are live microorganisms, when administered in adequate amounts conferring a health benefit on the host. It is believed that the maximum probiotics effect can be achieved if the organisms adhere to intestinal mucosal cells. Probiotics, perhaps in combination with prebiotics, may become an important means of preventing and treating disease. In fact, several types of diarrhea have been successfully treated with probiotics. The mechanisms behind favorable clinical outcome are still largely unknown, however, the potential benefits of probiotic therapy promise to be almost limitless. Organic acids offer therapeutic/health benefits, such as increasing of pancreatic secretion.

Prebiotics, such as oligosaccharides and polysaccharides, are defined as nondigestible food ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Shiitake mushroom (*Lentinus edodes*) is an edible and medicinal mushroom popular in Japan, with a prominent activity in very small dosages. Shiitake mushroom extract contains many of oligosaccharides and polysaccharides for this reason it is considered as prebiotics. *Lactobacillus* and bifidobacteria are thought to stimulate the immune system, produce B vitamins, inhibit pathogen growth, reduce blood ammonia and blood cholesterol levels, and help to restore the normal flora after antibiotic therapy. The probiotics are encouraged to grow by oligosaccharides and polysaccharides.

The overall goal of this project is to develop a new technique that could be applied in the food industry to ensure food-grade probiotics that extend the viability longer, express α-galactosidase/ β-galactosidase, and overall to enhance the quality of dairy products. Specific objectives to be accomplished are as follows: 1) to investigate the effect of mushroom extract on growth of

bifidobacteria and *lactobacillus in vitro*; 2) to determine the effect of shiitake mushroom extract on the pH, titratable acidity, viability and α-galactosidase/ β-galactosidase of probiotics in milk during refrigerated storage; 3) to study the impact of mushroom extract on viability and α-galactosidase/β-galactosidase activities of probiotics in yogurt products during refrigerated storage.

The proposed research will build and extend our knowledge to develop food-grade probiotics, which can extend probiotics viability, produce higher α-galactosidase/β-galactosidase activity and enhance the quality of the dairy products. Information obtained from this study will be valuable in developing strategies for the industrial application of this food grade bacteria and enzymes in dairy products.

CHAPTER 1

General Introduction

Human intestinal tract plays a vital role in overall health, not only allowing life-supporting nutrients to be absorbed, but also providing the first line of defense as a physical and immune barrier to food antigens or microorganisms that human may ingest. Within this environment is a highly active society of approximately 500 different species of bacteria that can have both harmful and beneficial effects on your health (Arunachalam, 1999; Isolauri, 2001). While it is imperative for one's overall health that the beneficial bacteria dominate, many factors can lead to an imbalance in favor of harmful bacteria, such as a poor diet, antibiotics, and contaminated food and water (Sulivan, 2001). Adding probiotics to food ingredients resulted in lowered blood cholesterol, and lowered blood pressure (Anderson & Gilliland, 1999; Seppo, Jauhiainen, Poussa, & Korpela, 2003).

Humanity is facing a progressive increase in immune-mediated, gut related health problems. Such as allergies and autoimmune and inflammatory diseases, and genetic factors are unlikely explanation for these rapid increases in diseases incidence (Sgouras et al., 2004; O'Toole & Cooney, 2008). Two environmental factors that related to the modern lifestyle in most world societies are hygiene and nutrition. There has been a decline in the incidence of microbial stimulation by infectious diseases as a result of improved hygiene, vaccination, and antimicrobial medicine. The development of probiotics, functional foods aims to provide a microbial stimulus to the host immune system by means of beneficial live microorganism cultures that are characteristic of healthy human gut microflora (Ibrahim, Hassan, Salameh, & Shahbazi, 2005) Therefore, the need from health conscious consumers has lead to be a high priority of various functional foods. Nowadays food science and technology has shown

development of prebiotics, which is dramatically improving the human gut microflora (Aida, Shuhaimi, Yazid, & Maaruf, 2009).

Probiotics bacteria were shown as gut defense lines. Therefore, we have to support our defense lines by consuming more of lactobacillus and bifidobacteria showing characteristics of high adherence to human gut. A proliferation of unhealthy bacteria can damage human intestinal lining and lead to the production of carcinogenic compounds and intestinal symptoms such as bloating, gas, diarrhea, and abdominal pain (Isolauri, Sutas, Kankaanpaa, Arvilommi, & Salminen, 2001; Schiller, 2007). A damaged intestinal lining allows infectious agents, toxic compounds, and macromolecules to pass through to the bloodstream. Symptoms of this increased intestinal permeability can include fatigue, diarrhea, and skin rashes. Ultimately, it can lead to many digestive disorders as well as seemingly unrelated illness, including chronic fatigue syndrome, eczema, migraine headaches, rheumatoid arthritis, and cancer.

Conversely, the healthy microflora provide protection against these harmful bacteria by strengthening the intestinal lining, competing with harmful bacteria for attachment to epithelial cells, producing antimicrobial compounds, and enhancing the intestinal immune system. Thus, maintaining a well-balanced intestinal microflora is important for reducing the risk of infections and supporting overall health. This may be accomplished through the therapeutic use of beneficial microorganisms, or probiotics such as bifidobacteria and lactic acid bacteria (Martinez-Villaluenga & Gomez, 2007).

Lactic acid bacteria and bifidobacteria exert various beneficial effects on host health by controlling undesirable intestinal bacteria. Among reported beneficial effects of probiotics, are their inhibitory effect to putrefactive bacteria, reduction of fecal enzymes involved in cancer initiation, and reduction of serum cholesterol (Ibrahim & O'Sullivan, 2000). The health

promoting benefits of these microorganisms is related to their ability to colonize in the intestinal tract. Attachment of a microorganism to intestinal tract is an important prerequisite for colonization. The adhesive ability to the intestinal mucus is one of the desirable properties that have to be selected for their specific use in commercial preparations (Bernet, Neeser, & Servin, 1993; Ibrahim & O'Sullivan, 2000; Kos et al., 2003). Probiotics, which can be used in animal feed and human food, are consumed microbial products containing abundant live and active cultures. They have been characterized as live microbial additive that provide beneficial effects to the host by improving its intestinal microflora balance.

Since the efficacy of the use of probiotics is directly related to the number of live active culture cells consumed, it is important for human health condition to have a high number of culture cells in the food product. Characteristics of these microorganisms that also must be included are resistance to gastrointestinal acids, high attachment to human intestinal surfaces, colonization of human intestine, and production of antimicrobial substances (Casas & Dobrogosz, 2000).

The health benefits and bifidogenic effect of prebiotics have been recognized from the last century (Martınez-Villaluenga & Gomez, 2007). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and in consequence improve host health (Gibson & Roberfroid, 1995; Huebner, Wehling, & Hutkins, 2007). Technique that combines both probiotics and prebiotics has been called synbiotic. Synbiosis is a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively

stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare (Gibson & Roberfroid, 1995).

Shiitake mushroom (*Lentinus edodes*) extract contains many of oligosaccharides and polysaccharides (Kawakami, Minato, Tokimoto, Fujitake, & Mizuno, 2004), for this reason it is considered as prebiotics. Probiotics improve immune system and encouraged to grow by oligosaccharides and polysaccharides. Shiitake mushroom is an edible and medicinal mushroom popular in Japan, with a prominent activity in very small dosages (Chihara, Humuro, Maeda, Arai, & Fukuoka, 1970). Shiitake mushroom contains antitumor polysaccharides (Guo et al., 2004). Most of clinical evidence for antitumor activity comes from polysaccharides lentinan (Maeda, Takahama, Kohara, & Yonekawa, 1996; Wasser, 2002). Many studies showed that polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host (Guo et al., 2004). Water-soluble extracts of shiitake mushroom was reported to inhibit the growth of Sarcoma 180 implanted in mice (Maziero, Cavazzoni, & Bononi, 1998). Therefore, colon bacteria may have an important role in carcinogenesis. The supply of indigestible oligosaccharides and polysaccharides is an important factor for the growth of intestinal microflora in human colon, such as lactobacillus and bifidobacteria (Gibson, 1999). According to that, shiitake mushroom is rich in oligosaccharides and polysaccharide; we suggest that it will be effective for the growth of intestinal lactic acid bacteria.

Many cultures worldwide have long recognized that hot water decoctions from certain mushroom fungi can have health-promoting benefits. In China and Japan, in particular, many of these mushroom extracts have become important ingredients in traditional Chinese medicine. At least 270 species of mushrooms are considered to possess therapeutic properties, including

anticancer activity (Ying, 1987), and the term “medicinal mushrooms” is now gaining worldwide recognition. Many edible mushrooms used in traditional folk medicine, including *Lentinus edodes*. Mushrooms have been used not only as a source of food but medicinal resources as well (Maeda et al., 1996; Wasser, 2002). The medicinal properties of mushrooms have been proved through an intensive research conducted many countries.

Intestinal bacteria can be divided on the basis of whether they can exert health promoting, benign or potentially harmful activities in their host (Gibson & Roberfroid, 1995). We have to consider the factors that may influence the flora composition in a manner than can impact upon health. The stimulated of probiotics bacteria (bifidobacteria and lactobacilli) should be a beneficial nature. To have these effects, prebiotics must be able to resistant digestive processes before they reach the colon and preferably persist throughout the large intestine such that benefits are apparent distally (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). Resistance to digestive processes as the criteria includes prebiotic resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption. Figure 1.1 illustrates the criteria for classification of food ingredient as prebiotics (Wang, 2009).

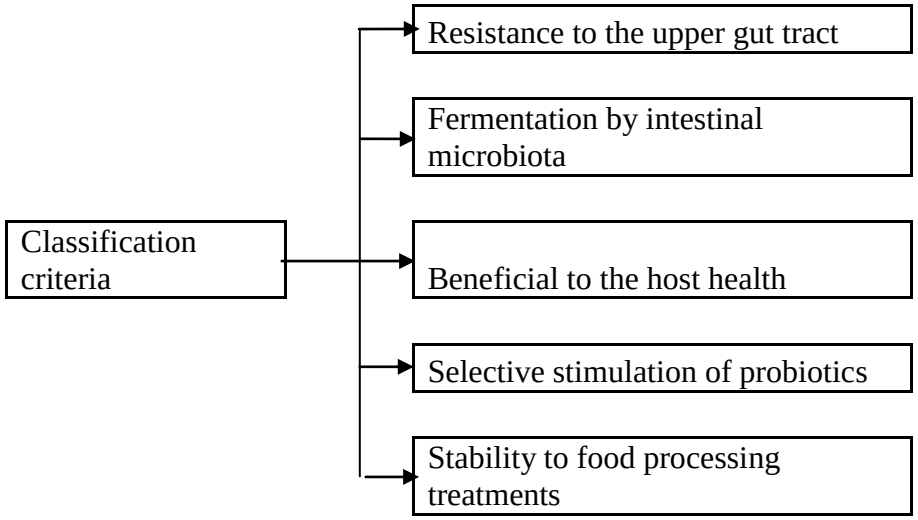


Figure 1.1. Criteria for classification of food ingredient as prebiotics

1.1. Objectives

The overall goal of this project is to develop a new technique that could be applied in the food industry to ensure food-grade probiotics that extend the viability longer, express α-galactosidase/β-galactosidase, and to enhance the quality of dairy products overall. Specific objectives to be accomplished are as follows:

1. To investigate the effect of mushroom extract/polysaccharides on growth of bifidobacteria and *Lactobacilli in vitro.*
2. To determine the effect of shiitake mushroom extract on the growth, viability and α-galactosidase/ β-galactosidase levels of bifidobacteria and *Lactobacilli* in milk during refrigerated storage.
3. To investigae the impact of mushroom extract on viability and α-galactosidase/β-galactosidase activities of bifidobacteria and *Lactobacilli* in yogurt products during refrigerated storage.

1.2. Organization of Dissertation

Chapter 2 of this dissertation presents background that mainly concerns with the probiotics properties of lactic acid bacteria and bifidobacteria with a focus on their use as a suitable vehicle for delivering health benefits to human. Chapter 3 details with the growth of *Lactobacillus reuteri* and bifidobacteria on MRS mixed with different level of SME. Chapter 4 investigates the effects of different concentrations of SME mixed with skim milk on enhancing the survival and viability. Chapter 5 describes enhancing the viability of *Lactobacillus reuteri* DSM 20016 and *Bifidobacterium breve* grown on yogurt in the present of SME. Chapter 6 summarizes the main conclusions of this dissertation and enumerates the recommendations for future researches.

CHAPTER 2

Literature Review

2.1. Milk Fermentation

Milk and dairy product has been used throughout the human life. Mostly all civilization have developed such type of fermented milk product such as yogurt, buttermilk, and drinking yogurt, although the fermentation process was not defined very well. Yogurt is defined as product obtained by the fermentation of milk with cultures of *streptococcus thermophilus* and *Lactobacillus delbrueckii* and. *Bulgaricus spp*, however, yogurt-like products are made by substituting *L. bulgaricus* by other *Lactobacillus* species for the fermentation of milk or yogurt containing probiotic bacteria (Nikawa et al., 2004; Guarner et al., 2005).

Recently dairy products process and its bacteria have received great attention after discovering the importance of viable and survival bacteria in food for health benefits. The first scientific study had been done by Metchnikoff to investigate the beneficial effects of fermented milk for human health. Later many studies have been spread all over the world describing the health benefit associated with the consumption of fermented milk and other products (Shin, Lee, Pestka, & Ustunol, 2000), LAB such as, *Lactobacillus delbrueckii, Bbulgaricus* and *Streptococcus thermophilus* do not adapted to human intestinal tract, and facing difficulty to pass through the digestive system (Klaver, Kingma, & Weerkamp, 1993). Therefore, to maximize the beneficial health effects by the fermented dairy products should be induce huge number in the human intestinal tract with *Lactobacillus* and *Bifidobacterium* species through fermented milk products (Reuter, 1990; Shah & Lankaputhra, 1997; Shah, 2001).

2.2. Probiotics

The human intestinal tract plays a vital role in overall health, not only allowing life-supporting nutrients to be absorbed, but also providing the first line of defense as a physical and immune barrier to food antigens or microorganisms that human may ingest. Within this environment is a highly active society of hundreds different species of bacteria that can have both harmful and beneficial effects on your health (Isolauri, 2001). While it is imperative for one's overall health that the beneficial bacteria dominate, many factors can lead to an imbalance in favor of harmful bacteria, such as a poor diet, antibiotics, and contaminated food and water (Sullivan, 2001).

A proliferation of unhealthy bacteria can damage human intestinal lining and lead to the production of carcinogenic compounds and intestinal symptoms such as bloating, gas, diarrhea, and abdominal pain (Isolauri, 2001). A damaged intestinal lining allows infectious agents, toxic compounds, and macromolecules to pass through to the bloodstream. Symptoms of this increased intestinal permeability can include fatigue, diarrhea, and skin rashes. Ultimately, it can lead to many digestive disorders as well as seemingly unrelated illness, including chronic fatigue syndrome, eczema, migraine headaches, rheumatoid arthritis, and cancer.

Conversely, the healthy microflora provide protection against these harmful bacteria by strengthening the intestinal lining, competing with harmful bacteria for attachment to epithelial cells, producing antimicrobial compounds, and enhancing the intestinal immune system (Sullivan & Nord, 2005). Thus, maintaining a well-balanced intestinal microflora is important for reducing the risk of infections and supporting overall health. This may be accomplished through the therapeutic use of beneficial microorganisms, or probiotics such as bifidobacteria and lactobacilli (Tuohy, Probert, Smejkal, & Gibson, 2003).

The term probiotics originated from the Greek word meaning “for life”, but is defined by the Food and Drug Administration of the United States (FDA) and the World Health Organization as follows: “live microorganisms when administered in adequate amounts confer a health benefit on the host by promoting positive physiological changes” (Broekart & Walker, 2006). Probiotics convey physiological changes in the gastrointestinal tract by increasing the amount of beneficial microflora in the stomach, colon, and intestines.

Bacteria with probiotic effects have been observed in genera Saccrohymes, Clostridium, Enterococcus, and Bacillus; however, most probiotic bacteria originate from the genera *Lactobacillus* and *Bifidobacterium* (Table 2.1). These two genera make up 90% of the beneficial bacteria found in the human gut. In addition, their positive effects on the host are due to their classification as lactic acid bacteria, which is due to their ability to produce lactic acid as a result of carbohydrate fermentation. The production of lactic acid allows for more efficient metabolism in the gastric environment of the gut which contains high levels of acidic bile and pancreatic enzymes (Parvez, Malik, Ah Kang, & Kim, 2006).

Probiotics organisms today have great benefit because they are alternative methods for the treatment of disease such as antibiotic. Balanced in intestinal microflora are necessary for maintaining the health of the host and the theory of probiotics suggest that one way to achieve this is to ingest exogenous bacteria and incorporate them into the colonic microflora. After antibiotic treatments, the frequency of dysbacteriosis is very high. The use of probiotic foods can maintain the balance of gut microflora and may help to control the effects of antibiotic therapy. Since the efficiency of the use of probiotics is directly related to the number of live and active culture cells consumed, it is important to increase the number of culture cells added to the food product. Characteristics of these microorganisms that must also be included are resistant to

gastrointestinal acids, highly attachment to human intestinal surfaces, colonization of the human intestine, and production of anti-microbial substances (Casas & Dobrogosz, 2000). Additionally, the culture should be active in terms of growth potential.

Table 2.1

Bacterial species reported to have probiotic applications

Lactobacilli	**Bifidobacteria**	**Other LAB**	**"Non-lactic"**
L. acidophilus	*B.adolescentis*	Enterococcus faecalis [a]	*Bacillus cereus* ('toyoi')[a, d]
L. casei	*B. bifidum*	*Enterococcus faeciu*	*Escherichia coli* ('Nissle,
L. crispatus	*B. animalis*	*Leuconostoc mesenteroides* [c]	1917')[d]
L. gallinarum[a]	*B. breve*		*Propionibacterium*
L. gasseri	*B. infantis*	*Pediococcus acidilactici* [c]	*reudenreichii*[a, d]
L. johnsonii	*B. lactis*[b]	*Lactococcus lactis*[c]	
L. paracasei	*B. longum*	*SporoLactobacillus inulinus*[a]	
L. plantarum		*Streptococcus thermophilus*	
L. reuteri			
L. rhamnosus			

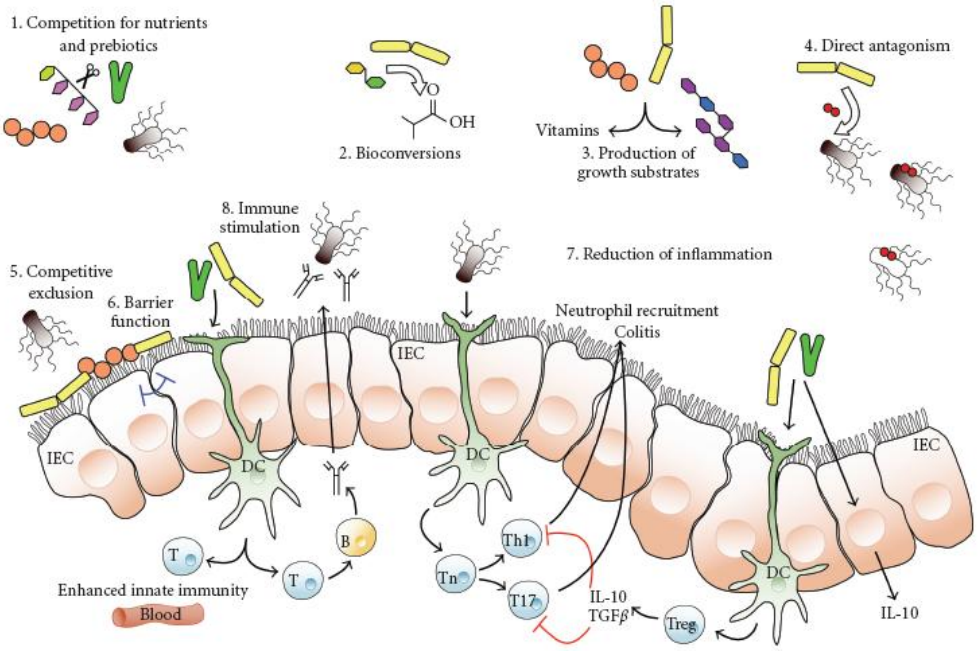
Adapted from Holzapfel et al., 1998

[a] Mainly used for animals.
[b] Probably synonymous with *B. animalis*.
[c] Little known about probiotic properties.
[d] Mainly as pharmaceutical preparations

2.3. Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) are gram-positive non-sporing bacteria, which are physiologically diverse and mostly used as probiotics. LAB belongs to a heterogeneous group of bacteria that are generally united by common morphological, physiological and metabolical characteristics (Axelsson, 2004). Although, probiotic products may contain a variety of beneficial microbes, Bifidobacteria and *Lactobacilli* are the predominant genera incorporated, independently or in mixed combinations. Products contain probiotics are sold in two forms: dairy

foods, primarily yogurt and milk, or as dietary supplements in the forms of capsules, powders, or tablets. Common *lactobacilli* used as probiotics include *Lactobacillus acidophilus, L. casei, L. bulgaricus, L. reuteri, L. brecis, L. cellobiosus,* and *L. fermentum*. The mechanism of the action of probiotics (e.g. bifidobacteria and *lactobacilli*) relies on their metabolic end products, mainly organic acids. Organic acids may lower the human gut pH at which pathogenic microbes are not able to compete effectively (Figure 2.1). The complex and dynamic microbial environment shows an overview of the potential activities of probiotic micro-organisms might influence the intestinal microflora (O'Toole & Cooney, 2008). Dietary intake supplemented with probiotic cultures may modulate the intestinal microflora or change its metabolic properties by competition for nutritional substrates Lactic acid bacteria are acid-tolerant, which enables them to continue fermentation of carbohydrates under low pH levels (Axelsson, 2004).



Adopted from O'Toole & Cooney, 2008

Figure 2.1. Interactions of probiotics and the normal microflora of the host to prevent colonization of opportunistic and pathogenic microorganisms

Probiotics bacteria are manufactured in an array of products, such as various dietary supplements and functional foods. Probiotic dietary supplements are easily purchased at grocery/health food stores or on the internet. The goal of these products is to maintain the balance of microflora in the human intestinal tract, by meeting the following criteria outlined by the FDA: (a) gastrointestinal adherence, (b) bacteriocin production, (c) assimilation of cholesterol, (d) high fecal enzyme activity, (e) antimutagenicity, and (f) small bowel bacterial growth (Doron & Gorbach, 2006). Probiotic supplements contain freeze dried bacteria administered to the host orally in tablet, powders, or capsule forms, which contain one to two billion viable bacteria per gram. Probiotic supplements often are packaged in nitrogen flushing glass bottles at 4°C, containing no hormones or other chemical additives (Walker, 2008).
This probiotic is generally recognized as safe (GRAS) by FDA (Speck, Dobrogosz, & Casas, 1993). *L. reuteri* has been incorporated into commercial probiotics and functional foods (Martoni & Bhathena, 2008). New applications have been proposed to use *L. reuteri* as a feed additive (Casas-Pérez, 1996) and food supplement (De Boever, Wouters, Vermeirssen, Boon, & Verstraete, 2001), as well as in fermented cereals (Charalampopoulos, Pandiella, & Webb, 2002) and buttermilk production (Rodas, Angulo, Cruz, & Garcia, 2002). It has been reported that the populations of *L. reuteri* strains are less variable because productions of reutericyclin appears to contribute markedly to their stability and persistence in sourdough fermentation (Ganzle & Vogels, 2003). *L. reuteri* had the highest viability after 30 d of storage and during fermentation of non-dairy, oat-based products (Martensson, Oste, & Holst, 2002). Although *L. reuteri* has a wide variety of beneficial effects on human health, it has a low occurrence in commercial dairy foods. Different studies on incorporation of probiotics microorganisms in dairy food have been reported. In this context, high growth rate and acidification ability in milk have been reported for

L. reuteri (Xanthopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2000). Also the report showed that if high cell concentrations are reached, better acidification may be attained (Kulozik & Wilde, 1999). In addition, a number of research groups have focused their attention on improving lactic acid bacteria high cell production via fermentation by exploiting novel bioreactors that permit the exchange of the medium to prevent lactic acid accumulation and therefore growth inhibition.

2.3.1. *Lactobacillus*. *Lactobacillus* is one of the most common genera of LAB found in nature. It is a gram positive, rod shaped, and facultative anaerobic genus of bacteria. The genus *Lactobacillus* is considered heterogeneous, (Schleifer & Stackebrandt, 1983) and the most acid-tolerant of LAB (Kashket, 1987). *Lactobacillus* has widespread applications in the food industry.

Previously, *Lactobacillus* was arranged into three groups: *Thermobacterium, Streptobacterium,* and *Betabacterium* (Orla-Jensen, 1921). However, since the previous groups did not represent formal subgenera, this classification was removed from Bergey's Manual (Kandler & Weiss, 1986). Instead, metabolic characteristics were used to divide the different species of *lactobacilli* into the three groups (obligatory homofermenters, facultative homofermenters and obligatory heterofermenters) described previously. Most species of *Lactobacillus* genus are homofermentive, but some are considered heterofermentive (Madigan & Martinko, 2006).

2.3.2. Habitats of *Lactobacillus*. *Lactobacilli* are considered very fastidious, and therefore they require complex media for growth. Fermentable carbohydrates, proteins, nucleic acid derivates, fatty acids, vitamins and metal ions are generally needed in the growth media of *lactobacilli*. Thus, in nature *lactobacilli* are associated with nutrient-rich media like plant material, milk and milk products, meat and meat products, fish, and the oral cavity, GI tract and

vagina of humans and animals (Kandler & Weiss, 1986). Certain species, such as *L. plantarum and L. casei*, can be found in almost any of the aforementioned habitats, whereas other species have restricted niches such as *L. reuteri*, which is found mainly in the GI tract of humans and animals. *Lactobacilli* are widely used in the food industry because they are considered GRAS by the FDA (Speck et al., 1993).

2.3.3. *Bifidobacteria.* *Bifidobacterium* is classified into hundreds different species. *Bifidobacterium* colonies are described as being shiny, with convex shape, circular form, and soft. They are cream to white in color (Valazquez & Feirtag, 1997). The morphology of bifidobacteria is characterized as rods of different shapes, and in single form or branched or "Y" groupings and as coccoidal regular cells (Reuter, 1963). Bifidobacteria are gram-positive anaerobic bacteria. The most commonly used and reported probiotics include two genera; *Lactobacillus* (*L. acidophilus, L. casei, L. bulgaricus*) and *Bifidobacteruim* (*B. bifidum, B. logum, B. breve, B. infantis, B. animalis,* and other *Bifidobacterium species*). Both genera are found in the normal intestinal flora at relatively low levels in healthy human adults. Bifidobacteria constitute up to quarter of the normal flora in adults and more than 90% in newborn. The increased level in infants has been associated with the practice of breastfeeding (Biavati, Castagnoli, & Trovatelli, 1986). Shah, (2001) mention that there are 56 species of *Lactobacillus* and 29 species of *Bifidobacterium,* used worldwide in dairy products The main species of *Lactobacillus* and Bifidobacteria had been reported in recent publications such as; *L. lactis, L. casei, L. paracasei* and more. *B. longum, B. adolesentis, B. breve,* and *B. bifidu*m (Shah, 2001). Bifidobacteria exert various beneficial effects on host health by controlling undesirable intestinal bacteria. Among reported beneficial effects of bifidobacteria, are their inhibitory effect to putrefactive bacteria, reduction of fecal enzymes involved in cancer initiation,

and reduction of serum cholesterol (Ibrahim & O'Sullivan, 2000; Koop, Valentijn-Benz, Nieuw, Amerongen, Roukem, & De Graaff, 1989). The health promoting benefits of these microorganisms is related to their ability to colonize in the intestinal tract. Attachment of a microorganism to intestinal tract is an important prerequisite for colonization. The adhesive ability to the intestinal mucus is one of the desirable properties that have to be selected for their specific use in commercial preparations (Bernet et al., 1993; Ibrahim & O'Sullivan, 2000; Koop et al., 1989; Petr & Rada, 2000).

2.3.4. Natural habitat of bifidobacteria. The typical habitat of bifidobacteria is human, warm-blooded animal and honeybee intestinal tract (Scardovi, 1986). *Bifidobacterium spp.*, are among the most common microorganisms in the human gut (Sghir et al., 2000). In breast-fed infant bifidobacteria form about 90% of their total microflora (Harmsen et al., 2000). Because the stomach is highly acidic, most bacteria settle in the small and large intestines, and are present in particularly large quantities in the colon. Under normal circumstances, bacteria in the digestive system live in balance and harmony, but factors such as poor diets and overuse of a variety of medications can throw the system off balance and lead to a host of difficulties (Walker & Duffy, 1998). Proper protection and nourishment of our healthy bacteria, and then, are very important to good overall health. Bifidobacteria in dairy product, are not growing as fast as lactobacillus, and they required certain growth factor, such as bifidogenic, these carbohydrates found in human milk (Liepke et al., 2002).

It has been said that there are more bacteria in our digestive systems than there are cells in our body. The colon alone contains trillions of bacterial cells. Bacterial cells can be classified in one of four hundred to five hundred types both healthy and dangerous. Twenty types make up three-quarters of the total and are present in large amounts.

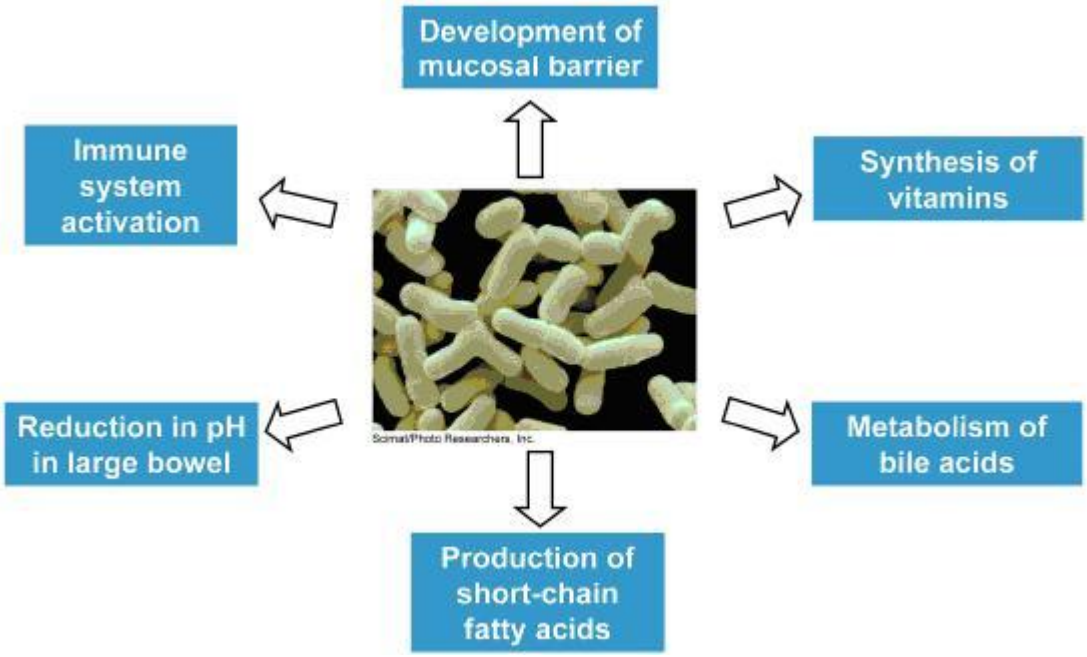
The two strains of bacteria recognized as the most important to health are *Lactobacilli* and bifidobacteria. *Lactobacilli* are concentrated in the small intestine and bifidobacteria reside primarily in the large intestine, mainly the colon. Our relationship with these bacteria can be described as symbiotic, we provide them with warm dark environments where they can flourish, and they provide us with disease protection and improved health.

Several authors studies the distribution of bifidobacteria during various period of life, from infants (breast-fed or not) to adult (Lerche & Reuter, 1962). New born are devoid of an intestinal flora but bifidobacteria appear on the first week of life and constitute one of the most numerous bacterial groups, amounting to 10^{10}/g of wet faeces (Mitsuoka, 1978; Mitsuoka, 1984) originally, it was thought that bifidobacteria between breast-fed and infants whereas in bottle-fed infant *Lactobacillus acidophilus* was supposed to be the most commonly found organism. However, according to Knol et al. (2005), the differences in occurrence and number of bifidobacteria between breast-fed and bottle-fed infants were not significant. The principle difference between both was that in bottle-fed infants the numbers of enterobacteriaceae, streptococci and anaerobes other than bifidobacteria were significantly higher than in breast-fed infants.

2.4. Probiotics and Associated Health Benefits

Research shows that probiotics have beneficial health effects which impact all ethnic groups and ages (Holzapfel, Haberer, Snel, Schillinger, & Huis, 1998) (see Figure 2.2). The first effect of probiotics can be shown within the first week of life where *Bifidobacterium* is the dominant bacterial genera passed to infants via breast feeding (Holzapfel et al., 1998). After the first month of life, the amount of probiotic bacteria begins to decrease and other genera of bacteria such as *Enterobacteriaceae*, *Lactobacillus*, and *Clostridia* become more dominant. Over

the average human lifespan there is an accumulation of over four hundred different strains of bacteria in the gastrointestinal tract (GIT) (Holzapfel et al., 1998).



Source: http://abbottnutrition.com/Education/Prebiotics

Figure 2.2. Effects of beneficial bacteria

Probiotics aid in proper functioning of the GIT by: (1) breaking down food components, (2) producing certain B vitamins and digestive enzymes, and (3) stimulating the immune response (Holzapfel et al., 1998). Probiotics are vital for immune modifications because of their role in the production of immunoglobulin's A and E, B and T cells, and the prevention of pathogenic microorganisms which can cause illness (Perdigon, 2005). Furthermore, the intake of probiotics benefits innate immune responses such as: (1) cell-mediated immunity, (2) the secretion of specialized gut antigens, and (3) the production of T helper, and T receptor cells (Perdigon, 2005). As researchers have begun to understand the importance of probiotics in the prevention of disease, it is important to closely examine some of the illnesses that the intake of probiotics is instrumental in preventing.

2.4.1. Probiotics in relief of lactose intolerance and diarrhea. The use of probiotics has been as a natural therapy for the alleviation of lactose intolerance and diarrhea. Lactose intolerance is a condition characterized by the inability of the body to metabolize the sugar lactose due to the absence of lactase which is a key enzyme located in the large intestinal tract (Vesa, Seppo, Marteau, Sahi, & Korpela, 1998). With lactase being absent, lactose, cannot be metabolized or absorbed by the intestinal wall where lactose enters the bloodstream to allow for digestion in the gastrointestinal tract (Matthews, 2005). If not metabolized, the sugar, lactose, remains in the intestines where fermentation occurs causing gas, cramps, bloating, and diarrhea (Garro, de Valdez, Oliver, & De Giori, 1996).

In one study published in 2002, where probiotics were used as a form of treatment against lactose intolerance (Levri, Ketvertis, Deramo, Merenstein, & D'Amico, 2005). The results of this investigation suggested 2002, where probiotics were used as a form of treatment against lactose intolerance (Levri et al., 2005). Levri and his team found that, oral probiotics do have a beneficial effect on lactose-induced symptoms and the amount of lactose present in the subject body. Of the studies, nine measured breath hydrogen where 66% showed positive results in the decrease of symptoms, while only 34% showed no improvement with probiotics. The investigation concluded that probiotic products containing a cocktail formula of *L. rhamnosus GG* and *L. reteuri* can alleviate the symptoms and signs of lactose intolerance in adults (Levri et al., 2005). Along with lactose intolerance, probiotics have also been used in the treatment ofdiarrhea. Diarrhea is a condition caused by viral infections and/or a combination of parasites or bacterial toxins (Schiller, 2007).

Diarrhea often must be treated medically when it occurs in infants and young children if it is moderate to severe. Medical attention is also required for diarrhea when accompanied with

fever, anemia, loss in weight, and serve abdominal pains (Alam & Ashraf, 2003). There are many types of diarrhea such as secretary, osmotic, inflammatory, infectious and motility-related diarrhea impacting a great number of people mostly (Alam & Ashraf, 2003).

Haung and colleagues (2002) found that probiotics can decrease the duration of acute diarrhea by one day in children. In addition, the study also showed that probiotics decrease the incidence of malabsorption associated with diarrhea in infants and young children. Furthermore, a meta-analysis study showed that probiotic products containing *Sacchomyces boularditi* decrease antibiotic-associated diarrhea in children, adults, and soldiers (Szajewska & Mrukowitz, 2001). The study concluded that the intake of probiotics have been shown to reduce risk and incidence in developing regions such as Africa, Asia, and the Caribbean.

2.4.2. Potential role of probiotics in colon cancer prevention. Colon cancer is a disease where the growth of abnormal cells proliferates and forms tumors. Colon cancer affects the large intestines centralizing in the colon and the rectal areas of the gastrointestinal tract. Although, for years researchers have hypothesized about the cause of colon cancer, little is known about the actual cause. There is a wide spread belief among cancer biologists that the disease is caused by polyps or small pre-cancerous growths that begin in the colon and spread into the rectal region. Colon cancer is prevalent in individuals that eat a high fat diet, smoke, and individuals that have a genetic predisposition (Rafter, 2003).

Colon cancer is diagnosed through a colonoscopy, which is a procedure that examines the inside of the colon and rectum. Furthermore, a biopsy can be performed where a pathologist examines the colon and rectum tissue under a microscope to see if the tissue is cancerous (Rafter, 2003). Colon cancer discovered at its early stages is treatable with radiation and chemotherapy, but in most cases surgery is required to remove the tumor (Rafter, 2003). With colon cancer

being the third most prevalent form of cancer, physicians believe that the ingestion of probiotics can reduce the risk of acquiring colon cancer. Researchers believe the ingestion of foods containing lactic acid bacteria aid in the formation of short chain fatty acids, which are vital in the production of the chemopreventive enzymes associated with the reduction of colon cancer (Wollowski, Rechkemmer, & Pool-Zobel, 2001).

In addition, probiotics assist in the reduction of colon cancer by the formation of metabolites. The process occurs through the breakdown of polysaccharides, starch, and fiber, which enhances the formation of short chain fatty acids which is vital in decreasing the pH of the colon, and then reducing the risk of tumor formation (Wollowski et al., 2001). Research shows that probiotics intake decreases the amount of somatic mutations associated with colon cancer. In vivo models supplemented with probiotics have also been shown to (1) deactivate genotoxic carcinogens (2) prevent DNA damage, and (3) prevent aberrant foci cell mutations associated with colon cancer (Rafter, 2003).

2.4.2.1. Impact of probiotics on immune function. An improperly functioning immune system can result in several immunological disorders such as autoimmune diseases, immune deficiencies and allograft rejection. These malfunctions can often cause fatal injuries and, in severe cases, death. In order to prevent the immunological disorders from occurring, researchers have performed in vitro, in situ, and in vivo studies to understand the chemical and physiological properties of the immune system and how probiotics affect organs. With the recent surge in popularity of probiotics, researchers have begun to investigate their importance on immune response. This is achieved through the study of passive immunity via saliva, gastric acid, mucus, intestinal flora, and several other factors that fight against antigens and aid the GIT main protective barricade, the gut mucosal barrier.

The importance of the gut mucosal barrier on the host and immune enhancement by probiotics can first be observed at birth. During postnatal development in the first months of life, there is a maturing of the gastrointestinal tract leading to the production of mucosal proteins, digestive enzymes, and secretion of hydrochloric acid occurs and allows for gastrointestinal tract immunity (Isolauri & Sutas, 2001). As a child ages and the body develops a tolerance against antigens, due to the growth of gut epithelial cells, which allow for mucosal immune response during the first years of life (Isolauri & Sutas, 2001). As a child matures, the intake of dairy products supplemented with probiotics has an added importance in their ability to develop large intestinal lymphoid tissue. These tissues represent the largest mass of lymphoid tissue in the human body, which affect the regulatory events of the intestinal immune response. Furthermore, this allows for the gastrointestinal tract to efficiently produce mucosa and secretory sites within the intestinal wall (Isolauri & Sutas, 2001). The gut lymphoid tissue also allows for the production of antibodies in the respiratory tract, salivary, and mammary glands. As a child matures to adulthood, probiotics play an equally important role in immune enhancement (FAW, 2002). *Bifidobacterium spp*. increase adherence of gut lymphoid tissue and gastrointestinal microflora in the maintenance of normal immune function (FAW, 2002).

In addition, probiotic products supplemented with bacteria from the genus *Lactobacillus* have been shown to have the most wide spread effect influencing chronic vaginitis, urogenital infections, Crohn's disease, and diarrhea. Moreover, supplementing products containing *Lactobacillus* can increase: (1) the secretion of IgA, (2) interleukin-6 and 10, (3) the production of cytotoxic T cells, (4) T helper cells 1 and 2, and (5) the production of dendritic cells (Marteau, 2001). In addition, the intake of probiotics is vital in the development of a healthy and efficient immune system in children and adults. Without the intake of probiotics the immune system will

not be able to productively and efficiently fight against disease as efficiently without the intake of probiotics.

2.4.2.2. Possible treatment of inflammatory bowel disease (IBS). One of the most common gastrointestinal disorders that affect individuals in the United States is IBS. The intestinal disorder is characterized by abdominal pain, cramping or bloating, diarrhea, and/or constipation (Gilkin, 2005). IBS is distinguished from other gastrointestinal disorders because physicians find no change in physiological structure of the body such as inflammation or tumors. However, physicians notice that the disorder is related to an abnormality between the brain and the intestinal tract where abnormal contractions result from different signals between the brain and the intestines. Once diagnosed, IBS is very manageable.

One aspect that has been researched is the importance of probiotics in the treatment and reduction of IBS (Camilleri, 2006; Alazzeh, Ibrahim, Song, Shahbazi, & AbuGhazaleh, 2009). Probiotics such as *Lactobacillus acidophilus* and *Bifdobacterium infantis* found in milk products reduce the symptoms of IBS by decreasing the amount of gas, cramping, and bloating associated with the disorder (Holzapfel et al., 1998; Krammer & Schlieger, 2005; Heyman, 2006). Furthermore, probiotics along with antibiotics such as ciprofloxacin show a decline in the amount of symptoms associated with IBS (Camilleri, 2006). A study performed at the University of Helsinki showed that a combination of probiotic strains *Lactobacillus rhamnosus*, and *Bifidobacterium breve* showed a 42% decrease in abdominal pain, distension, flatulence, and rumbling of the stomach compared to patients that did not have the probiotic treatment (Penny et al., 2008). Probiotic intake decreases the production of toxic short chain fatty acids, as well as the formation of intestinal hydrogen in IBS patients (Penny et al., 2008). Furthermore, probiotic intake can reduce side effects of a decrease in bacterial enzymes by reducing the amount of small

intestine bacterial overgrowth and increase of probiotic bacterial adherence to the gut (Korpela, 2006). The influence of probiotics on IBS can also be noted in the reduction of symptoms associated with the disease such as abdominal pain, bloating, gas, and constipation (Gilkin, 2005).

Technique that combines both probiotics and prebiotics has been called symbiotic, which is a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract. By selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria and thus improving host welfare (Gibson & Roberfroid, 1995).

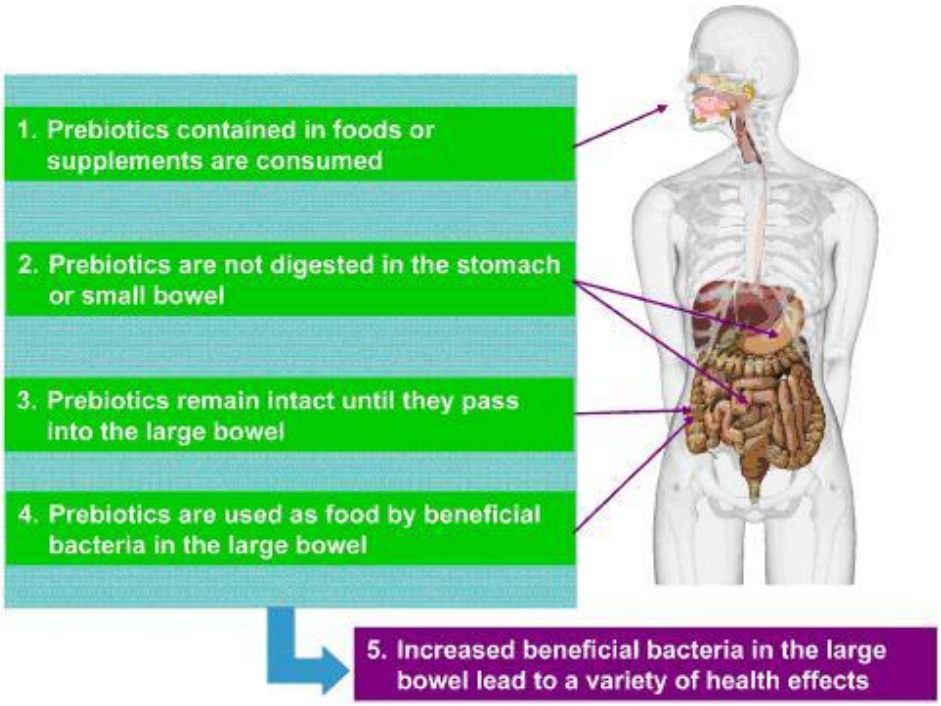
2.5. Prebiotics

A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health (Roberfroid, 2007). There are some factors affect the composition of gastrointestinal tract microflora in humans, such as age, colonization, health, nutritional requirements, and immune system condition of the host, the large intestinal tract pH, and the correlation between flora components and availability of prebiotic material in the human gastrointestinal tract. According to these factors, it is probably the amount and type of growth substrate that has the most effect. Non-digested food residues or prebiotics in the upper part of gastrointestinal tract, as well as endogenous materials like mucins, epithelial cells, and bacterial remaining products, contribute to the pool of metabolizable substrates (Gibson & Roberfroid, 1995). Dietary intake may exert a major influence on gastrointestinal tract microflora and their development.

2.5.1. Concept of prebiotics. The human gastrointestinal tract microflora influences the health and happiness. The use of diet to fortify certain gastrointestinal flora components is a popular current aspect of functional food sciences. The food containing fibers plays as major fermentable diet in the human gastrointestinal. Examples for these kinds of food are carbohydrate-based materials such as, lactose, oligosaccharides, food sweeteners, and other non-absorbed sugars. There is a small contribution from nitrogen-based materials like legumes, amino acids, and some dietary lipids may also passed to the large intestinal tract in a metabolizable processing. Prebiotics are foods that stimulate the growth of bifidobacteria and lactobacilli in the gastrointestinal tract, and thereby, increase the body's natural resistance to fight harmful bacteria (Kim & Rajagopal 2000; Ibrahim et al., 2009). Also prebiotic carbohydrates may have less specific benefits because they are fermented in the human large intestinal tract. The prebiotic carbohydrates that have been evaluated in humans at the present time largely consist of oligosaccharide and polysaccharide. There is proved evidence that these are not digested by normal human enzymes, but are readily fermented by anaerobic bacteria in the large intestine. Through fermentation in the large intestine, prebiotic carbohydrates yield short-chain fatty acids, stimulate the growth of many bacterial species in addition to the selective effects on lactobacilli and bifidobacteria, they can also produce gas (Cummings & Macfarlane, 2002). Potentially, the most important effect of prebiotics carbohydrates is to support the human body's defense of invading pathogens and, thereby, prevent of diarrhea (Figure 2.3).

The gastrointestinal tract of infants is cultured mostly by organisms oriented from the mother's vagina and feces and from the environment. After birth, the digestive system tract is rapidly colonized by bacteria, but probiotic bacteria gradually become a dominant as main organisms, and continue to be super bacteria in the first few months after delivery, in breast-fed

infants (Ishibashi & Hayasawa, 1997). The population of probiotics in gastrointestinal tract in bottle- fed infants, gradually decreased in number from the time of weaning, and bacteria such as anaerobic streptococci and *E. coli* will exist in highest numbers (Rotimi & Duerden, 1981). These bacteria may subsequently create a highly new environment that allows the growth of competitive an aerobic species. Differences in microflora are also observed by culturing the fecal flora of breast-fed and bottle-fed infants (Mitsuoka, 1978). The high number of *Lactobacillus* and *Bifidobacterium* is a sign of possible explanation for the health advantages. The change of dietary intake may influence the microflora composition and consequences of that is the health status of human. The use of probiotics, prebiotics, and synbiotics may all be feasible. Ben et al. (2004) proved that the addition of prebiotics to infant formula can increase beneficial bacteria in the digestive system to levels similar to that mother milk. In addition, prebiotics can help in laxation to be like those of breastfed infants.



Source: http://abbottnutrition.com/Education/Prebiotics

Figure 2.3. How prebiotics work

2.5.2 Prebiotics criteria and hypothesis for mechanisms. The criteria used for classification of a food component as a prebiotic should be resistance to digestion processing, hydrolysis and fermentation by colonic microflora, and most importantly, selective stimulation of growth of one or a few number of bacteria in the feces. Resistance to digestion should be proved in the laboratory (Macfarlane & Cummings 1999; Thibault, Aubert-Jacquin & Goulet, 2004; Roberfroid, 2007). To show and quantify hydrolysis and fermentation by colonic microflora, human fecal samples are a valuable surrogate for colonic content. Prebiotics are a unique concept in human nutrition and health condition for which, as is often the case with a new application, many health and physiological claims have already been made. Table 2.2 shows these claims. Central to all claims is the effect on the microflora, which in turn should work hardly and strong to resist the pathogen invasion in the large bowel, and a reduction in diarrheal diseases (Cummings & Macfarlane, 2002).

Table 2.2

Claimed gastrointestinal effects of prebiotics

Gastrointestinal	Effects
Through fermentation in the large bowel	• Production of short-chain fatty acids and lactate • Gas, mainly CO_2 and H_2 • Increase in biomass • Increased fecal energy and nitrogen • Mild laxative properties
On the microflora	• Selective increases in bifidobacteria and lactobacilli in planktonic and biofilm communities • Reduction in clostridia • Increase in colonization resistance to pathogens • Potential benefit in preventing pathogen invasion

Table 2.2 *(cont)*

Gastrointestinal	Effects
Small intestine	• Osmotic effect of low molecular weight prebiotics (DPS, 4) which occasionally causes diarrhea • Improved calcium, magnesium and iron absorption • Interaction with mucus to change binding sites for bacteria, lectins etc.
Mouth	• Protection against caries
Other effects	• Bile acid metabolism-no consistent changes reported • Variable effects on microbial enzymes with potential to affect carcinogenesis • Stimulation of apoptosis

The composition and activities of the large intestinal tract microflora is essential for health-promoting activities are optimized remains as a major in functional food development. The prebiotics effect is an environmental effect and needs to be treated as such. The demonstration of this effect requires a much more qualitative and quantitative analysis of the gastrointestinal tract microflora and its modulation by the prebiotics treatment. These methods are being extensively applied to human large intestine tract's environment to detect fundamental changes in colonic microflora composition and to correlate them with health benefits that are possibly not to be limited to gastrointestinal physiology. Systemic effects of prebiotics should be identified and followed by further investigation.

As long as shiitake mushrooms consider as prebiotics, the potential application of shiitake mushroom extract is required to be investigated as functional food for reducing the risk of colon carcinogenesis as well as to improve. Lentinan from Shiitake has been proved as an anticancer therapy by the Japanese FDA. Lentinan as extract of shiitake extract has shown some effect on cancer cells affected bowel, liver, stomach, ovarian, and lung (Mohammad, Faruqi, & Mustafa, 2009). In many studies lentinan stimulated the production of T lymphocytes and natural

killer cells (Mizuno, Kawakami, Hashimoto, Ashida, & Minato, 2001; Wesser, 2002). Shiitake is rich in several antioxidants like selenium, uric acid, and vitamin A, E, and C as well as vitamin D. The high proportion of people susceptible to colon cancer, are those have high population of somatic mutations occurring during the lifetime of an individual, could be prevented by eliminating these mutations.

2.6. Shiitake Mushroom

2.6.1. Overview. When most people hear the word "mushroom" they picture just one variety: "the cultivated white mushroom, fruit of the fungus Agaricus bisporus." But other varieties are appearing in local markets, including shiitake, a darker and stronger-flavored cousin of the common button mushroom (see Figure 2.4). The shiitake (*Lentinula edodes*), begins life as an invisible network of thin, spidery threads, which grow through the dead tissue of various hardwoods such as oak, beech, or chestnut. The mycelia digest the wood and convert it into fungal tissue (Witzany, 2008). It is believed that Chinese growers introduced shiitake cultivation techniques to Japanese farmers, who named the mushroom and were later responsible for its spread eastward (Chang, 1999). Composed of sawdust and supplemented with millet and wheat bran are a good medium for shiitake to grow. Also, environmentally controlled houses allow for the manipulation of temperature, humidity, light, and the moisture content of the medium to produce the highest possible yields. Mushrooms consume water and nutrients from their environment and begin to reproduce. The medium that mushroom mycelium grows on is usually called substrate. Before the mushroom can start to form fruit bodies the mycelium colonizes the substrate fully and when the environmental conditions are right the mushroom emerges to produce more spores. Mushroom cultures can be stored for prolonged periods of time to preserve

the unique strains that mushrooms produce; some strains are desired for their rapid growth, resistance to disease and other characteristics. Mushrooms are truly unique organisms. Their full potential in recycling agricultural wastes and tree stump decomposing has yet to be taken advantage. Some mushrooms like the Shiitake even boast cancer fighting properties.

Photo courtesy Dr. Omon Isikhuemhen, NC A&T

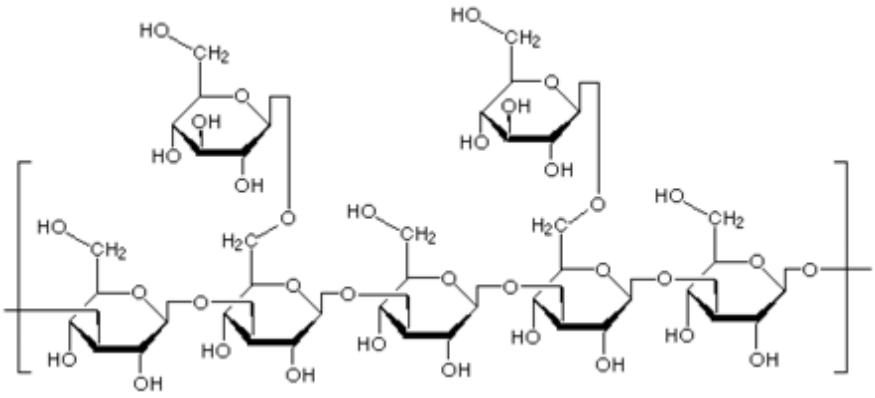
Figure 2.4. Shiitake mushroom

2.6.2. Production and consumption of shiitake mushroom. Shiitake were originally treasured Asian mushrooms with great flavor, taste and an enticing aroma. They are also known for their medicinal benefits in strengthening the immune system. Asian populations worldwide continue to favor Shiitake, whether fresh or dried. Factors that may affect Shiitake consumption include consumers' ethnic and cultural backgrounds, tastes for food, ages, sex, perception and knowledge of mushroom products, mushroom image and consumers' income. Shiitake appeals to health-conscious populations as well as vegetarians for its attributes of high nutrition, quality protein, essential amino acids, low calorie content and health benefits. In regions where edible

mushrooms are not a traditional food, demand for mushrooms such as Shiitake depends on the continuous effort of mushroom producers to educate consumers.

The benefits of modern sawdust cultivation include a consistent market supply through year round production to meet consumer demand. Moreover, sawdust cultivation is a means to use agricultural wastes (Lopez, Valencia, & Chang, 2004) and spent mushroom substrates to generate food. World production of mushrooms is dominated by species that are edible and have medicinal benefits. Shiitake now ranks number one in specialty production. Current trends in mushroom production and consumption turn toward fresh product, particularly in the main markets of the U.S. and Europe (Zhang, 2004).

2.6.3. Lentinan. Lentinan is a polysaccharide derived from the vegetative parts of the edible shiitake mushroom. It is the cell wall constituent extracted from the fruiting body or mycelium of *L. edodes* and is not generally associated with side effects. See Figure 2.5 for the chemical structure of β-glucan lentinan.



Adopted from Nakano et al., 1999.

Figure 2.5. Chemical structure of β-glucan lentinan

2.6.4. Active hexose correlated compound (AHCC). Active hexose correlated compound (AHCC), extracted from shiitake mushrooms, which contains α-glucan rich in ploysaccahrides and fibers. AHCC has been shown to induce enhancement of human health, by

immune responses, including enhanced natural killer activity (Aviles et al., 2003; Weisbuger, 1977). Research proved that AHCC possesses many physiological activities, such as Increasing resistance to pathogens, producing an anti-cancer, and enhancing immune function.

2.6.5. Medicinal value. An extract (Lentinan) from Shiitake has been licensed as an anti-cancer drug by the Japanese FDA. Lentinan has shown some effect on bowel cancer, liver cancer, stomach cancer, ovarian cancer and lung cancer (Maeda et al., 1996; Wasser, 2002). Lentinan stimulates the production of T lymphocytes and natural killer cells and can potentiate the effect of AZT in the anti-viral treatment of A.I.D.S (Guo et al., 2004). Shiitake is rich in several anti-oxidants (Selenium, Uric acid and Vitamin A, E, and C) as well as Vitamin D. Shiitake mushrooms may also lower blood pressure in those with hypertension, lower serum cholesterol levels, increase libido, and stimulate the production of interferon which has anti-viral effects, and has proven effective against hepatitis in some cases. Shiitake mushroom (*Lentinus edodes*) extract contains many of oligosaccharides and polysaccharides (Kawakami et al., 2004), for this reason, is considered as prebiotics. Probiotics improve immune system and are encouraged to grow by oligosaccharides and polysaccharides. Most of clinical evidence for antitumor activity comes from polysaccharides lentinan (Maeda et al., 1996; Wasser, 2002). Many studies showed that polysaccharides from mushrooms produce their antitumor effects by activating different immune responses in the host (Guo et al., 2004). Water-soluble extracts of shiitake mushroom was reported to inhibit the growth of Sarcoma 180 implanted in mice (Maziero et al., 1998). Therefore, colon bacteria may have an important role in carcinogenesis. The supply of indigestible oligosaccharides and polysaccharides is an important factor for the growth of intestinal microflora in human colon, such as lactobacillus and bifidobacteria (Velazquez & Feirtag, 1997; Gibson, 1999).

Shiitake mushroom has been used in Japan and China as both a food and medicine for thousands of years. It is now commonplace throughout the world. Extracts of these mushrooms are now being incorporated into over-the-counter dietary supplements designed to improve the status of the immune system. According to that, shiitake mushroom is rich in oligosaccharides and polysaccharide; we suggest that it will be effective for the growth of lactic acid bacteria.

Many cultures worldwide have long believed that hot water decoctions from certain mushroom fungi could support health-promoting benefits. In China and Japan, in particular, many of these mushroom extracts have become important ingredients in traditional Chinese medicine. At least 270 species of mushrooms are considered to possess therapeutic properties, including anticancer activity (Ying, 1987), and the term “medicinal mushrooms” is now gaining worldwide recognition. Many edible mushrooms used in traditional folk medicine, including *Lentinus edodes*. Mushrooms have been used not only as a source of food but medicinal resources as well (Maeda et al., 1996; Wasser, 2002). The medicinal properties of mushrooms have been proved through an intensive research conducted many countries.

CHAPTER 3[1]

Shiitake Mushroom (*Lentinus Edodes* (Berk.) Singer) Extract Enhance the Growth of Lactic Acid Bacteria and Bifidobacteria *in vitro*

3.1. Introduction

Probiotics create a balance between beneficial microflora and pathogens that could invade the gastrointestinal tract such as *Clostridium difficile, Escherichia coli,* and *Salmonella* spp. Probiotics are used in the alleviation of gastrointestinal disorders, such as infectious diarrhea and irritable bowel syndrome cramps (Garro et al., 1996). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and in consequence improve host health (Gibson & Roberfroid, 1995). Technique that combines both probiotics and prebiotics has been called synbiotic (Indrio et al., 2009). Synbiosis is a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract.

Recently, public media got attention for some valuable foods and health-enhancing effects of using shiitake mushroom and beneficial bacteria (Vargas & Ohashi, 1996). Shiitake mushroom (*Lentinus edodes* (Berk.) Singer) extract contains many oligosaccharides and polysaccharides (Kawakami et al., 2004), and is considered a prebiotic. Probiotics growth is stimulated by oligosaccharides and polysaccharides similar to those found in mushrooms (Vargas & Ohashi, 1996). Shiitake mushroom popular for its edible and medicinal mushroom has become the 2nd most cultivated mushrooms in the world (Chang, 1993). Shiitake mushroom contains antitumor polysaccharides (Guo et al., 2004). Most clinical evidence for antitumor

[1] Parts of this chapter were adapted from: Hassan, O.A., Ibrahim S.A., Song D., Isikhuemhen, O., Shahbazi, A. & AbuGhazaleh A.A. *Milchwissenschaft*, 66(3) (2011).

activity comes from polysaccharides lentinan (Wasser, 2002). Many studies showed that polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host (Guo et al., 2004). In this study we investigate the effect of shiitake mushroom extract on the growth of bifidobacteria and lactobacilli in vitro.

3.2. Materials and Methods

3.2.1. Preparation of shiitake mushroom. Fresh shiitake mushroom was obtained from a local market in Greensboro, North Carolina. The mushroom was dried under a freeze drying machine (Labconco Freeze dryer, Kansas city, Mo, USA), powdered, and then sieved (particle size 1 mm). Mushroom extract (1%, 2%, and 4%) was heated on hot plates at 80°C for three hours (Minato, Mizuno, Terai, & Tsuchida, 1999). After cooling to room temperature, mushroom extract was filtered twice first through Whatman number 1 filter paper (Whatman Corp., Clifton, NJ, USA), and then through Nalgene filter unit with 0.2 um pore size (Nalge Nunc International, Rochester, NY, USA). The extract was then stored in a freezer at -20°C.

3.2.2. Chemical analysis of shiitake mushroom.

3.2.2.1. Proximate analysis of mushroom. Proximate chemical composition of mushroom sample powder, including moisture, ash, protein, and lipid were determined according to the Association of Official Analytical Chemists methods (AOAC, 1995). Carbohydrate content on a dry weight basis was calculated by following the formula: % carbohydrate = 100% - % ash - % protein - % lipid (on a dry matter basis).

3.2.2.2. High performance liquid chromatography (HPLC) analysis. One milliliter of shiitake mushroom extract was taken for sugars analysis. Samples were extracted with hot water (85ºC) for three hours. Samples were prepared for HPLC filtered by micro-filter (0.45 µm). The

samples were applied to a shodex KC-811 fermentation column (Water corp., Milford, MA, USA), a column (8x 300mm) used in conjunction with a Waters 410 differential refractive index detector for identification specific carbohydrate. The mobile phase was 0.1% H_3PO_4 at a flow rate of 1 mL/min.

3.2.3. Media preparation. Fifty-five grams of MRS (de Man Rogosa Sharpe broth) from Difco Laboratories (Becton Dickinson, Sparks, MD) was dispensed in 1 L of deionized water and allowed to stir moderately for 10 min to allow media to dissolve. Cysteine-hydrochloride (0.5 g/L) was added as the reducing agent and allowed to stir at low speed (5-10 rpm) for an additional 10 min to dissolve (Poch & Bezkorovainy, 1991).

3.2.4. Culture conditions. Four strains of *Lactobacillus* and *Bifidobacterium sp.* obtained from the culture collection in the Food Microbiology Laboratory at North Carolina A&T State University were tested in this study (Table 3.1). All strains were maintained on *Lactobacillus* MRS agar Petri dishes at 4ºC. Before use, the strains were activated by taking one bacterial colony of each strain and separately inoculated the colony into *Lactobacillus* MRS broth in anaerobic culture tubes at 37°C for 18 hour.

Table 3.1

Probiotics strains that were used in this project

Strains	Original sources
Lactobacillus reuteri DSM20016	Mother's milk
Lactobacillus reuteri CF2-7F	Child fecal isolate
Bifidobacteria breve ATCC 15701	Infant stools
Bifidobacteria adolescentis ATCC 15704	Human feces

3.2.5. Sample preparation and treatment. Batches of MRS broth containing different concentrations of shiitake mushroom extract were prepared. Bacterial strains were inoculated into individual test tubes with MRS medium and shiitake mushroom extract at different concentrations (0, 1, 2, and 4% w/v) and incubated at 37ºC for 8 hr.

3.2.6. Bacterial growth. Bacterial growth was monitored by measuring optical density (O.D. 610 nm) at different time intervals (0, 2, 4, 6, and 8 hours) using a spectronic 21 Milton Roy spectrophotometer (Model Genesys 10 Vis, Thermospectronic, Rochester, NY, USA).

3.2.7. Bacterial enumeration. Bacterial populations were determined by plating onto MRS agar plates. Samples (1mL) were withdrawn from inoculated samples at 8 hours, serially diluted in 1% peptone water, and then appropriate dilutions were surface plated (100 µL) onto duplicate plates of MRS agar. Colonies were counted after incubation at 37°C for 72 h to determine bacterial population.

3.2.8. Determination of pH and titratable acidity. Samples were withdrawn at different time intervals (0, 2, 4, 6, and 8 hours) to measure pH values. The pH meter (model 410A, Orion, Boston, MA) was calibrated with pH standard buffers 4.0 and 7.0. After calibration, sample pH was taken and recorded. Between different samples, electrode was rinsed with distilled water. The titratable acidity was determined after mixing the 1 mL of sample with 9 mL of distilled water by titrating with 0.1 N NaOH to the pH 8.6 using a pH meter (model 410A; Orion, Boston, MA) and titrator (Dejardins & Roy 1990).

3.2.9. Statistical analysis. Data analyses were focused on determining if the addition of shiitake mushroom extract resulted in significantly greater growth over that of the control samples. Statistical analysis of data was performed using the SAS General Linear Model (GLM) program (1999). Duncan's Multiple Range Test was used to determine significant ($P < 0.05$)

differences. For each growth condition, triplicate samples were tested for the growth of all strains.

3.3. Results and Discussion

3.3.1. Composition of shiitake mushroom. Table 3.2 shows the chemical composition of the shiitake mushroom extract. Shiitake mushroom extract contained 22% dry matter, 6.66% ash, 60% carbohydrate, 29.06% protein, and 1.5% lipid (dry matter basis). According to analysis by HPLC, shiitake mushroom extract contains 0.033% raffinose, 0.035% glucose, 0.045% trehalose and 0.069% arabinose (Figure 3.1). The large portion of protein content could be in the form of amino acids, small peptides and nucleic acids material (Vargas & Ohashi, 1996).

Table 3.2

Chemical composition of shiitake mushroom

Content	g/100 g DM weight
Ash	6.66
Protein	29.06
Carbohydrate	54.00
Lipid	1.5
pH	5.59

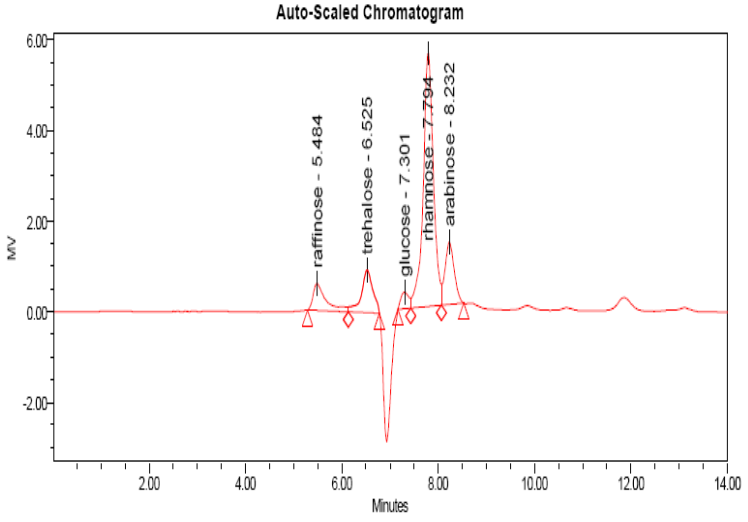


Figure 3.1. Simple sugars in shiitake mushroom extract

3.3.2. Change in pH value. The results provided in Figure 3.2 are the mean triplicate pH measurements for shiitake mushroom extract samples. Figure 3.2 shows the acid production of each strain in combination with shiitake mushroom extract. As time increased there was an increase in the amount of acid produced in all strains causing a decrease in pH. Strains supplemented with the appropriate concentration of shiitake mushroom extract (4%) had the highest amounts of acid production after 8 hour. Strains supplemented with 1 or without SME had minimal acid production in comparison to the 2 and 4% of concentration of mushroom extract used in this study.

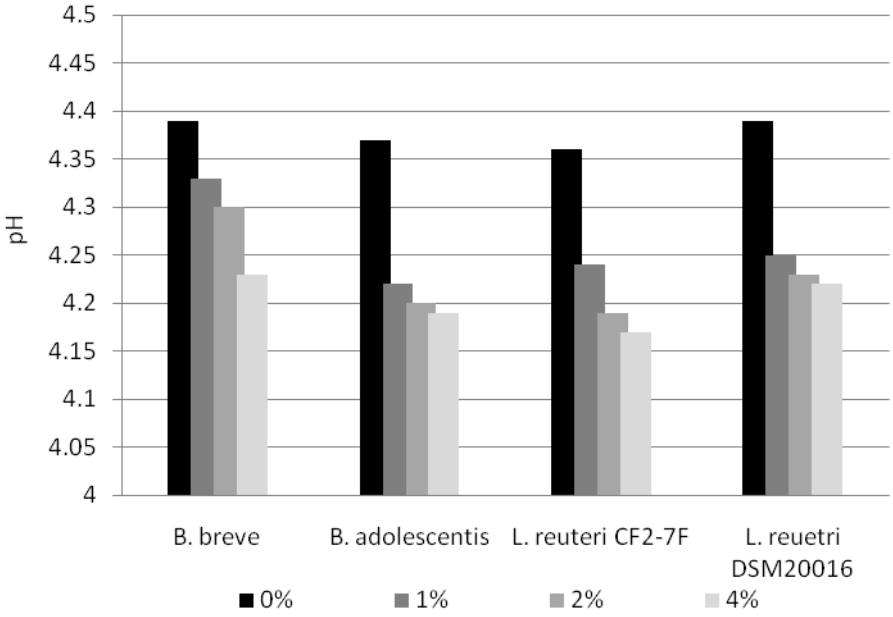


Figure 3.2. Stimulatory effect of shiitake mushroom extract on pH variation in MRS culture media after 8 hour incubation

The strains that produced more acid were *L. reuteri* DSM20016 and *L. reuteri* CF2-7F and *B. adolescentis*. *B. breve* had lower amount of acid production when supplemented with mushroom extract. The data showed over time intervals strains *L. reuteri* DSM20016 and *L. reuteri* CF2-7F had higher amounts of acid production versus control samples not supplemented

with mushroom extract. Lactic acid bacteria contain specific enzymes that are able to hydrolyze sugars. The continuous fermentation activity of lactic acid bacteria gradually produced more acid in the medium.

3.3.3. Titratable acidity. During initial period there were minimal amounts of acid production. As time of incubation increased, there was an increase in the amount of acid produced in all strains. Strains supplemented with 4% of mushroom extract had higher amounts of acid production, while strains supplemented with 1 and 0% of mushroom extract had minimal acid production (P<0.001) (Figure 3.3).

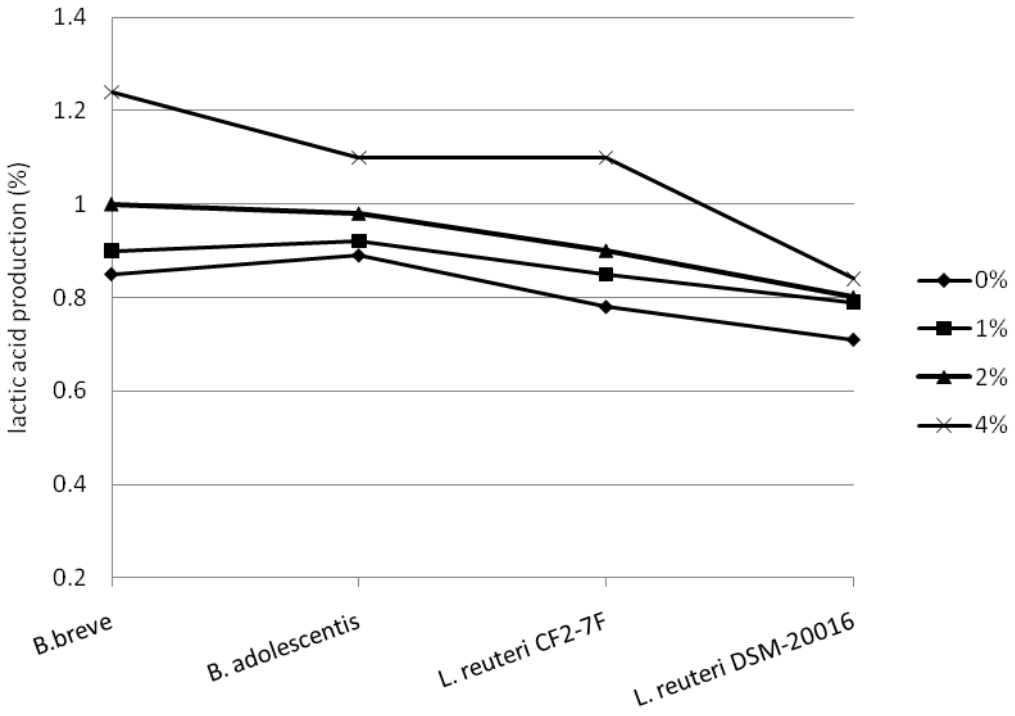


Figure 3.3. Stimulatory effect of shiitake mushroom extract on lactic acid production in MRS culture media after 8 hours incubation

3.3.4. Bacterial enumeration. The growth rate of all strains used was enhanced with an increase in the concentration of mushroom extraction in MRS broth samples (P<0.001). Addition of mushroom extract at 2 and 4% were most effective in enhancing growth of all four strains

used in this study compared to control sample (without mushroom extract). The highest concentration (4%) of mushroom extract was the most effective on stimulate the growth of bifidobacteria and *Lactobacilli* (Table 3.3). The effect of shiitake mushroom extract on the growth rate of both *Bifidobacterium* (*adolescentis* and *breve)* and *Lactobacillus retueri* (DSM20016 and CF2-7F) was dependent on strain used and concentration of shiitake mushroom extract in the growth medium.

Table 3.3

Bacterial counts (log cfu/ml) of L. reuteri and Bifidobacterium spp grown in MRS culture media with different shiitake extract concentrations

MRS + shiitake extract	***B. breve***	***B. adolescentis***	***L. reuteri CF-2F***	***L. reuteri DSM20016***
0%	8.70[a]	7.63[a]	8.90[a]	8.23[a]
1%	8.88[a]	7.74[b]	9.00[b]	8.36[b]
2%	8.94[b]	7.79[b]	9.08[b]	8.37[b]
4%	8.99[c]	7.83[c]	9.13[c]	8.43[c]

[abcd] Means with different superscripts in the same column indicate significant differences ($P<0.05$)

3.4. Conclusions

Our results indicated that shiitake mushroom extract could enhance the growth of lactic acid bacteria and bifidobacteria. The growth rate of all strains used was improved with an increase in concentration of SME in MRS broth. The strains that produced high population after 8 h of incubation at 37°C were *L. reuteri* CF2-7F, *B.breve* and *L. reuteri* DSM20016 (9.13, 8.99 and 8.43 log CFU/ml respectively). This may suggest the potential use of shiitake mushroom as a functional food product to promote the growth of probiotic strains in the human intestinal tract. Further research is needed to test efficacy in combination of shiitake mushroom extract with probiotic bacteria as a dietary supplement.

3.5 Acknowledgments

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CHAPTER 4[1]

Growth and Viability of *Lactobacillus reuteri* and *Bifidobacterium* in Skim Milk in the Presence of Shiitake Mushroom Extract during Refrigerated Period

4.1. Introduction

Lactic acid bacteria and bifidobacteria have been used as a functional food for human consumption with the purpose of enhancing human health. These bacteria called probiotics, defined as living organisms, when used in certain amounts, confer health benefits to the host (FAO, 2001). Dairy products are commonly used as a vehicle to carry probiotic bacteria. Recently, a number of research work showed significant health effects due to regular consumption of bacterial cultures, including lactic acid bacteria (Fonden, Mogensen, Tanaka, & Salminen, 2000). Therefore, due to the health promoting effects of lactic acid bacteria, there is an increasing interest in incorporating lactic acid bacteria and probiotics into milk and milk products (Broekart & Walker, 2006). The growth and viability of lactic acid bacteria are key factors required for developing probiotics in milk. The amount of bacterial cells required to produce a functional food is not well known and might vary as a characteristic of the strain and the health effect desired. A minimum level of more than 10^6 viable probiotic bacteria per gram of food product is accepted (Ouweh & Salminen, 1998). Lactic acid bacteria are fastidious organisms and regular certain growth factors. In previous studies, investigators examined various supplements that stimulated the growth of lactic acid bacteria in artificial media and cow's milk (Poch & Bezkorovainy, 1988; Ibrahim & Bezkorovvainty, 1994; Rada, 1997; Farnworth, Mainville, Desjardins, Gardner, & Champagne, 2006). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or

[1] Parts of this chapter were adapted from: Hassan, O.A., Ibrahim S.A., Song D., Isikhuemhen, O., Shahbazi, A., & AbuGhazaleh, A.A. *International Probiotics and Prebiotics* (2011). (*submitted).*

activity of one or a limited number of bacteria in the colon and improve host health (Gibson & Roberfroid, 1995). The production of lactic acid allows efficient metabolism in the gastric environment of the gut, which contains high levels of acidic bile and pancreatic enzymes (Parvez et al., 2006; Camilleri, 2006).

Recently, there has been more concern on a combination of prebiotics and probiotics in many dairy products (Hammes & Hertel, 2002). Using shiitake mushroom and beneficial bacteria for some valuable foods and health enhancing effects is a very unique idea of Varagas and Ohash (1996). Shiitake mushroom (*Lentinus edodes* (Berk.) Singer) extracts contain mono and polysaccharides (Kawakami et al., 2004), and is considered as a prebiotic (Wasser, 2002; Guo, 2004). Probiotics growth is stimulated by oligosaccharides and polysaccharides similar to those found in mushrooms (Varagas & Ohashi, 1996). In this study, we investigated the effect of shiitake mushroom extract on the viability and survival of bifidobacteria and lactobacilli in skim milk during refrigerating storage.

4.2. Materials and Methods

4.2.1. Samples preparation and treatment. Skim milk was obtained from local market and sterilized at 116ºC for 10 min. To prepare each sample, 100 µL of shiitake mushroom extract at different concentrations (0, 1, 2, and 4%) active strains were added to skim milk (9.8 mL) previously inoculated (Table 3.1), and then kept at 4 ºC for four weeks (Alazzeh et al., 2009). Experiments were conducted to determine bacterial count, pH, and titratable acidity of the skim milk samples at one week intervals during storage at 4ºC for four weeks.

4.2.2. Bacterial enumeration. Samples (1 mL) were withdrawn weekly for four weeks and serially diluted in 1% peptone water. Appropriate dilutions were surface plated onto MRS

agar plates. Bacterial colonies were counted after incubation at 37°C for 48 hrs to determine bacterial population.

4.2.3. Determination of pH. The pH values were measured at one week intervals for up to 4 weeks. The pH meter (model 410A; Orion, Boston, MA) was calibrated with fresh pH 4.0 and 7.0 standard buffers. The pH values of the samples were recorded and the electrode was rinsed with distilled water in between measurements of each sample.

4.2.4. Lactic acid production. The amount of lactic acid production was determined after mixing the sample of appropriate concentration of shiitake mushroom and bacterium samples with 9 mL of distilled water. Samples were titrated with 0.1 N NaOH to the endpoint of pH 8.6 using a pH meter and titrator. The total of titratable acidity was expressed in term of lactic acid (%) according to the method used by Vargas & Ohashi (1996).

4.2.5. Enzyme assay.

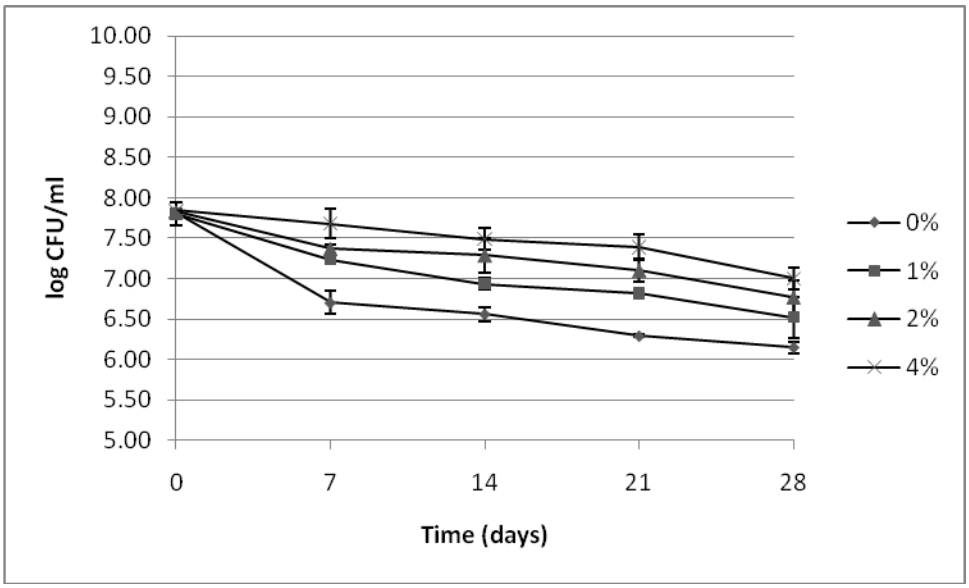
4.2.5.1. α-Galactosidase assay. α-Galactosidase activity was assayed according to the Food Chemicals Codex (2003) protocol. α-Galactopyranoside activity was tested by adding 1 mL of p-nitrophenyl-α-D-galactopyranoside substrate to 0.5 mL of each sample and transfer all the samples into a shaker water bath at 37ºC for 15 minutes. All reactions were stopped by adding 2.5 mL of borax buffer. A blank for each sample was prepared by sequentially adding 0.5 mL sample, 2.5 mL borax buffer and 1 mL of substrate solution. Spectrophotometer (Model Genesys 10 Vis, Thermospectronic, Rochester, NY, USA) were used to measure absorbance at 405 nm.

4.2.5.2. β-Galactosidase assay. β-Galactosidase activity was assayed according to the method described by Nagy et al. (2001). The reaction mixture composed of 0.5 mL of enzyme sample and 0.5 ml of 15 mM *o*-nitrophenyl-β-D-galactopyranoside in 0.03 M sodium phosphate buffer (pH 6.8) was maintained on a shaker water bath at 37^{o}C for 10 minutes, and then 2.0 mL

of 0.1 M sodium carbonate were added to stop the reaction. The optical density for each of the samples was measured at 420 nm with spectrophotometer (Model Genesys 10Vis, Thermospectronic, Rochester, NY, USA). Units of activity are expressed as micro-moles of *o*-nitrophenyl released per minute, 1U=1μmol/min (Hughes & Hoover, 1995).

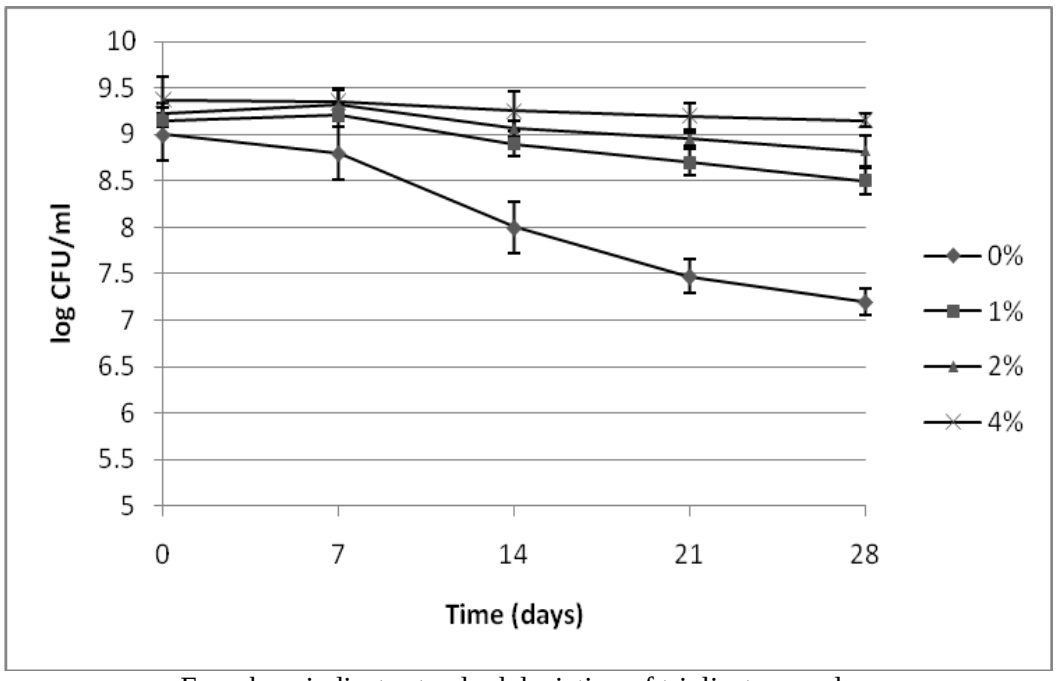
4.3. Results and Discussion

4.3.1. Bacterial enumeration. At initial storage period, the mean bacterial population among skim milk samples ranged from 7.5 to 9.38 log cfu/ml among all strains used in this study (Figures 4.1-4.4). The bacterial count ranged between 6.7 to 9.35 log cfu/ml for all samples at the 7 day storage period, showing a significant decrease among samples treated without or 1%, and stable among others treated with 2 or 4%. Comparison between strains showed that B. adolescentis did not grow as fast as other strains (Figure 4.1). Examining the results of strains for different concentration of mushroom extract showed that there were minimal differences between mushroom extract affecting the survival and viability of lactic acid bacteria (Figures 4.1-4.4). Adding mushroom extract at 2 and 4% concentration was most effective in enhancing the survival and viability of all the four strains used in this study compared to control samples. At the 28 day storage period, the bacterial population for 0, 1, 2, and 4% supplementation was 7.20, 8.50, 8.82, and 9.15 log cfu/ml respectively when *B. breve* was used (Figure 4.1). The highest concentration (4%) of mushroom extract was the most effective in extending the viability of all strains (Figures 4.1, 4.2, 4.3 and 4.4). Results showed that energy source is an essential factor for the growth of bifidobacteria and *L. reuteri* strains. The growth and viability of lactic acid bacteria was enhanced with an increase in the concentration of prebiotics, such as mushroom extract (Hassan et al., 2011; Desai, Powell & Shah, 2004).



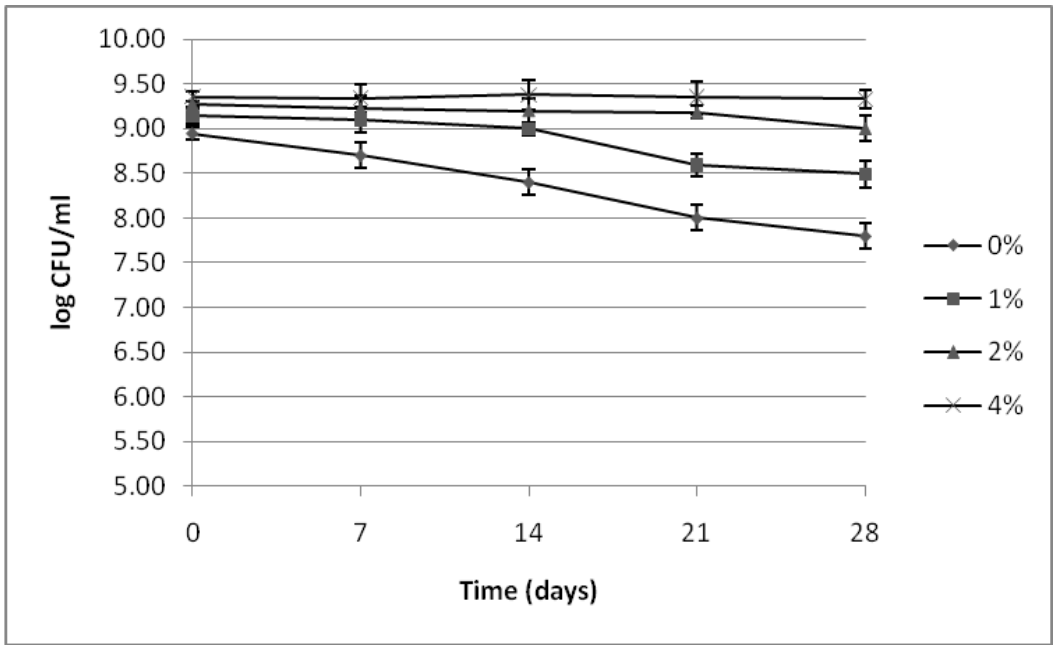
Error bars indicate standard deviation of triplicate samples.

Figure 4.1. Viability of *Bifidobacterium adolescentis* grown in skim milk containing different concentrations of shiitake mushroom after 28 days of refrigerated storage at 4°C



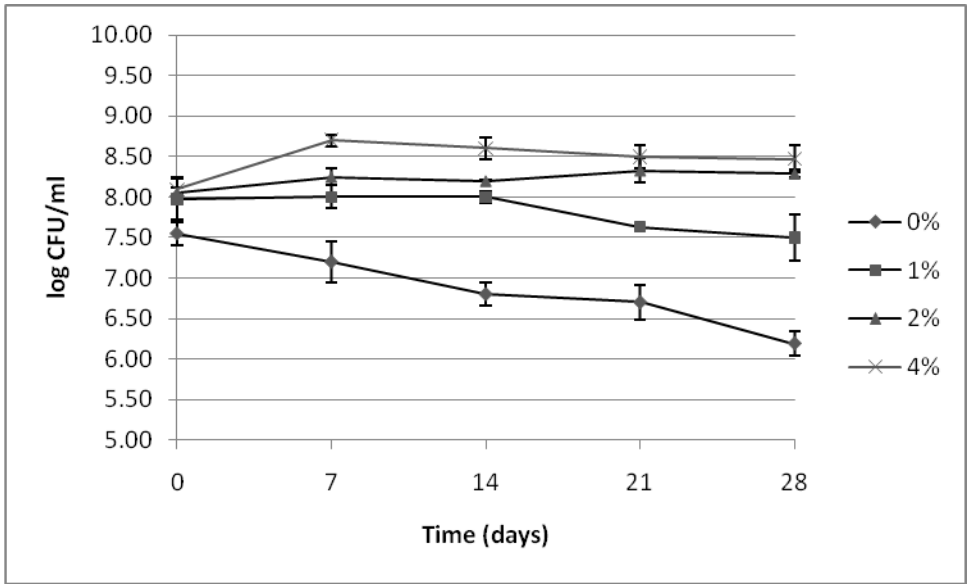
Error bars indicate standard deviation of triplicate samples.

Figure 4.2. Viability of *Bifidobacterium breve* grown in skim milk containing different concentrations of shiitake mushroom after 28 days of refrigerated storage at 4°C



Error bars indicate standard deviation of triplicate samples.

Figure 4.3. Viability of *Lactobacillus reuteri* CF2-7F grown in skim milk containing different concentrations of shiitake mushroom after 28 days of refrigerated storage at 4°C



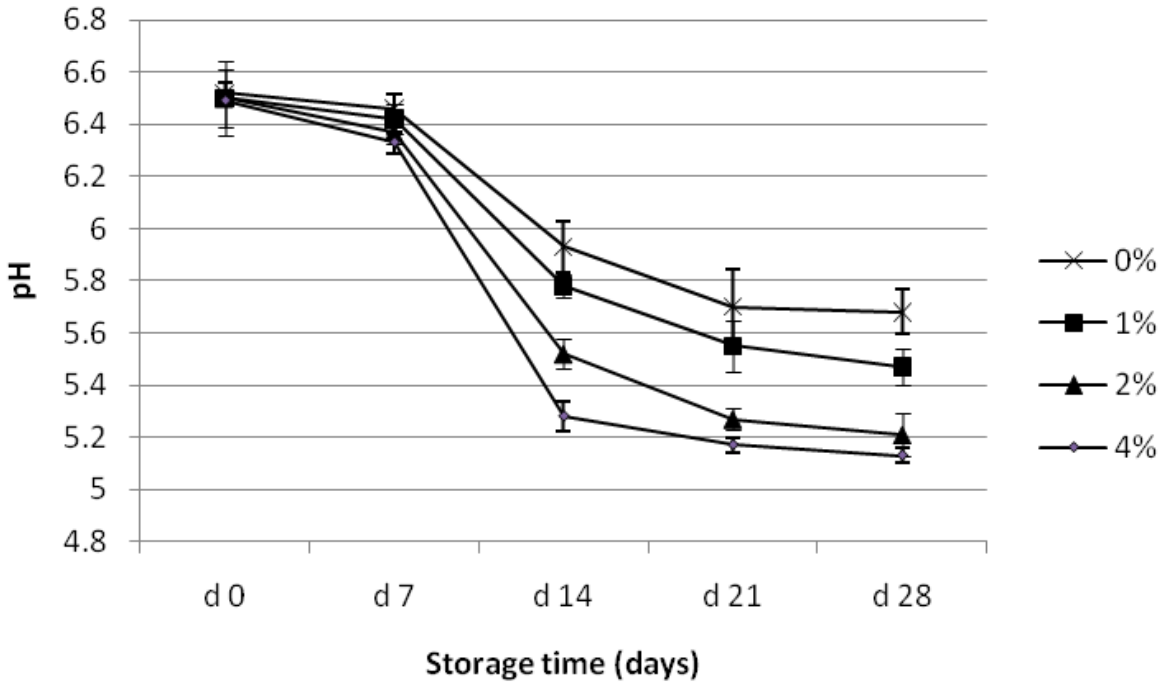
Error bars indicate standard deviation of triplicate samples.

Figure 4.4. Viability of *Lactobacillus reuteri* DSM 20016 grown in skim milk containing different concentrations of shiitake mushroom after 28 days of refrigerated storage at 4°C

In general, bifidobacteria and *L. reuteri spp*., grown on skim milk (without mushroom extract) reduced the survival and viability than those with mushroom extract (P<0.001).The effect of shiitake mushroom extract on the survival and viability of both *B.* (*adolescentis* and *breve*) and *L. reuteri* (DSM20016 and CF2-7F) was dependent on the strain used and the concentration of shiitake mushroom extract added to the storage medium. However, the viability and survival were significantly higher (P<0.05) than that of control, when no prebiotics were used, which could be attributed to enriched carbon, nitrogen and other mineral in the samples with mushroom extract.

4.3.2. Determination of pH. In this study the growth and acid production of *L. reuteri* strains and *Bifidobacterium spp*. were measured. Readings were taken over 28 days at 7 days intervals (0, 7, 14, 21, and 28 days). During the initial incubation periods of 0 and 7 days there was drop in pH ranging from 6.51-6.3 (Figures 4.5-4.8). As time increased, there was an increase in the amount of acid produced in each *L reuteri* and *Bifidobacterium* strains. Samples containing the appropriate amount of mushroom extract showed high amounts of acid production over time, while in contrast, control samples that contained no mushroom extract showed minimal acid. Mushroom extract concentration at 2 and 4% yielded high amounts of acid production, while 0 and 1% had minimal acid production in comparison to other concentrations used in this study (Figures 4.5, 4.6, 4.7, and 4.8). The most prevalent amount of acid production was observed in strain *L. reuteri* CF2-7F and DSM 20016 (Figures 4.5 and 4.6). *B. breve* and *adolescentis* were sensitive to acid production in the presence of mushroom extract (Figures 4.7 and 4.8). The results provided in Figures 4.5, 4.6, 4.7 and 4.8 are the mean of triplicate pH measurements for mushroom extract with skim milk samples. As indicated in these figures, milk with different prebiotic concentrations required different fermentation times to reach lowered

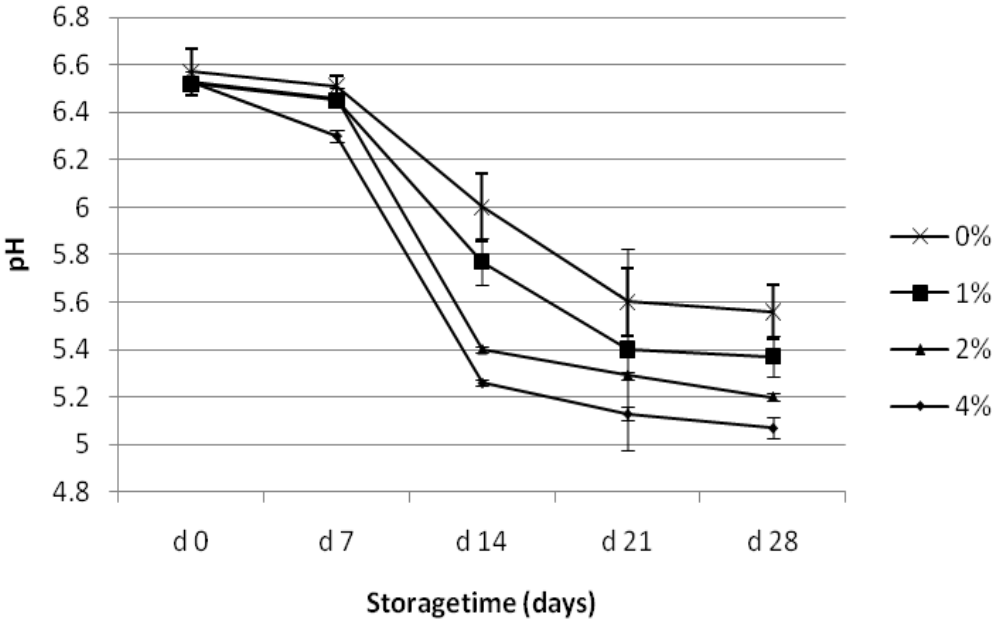
pH. Data displayed in Figures 4.5, 4.6, 4.7 and 4.8 showed that over time all strains produced higher amounts of acid when supplemented with mushroom extract versus control samples, and this dropped pH to the range of 5.0-5.13. Although there was high amount of growth observed, statistical analysis showed that there was a statistically significant difference in acid production of *L. reuteri* ($P<0.05$). *L. reuteri* DSM 20016 showed a significant difference in acid production between control samples and those containing mushroom extracts.



Error bars indicate standard deviation of triplicate samples.

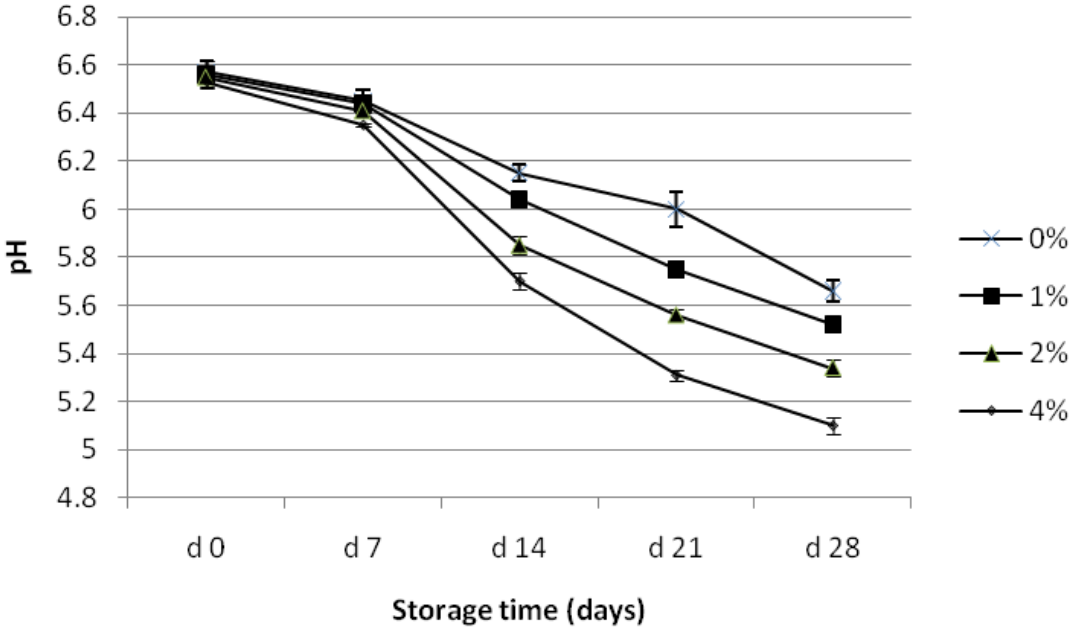
Figure 4.5. pH change in skim milk with *Lactobacillus reuteri* CF2-7F and different concentrations of shiitake mushroom extracts stored at 4ºC during 28 days

4.3.3. Titratable acidity. The analytical study measured the percentage of lactic acid production of *L. reuteri* and bifidobacteria by titratable acidity. Figures 4.9-4.12 shows the acid production rankings for *L. reuteri spp.* and *Bifidobacterium spp.* used in this study by measuring the total percentage of lactic acid.



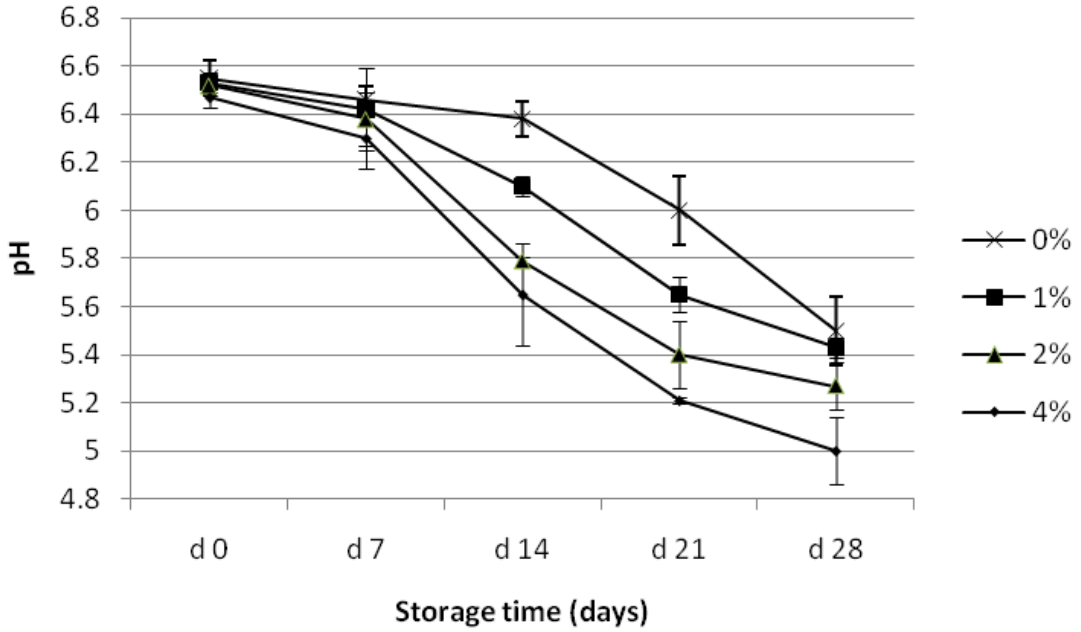
Error bars indicate standard deviation of triplicate samples.

Figure 4.6. pH change in skim milk with *Lactobacillus reuteri* DSM 20016 and different concentrations of shiitake mushroom extracts stored at 4ºC during 28 days



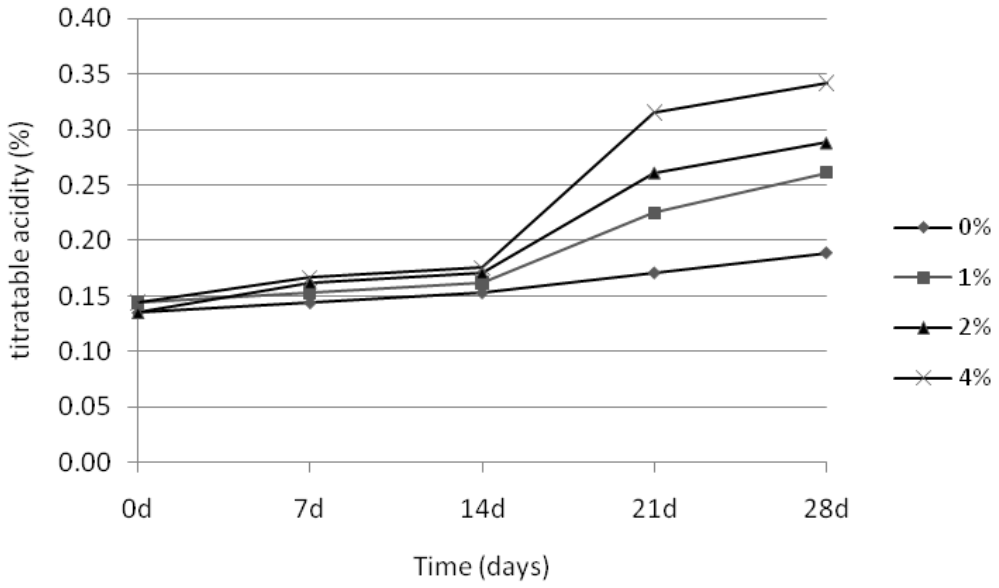
Error bars indicate standard deviation of triplicate samples.

Figure 4.7. pH change in skim milk with *Bifidobacterium breve* and different concentrations of shiitake mushroom extracts stored at 4ºC during 28 days



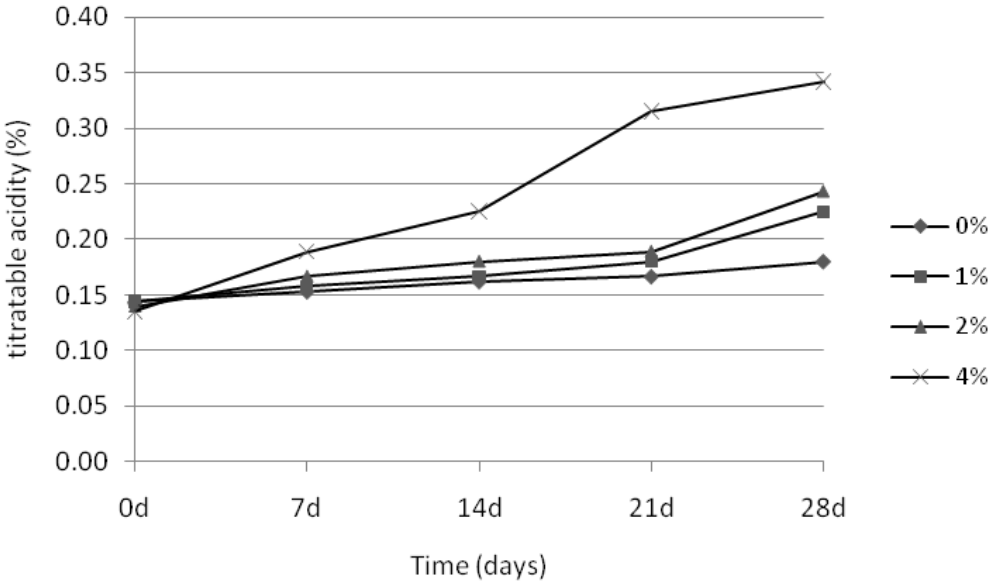
Error bars indicate standard deviation of triplicate samples.

Figure 4.8. pH change in skim milk with *Bifidobacterium adolescentis* and different concentrations of shiitake mushroom extracts stored at 4ºC during 28 days



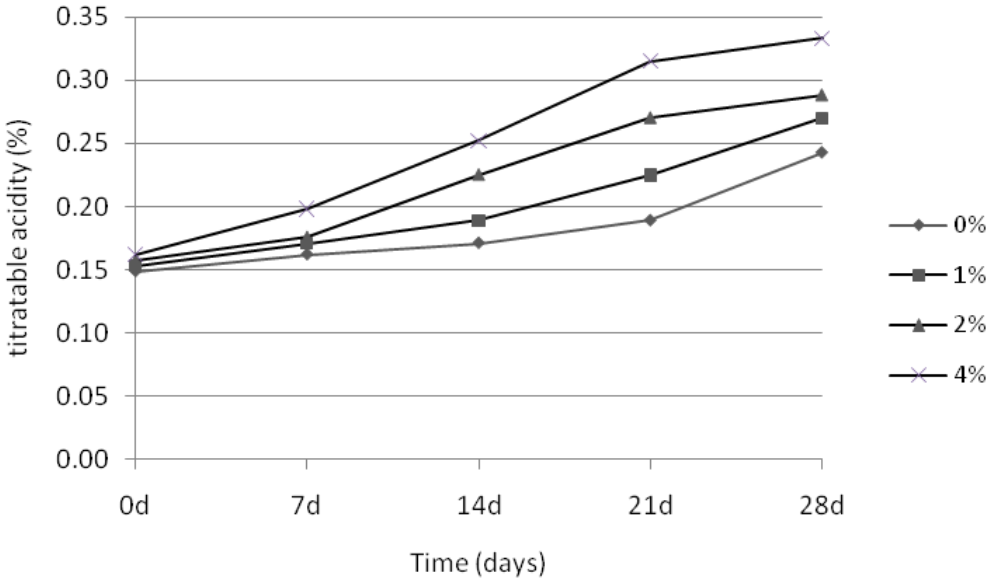
Error bars indicate standard deviation of triplicate samples.

Figure 4.9. Stimulatory effect of shiitake mushroom extract on lactic acid production by *Bifidobacterium breve* in skim milk after 28 days stored at 4ºC



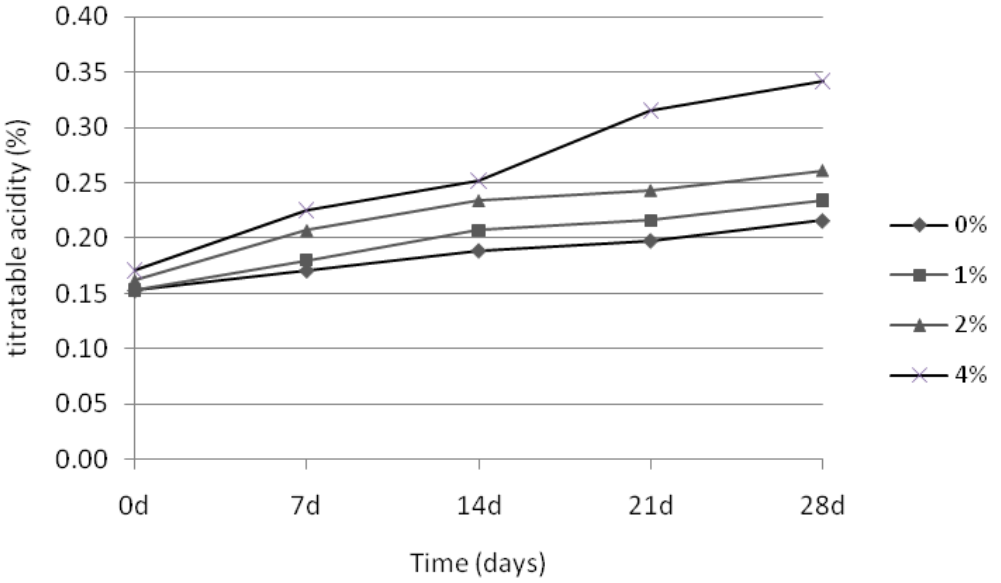
Error bars indicate standard deviation of triplicate samples.

Figure 4.10. Stimulatory effect of shiitake mushroom extract on lactic acid production by *Bifidobacterium adolescentis* in skim milk after 28 days stored at 4ºC



Error bars indicate standard deviation of triplicate samples.

Figure 4.11. Stimulatory effect of shiitake mushroom extract on lactic acid production by *Lactobacillus reuteri* CF2-7F in skim milk after 28 days stored at 4ºC



Error bars indicate standard deviation of triplicate samples.

Figure 4.12. Stimulatory effect of shiitake mushroom extract on lactic acid production by *Lactobacillus reuteri* DSM 20016 in skim milk after 28 days stored at 4ºC

Readings of titratable acidity were taken over 28 days at five 7 days intervals. During the initial time intervals, there was minimal amount of lactic acid production. As storage time increased, there was an increase in the amount of lactic acid produced in each *L. reuteri spps* and *Bifidobacterium spp*. Samples supplemented with shiitake mushroom extract (2 and 4%) had high amounts of lactic acid production ranging from 0.24-34% by the end of 28 days, while strains supplemented with the 0 and 1% had minimal acid production of 0.14-27% (see Figures 4.9-4.12).

The strains that produced the most lactic acid were DSM 20016 and CF2-7F. *Bifidobacterium breve* and *adolescentis* had low amounts of acid production when supplemented with shiitake mushroom extract. The data showed that over time, strains DSM 20016 and CF2-7F produced higher amounts of lactic acid versus control samples not supplemented with mushroom extract.

There were high amounts of growth observed, and statistical analysis showed a significant statistical difference in acid production of *L. reuteri* strains ($P<0.05$) than *Bifidobacterium spps*. *L. reuteri* DSM 20016 showed a significant difference in acid production between samples supplemented with mushroom extract and the control ($P<0.05$), (Figures 4.9-4.12). In addition, data showed that there was a statistically significant difference between strains. All strains supplemented with mushroom extract had generated more lactic acid production than control ($P<0.01$). Strains supplemented with 2 and 4% of shiitake mushroom extract observed high levels of acid production whereas strains supplemented with 0 and 1% of mushroom extract had less acid production in comparison to other concentrations (see Figures 4.9-4.12).

Generally, in this study the survival of probiotics was improved by the addition of mushroom extract to skim milk. This study agrees with the previous investigation that indicated shiitake mushroom extract has improved the bioavailability of minerals such as calcium, magnesium and iron and they increase the absorption of calcium and magnesium in the large intestine (Frank, 2000; Scholz-Ahrens, Schaafsma, Vanden Heuvel, E. G. & Schrezenme, 2001). Use of polysaccharides and monosaccharide in the diet on daily basis produce substantial changes in the intestinal microflora and increase the faecal bifidobacteria count and reduce the potential pathogens such as *Clostridium, E. coli* (Gibson & Roberfroid, 1995).

4.3.4. Enzyme activity. β-Galactosidase and α-galactosidase activities varied considerably among the strains used in this study. The magnitude of the differences in the enzyme activity, between *Bifidobacterium* and *Lactobacillus*, was varied. Bifidobacteria exhibited unique characteristics in its growth in skim milk with mushroom extract. β-Galactosidase and α-galactosidase activities were detected in all strains. β-Galactosidase activity

of *Bifidobacterium spp*., was much greater than that of *Lactobacillus spp.* During refrigerated storage, there was a significant reduction in enzyme activity of all strains depending on the amount of mushroom extract added to the sample. Samples treated with 2 and 4% of mushroom extract showed higher amount of enzyme activity and stability during incubated time at 4ºC.

4.3.4.1. α-Galactosidase assay. Results showed that *L. reuteri* strains DSM20016 and *B. adolescentis* (1.8 and 1.6, Gal U/ml, respectively) (Table 4.1) had a significantly higher ($P < 0.05$) α-galactosidase activity than *Bifidobacteria breve* and *L. reuteri* CF2-7F (1.3 and 1.25 Gal U/ml, respectively) on the first week when 4% of shiitake mushroom extract was used. Generally, all strains used in this study showed stability and significant production of α-galactosidase activity until the fourth-week when 2 and 4% of shiitake extract was used.

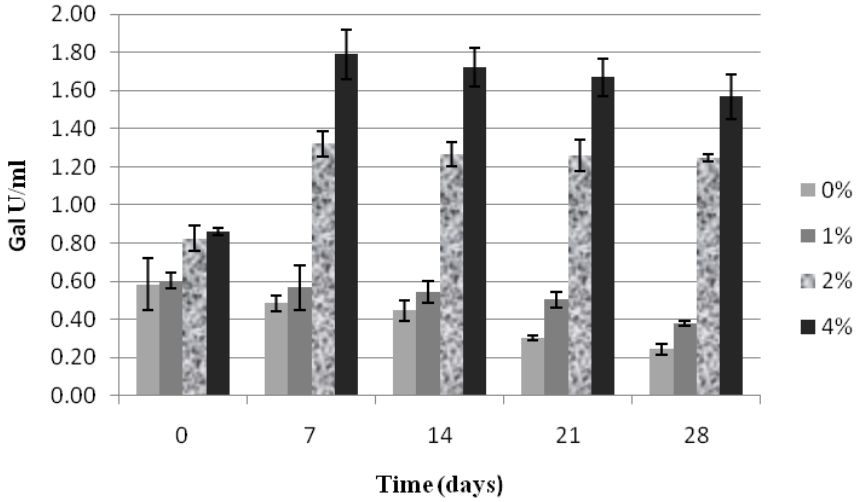
Table 4.1

The effect of mushroom extract on α-galactosidase activity by Bifidobacterium spp and Lactobacillus reuteri during storage at 4ºC for 28 days

	day 7				**day 28**			
Shiitake Extract	**DSM**	**CF2**	***B. breve***	***B. adolescentis***	**DSM**	**CF2**	***B. breve***	***B. adolescentis***
0%	0.48±.03	0.66±.03	0.84±.01	0.51±.03	0.24±.03	0.48±.01	0.51±.05	0.24±.01
1%	0.57±.12	0.69±.03	0.90±.01	0.63±.03	0.38±.01	0.57±.06	0.87±.01	0.33±.05
2%	1.32±.07	0.94±.02	1.10±.03	1.14±.10	1.25±.02	0.96±.01	1.20±.01	1.05±.12
4%	1.79±.13	1.31±.07	1.24±.17	1.64±.25	1.57±.12	1.27±.01	1.30±.01	1.49±.10

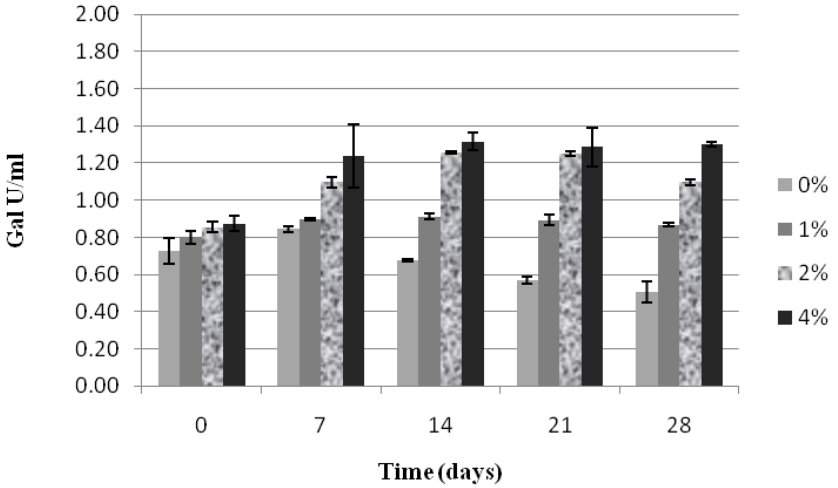
Figures 4.13-4.16 show α-galactosidase activity production of each strain in combination with shiitake mushroom extract. On the first week of storage at 4ºC, as time increased, there was stability in the amount of α-galactosidase activity in all strains when supplemented with the appropriate concentration of shiitake mushroom extract. The highest amount of α-galactosidase

activity was observed on 28th day when 4% of mushroom extract (1.57 Gal U/ml) was used. Strains supplemented with 0 or 1% of mushroom extract had minimal α-galactosidase activity (0.24 and 0.33 Gal U/ml respectively) in comparison to the 2 and 4% concentration used in this study.



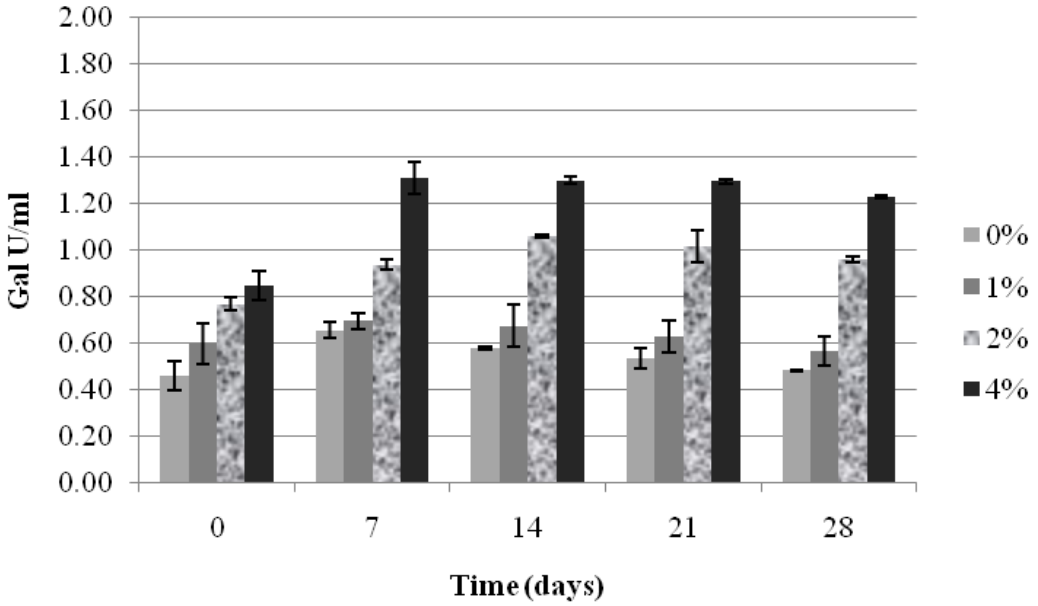
Error bars indicate standard deviation of triplicate samples.

Figure 4.13. α-Galactosidase activity (Gal U/ml) of *L. reuteri* DSM 20016 in skim milk containing different concentrations of shiitake mushroom extract



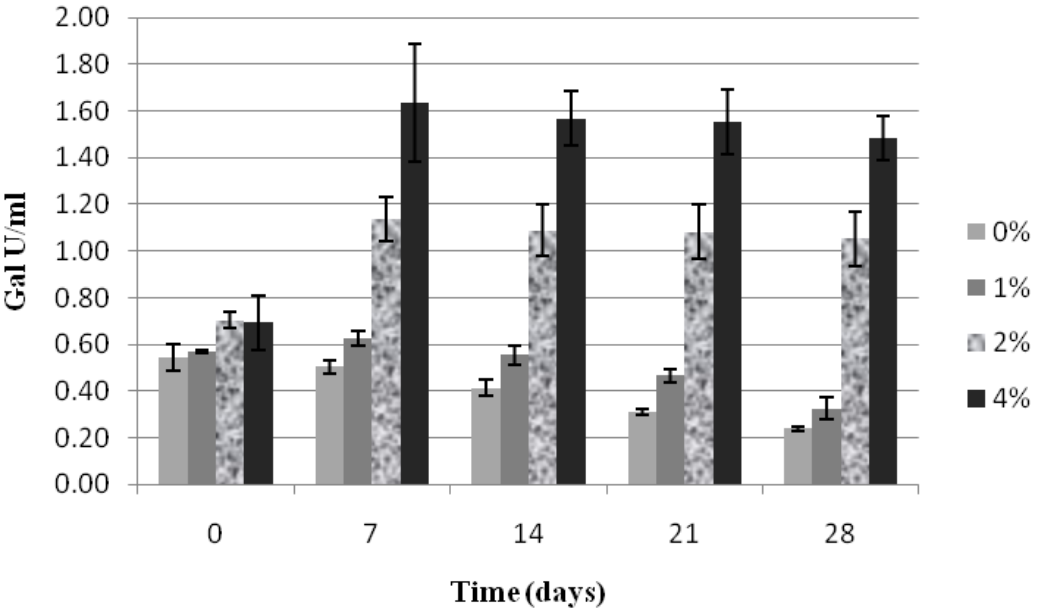
Error bars indicate standard deviation of triplicate samples.

Figure 4.14. α-Galactosidase activity (Gal U/ml) of *Bifidobacterium breve* in skim milk containing different concentrations of shiitake mushroom extract



Error bars indicate standard deviation of triplicate samples.

Figure 4.15. α-Galactosidase activity (Gal U/ml) of *Lactobacillus reuteri* CF2-7F in skim milk containing different concentrations of shiitake mushroom extract



Error bars indicate standard deviation of triplicate samples.

Figure 4.16. α-Galactosidase activity (Gal U/ml) of *Bifidobacterium adolescentis* in skim milk containing different concentrations of shiitake mushroom extract

Our results and that of Alazzeh et al. (2009) are comparable. Alazzeh et al. (2009) found that the α-galactosidase activity of *Lactobacillus reuteri* was in the range of 0.92-10.55 Gal U/ml with the highest enzymatic activity when different carbohydrates sources were used. Garro et al. (2004) also reported that raffinose in a high pH environment leads to an increase of α-galactosidase activity in *Bifidobacterium longum.* This was expected since α-galactosidase hydrolyzes the α-galactoside bonds in oligosaccharides such as raffinose. Shiitake extract rich in carbohydrate, such as arabinose, glucose, trehalose and raffinose (Hassan et al., 2011).

4.3.4.2. β-Galactosidase assay. *L. reuteri* strains had generally higher β-galactosidase activity than bifidobacteria (see Table 4.2 and Figures 4.17-4.20). DSM20016 and CF2-7F had significantly higher ($P < 0.05$) β-galactosidase activity (5.39 and 4.8 Gal U/ml, respectively), than *B. breve* and *B. adolescentis* (4.36 and 3.7 Gal U/ml, respectively).

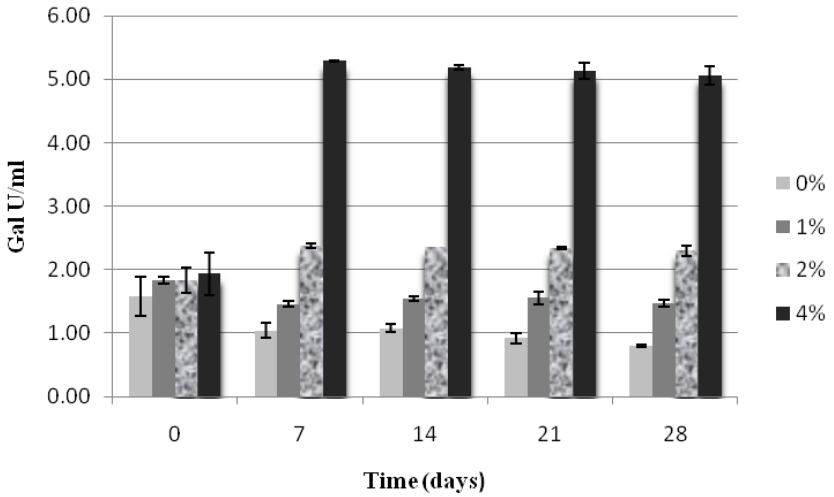
Table 4.2

The effect of mushroom extract on β-galactosidase activity by Bifidobacterium spp and Lactobacillus reuteri during storage at 4ºC for 28 days

	day 7				day 28			
Shiitae Extract	**DSM**	**CF2**	***B. breve***	***B. adolescentis***	**DSM**	**CF2**	***B. breve***	***B. adolescentis***
0%	1.04±.12	0.79±.0	2.22±.05	1.91±.06	0.80±.02	0.56±.06	1.70±.11	1.72±.15
1%	1.46±.04	0.93±.17	2.39±.0	2.18±.04	1.47±.06	0.77±.02	2.12±.03	1.93±.04
2%	2.38±.04	3.84±.18	3.30±.02	2.87±.07	2.30±.07	3.54±.2	3.20±.13	2.77±.08
4%	5.29±.01	4.8±.22	4.15±.04	3.82±.15	5.06±.15	4.93±.03	4.06±.13	3.73±.17

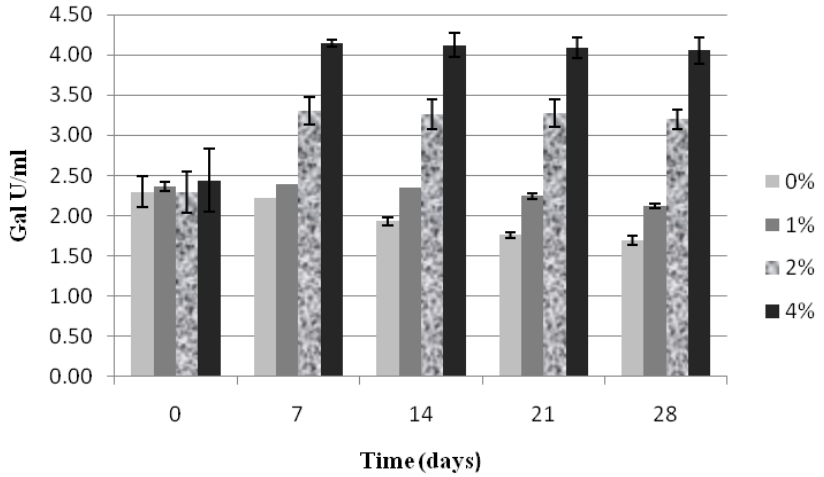
Several studies have investigated the possibility of increasing the activity of α- and β-galactosidases in different bacterial strains. Dumortier, Brassart, and Bouquelet (1994), observed that β-galactosidase activity of *B. bifidum* occurred at higher temperature. Others got it back to the differences in cell mass, cell growth, or variable number of cell per gram of culture (Occhino,

Morris & Savaiano, 1986; Hsu, Yu & Chou, 2005). Hsu et al. (2005) indicated that carbohydrate and nitrogen source, pH of the media, and mineral additive have a significant effect on increasing the β-galactosidase activity in bifidobacteria.



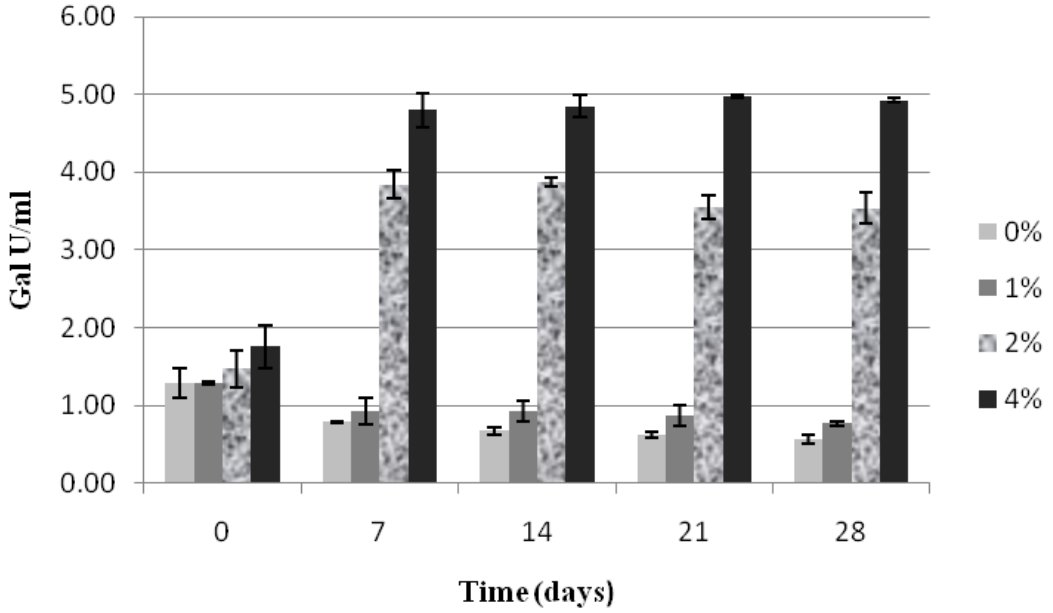
Error bars indicate standard deviation of triplicate samples.

Figure 4.17. β-Galactosidase activity (Gal U/ml) of *Lactoacillus reuteri* DSM 20016 in skim milk containing different concentrations of shiitake mushroom extract



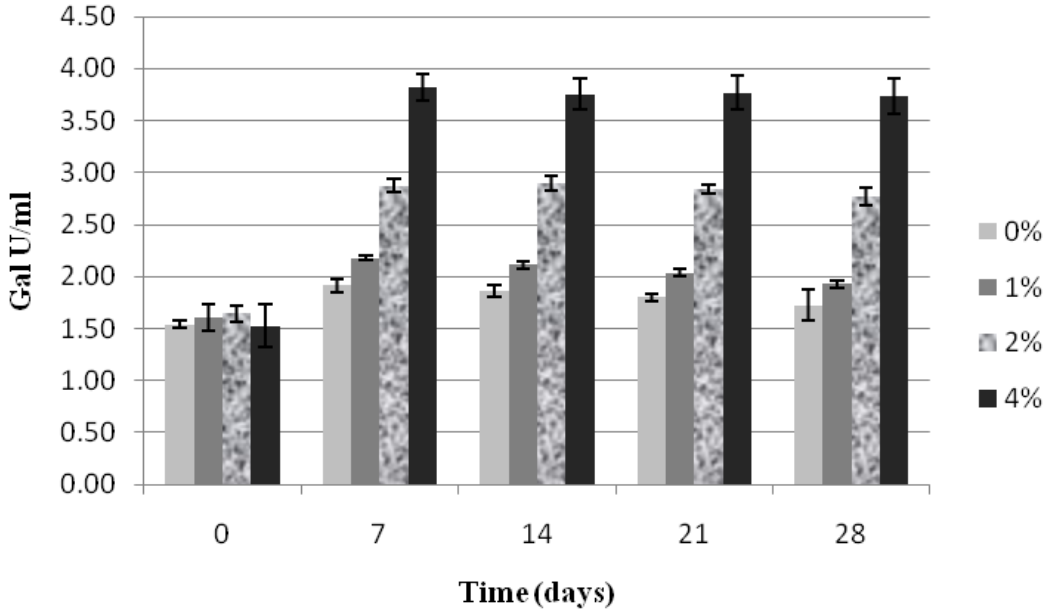
Error bars indicate standard deviation of triplicate samples.

Figure 4.18. β-Galactosidase activity (Gal U/ml) of *Bifidobacterium breve* in skim milk containing different concentrations of shiitake mushroom extract



Error bars indicate standard deviation of triplicate samples.

Figure 4.19. β-Galactosidase activity (Gal U/ml) of *Lactobacillus reuteri* CF2-7F in skim milk containing different concentrations of shiitake mushroom extract



Error bars indicate standard deviation of triplicate samples.

Figure 4.20. β-Galactosidase activity (Gal U/ml) of *Bifidobacterium adolescentis* in skim milk containing different concentrations of shiitake mushroom extract

The high α-galactosidase enzymatic activity of *L. reuteri* DSM20016 and *B. adolescentis* and the high β-galactosidsase activity DSM20016 and CF2-7F are promising and the conditions for growing these bacteria to obtain hyper productivity levels of these enzymes could be further investigated.

4.4. Conclusions

Adding SME at 2 and 4% to skim milk was most effective in enhancing the viability of all strains used in this study during 28 days of storage period at 4°C. Generally bifidobacteria and L. reuteri strains grown on skim milk without SME reduced the survival and viability than those with SME ($P<0.001$). The result showed that over time all strains produced higher amounts of acid when supplemented with SME compared to control samples, and this dropped pH to the range of 5.0 to 5.13. The strains that produced the most lactic acid were *L. reuteri* DSM20016 and CF2-7F whereas *Bifidobacterium breve* and *adolescentis* had low amounts of acid production when supplemented with SME.

L. reuteri DSM 20016 and *B. adolescentis* gave best α galactosidase activity when treated with 4% SME at 7 days (1.8 and 1.6 Gal U/ml, respectively) had significantly higher ($P<0.05$) than *Bifidobacterium breve* and *L. reuteri* CF2-7F (1.3 and 1.25 Gal U/ml, respectively). L.reuteri strains had generally higher β-galactosidase activity than *Bifidobacterium spp*. The results showed that L. reuteri strains grown in skim milk supplemented with SME provid good condition as good probiotic bacteri. This may suggest the potential use of shiitake mushroom as a functional food product to promote the growth of probiotic strains in the intestinal tract.

4.5 Acknowledgments

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CHAPTER 5[1]

Viability and α/β-Galactosidase Activities of *Bifidobacterium Breve* and *Lactobacillus Reuteri* DSM 20016 in Yogurt Products Supplemented by Shiitake Mushroom Extract during Refrigerated Storage

5.1. Introduction

The human gut contains over 500 different microbial species. The original microorganisms, which are the dominant microflora in the large intestine, reduce the ability of pathogenic genera including, *Salmonella, Clostridium, Escherichia* and *Campylobacter* to attach to the intestinal surface (Ziemer & Gibson, 1998). When the microbial balance is disturbed, intestinal bloating and diarrhea may occur. The popularity of probiotics has been increasing rapidly worldwide (Sanders, 2000; Sanders 2003; Rafter, 2004; Benkouider, 2004 (a, b); Kotilainen, Rajalahti, Ragasa, & Pehu, 2006). The probiotic genera of bifidobacteria and lactobacilli have been widely studied and established as valuable native inhabitants of the human intestinal tract. Probiotics are defined as live microbial feed supplements which beneficially affect the host by improving the intestinal microbial balance (Fuller, 1989). In the recent years, probiotics have caught the attention of the food industry (Saarela et al., 2002; Salminen & Gueimonde, 2004). Food industries are increasingly manufacturing different kinds of foods containing probiotic bacteria, which are called functional foods. Dairy products incorporated with probiotics such as yogurts containing *L. acidophilus* and *Bifidobacterium spp.* constitute a significant amount among the commercially available probiotic foods (Reid et al., 2003). However, in recent days, the increasing use of probiotic dairy products, especially probiotic yogurt, has attracted the market for probiotic yogurt and other foods where probiotics can be

[1] Parts of this chapter were adapted from: Hassan, O.A., Ibrahim S. A., Song D., Isikhuemhen, O. Shahbazi A., & AbuGhazaleh A. A. *Journal of Dairy Science* (2011). (*To be submitted).*

incorporated (Playne, 2002). Maintaining the growth and viability of probiotic bacteria in dairy products remains a major challenge to the fermented dairy producers because of the inability of many strains to grow well in milk. Therefore, there were many suggestions to use some nutrients to enhance the growth and survival of different probiotic strains.

Considerably viable probiotic organisms reduce or eliminate sickness such as colon infection, irritation, constipation and traveler's diarrhea. Also, other health benefits include inhibition of pathogenic bacteria, lowering of blood pressure, synthesis of B vitamins, cholesterol absorption, high of ammonia levels, and inhibition of tumor formation (Roberfroid, 2000; Ziemer & Gibson, 1998). However, in order to provide health benefits, it is essential that there is a minimum of 6 log cfu of viable probiotic organisms per gram of a product (Shah, 2002; IDF, 1992; Lourens, Viljoen, & Jooste, 2000; Lourens-Hattingh & Viljoen, 2001). Some reports have shown probiotic growth and survival numbers to be stable during the shelf life of the product (Dinakar & Mistry, 1994), others have cited a rapid decline in the number of viable probiotic bacteria over shelf life (Ibrahim & Carr, 2005; Stanton, Desmond, Fitzgerald, & Ross, 2003). Using prebiotic or hydrolyzed milk to improve the growth and survival of probiotic bacteria, especially bifidobacteria, the results showed a variety of effects depending on the strain and the dairy product used (Alkalın, Fenderya, & Akbulut, 2004). However, a limited number of studies have examined the effect of prebiotic on the growth and survival of *Bifidobacterium spp.*

Ibrahim and Carr (2006) reported that in many commercial yogurt brands, probiotic organisms were not viable after 3-4 weeks of storage at 4ºC. It has been known that using mushroom extract help in maintaining the viability of probiotics. Also, using mushroom extract in dairy products could help to carry out natural substrates with probiotics to reduce cell losses during processing and storage at low temperature (Hassan et al., 2011a).

The survival of probiotics including *L. bacillus returi* and *Bifidobacterium spp*. was improved by the addition of mushroom extract at 4% w/v to skim milk (Hassan et al., 2011b). Therefore, there is a need to study the effect of shiitake mushroom extract a prebiotic, to improve the viability of probiotic organisms in yoghurt during refrigerated storage. Gibson and his team proved that prebiotics showed resistance to gastrointestinal infection because of their stimulatory effect on *Bifidobacterium* spp., by producing several anti- microbial mechanisms (Gibson et al., 2005). Prebiotics can help to increase the beneficial bacteria in the gastrointestinal tract. Alazzeh et al. (2009) showed that *Lactobacillus reuteri* utilizes lactose, raffinose, and galactose besides glucose, and has the ability to metabolize oligosaccharides and the simple sugars. This ability is strain related, for example it has been found that *Bifidobacterium spp and L. reuteri* possessed the enzymes required to utilize some kind of sugar such as raffinose family and lactose (Martınez-Villaluenga & Gomez, 2007; Hassan et al., 2011b). A general increase in the beneficial bacterial population may not necessarily contribute to increased health effects, as it is strain related in this investigation, probiotic organisms were selected based on their ability to grow well and survive longer in yoghurt at 4ºC. The objective of this study was to investigate the effectiveness of mushroom extract, on the viability of *Lactobacillus reuteri* DSM20016 and *Bifidobacterium breve* after processing and storage of yoghurt for 35 days.

5.2. Materials and Methods

5.2.1. Production of freeze-dried starters. Four batches of de Man Rogosa Sharpe (MRS) broth were inoculated with each strain (Table 3.1) and were incubated for 24 h at 37°C. Samples were centrifuged at 8000 g for 15 min. Pellets were washed and re-suspended in peptone water to its original volume. The cells were inoculated at 5.0 log cfu/mL to skim milk and allowed to ferment overnight at 37°C. Yoghurt produced were transferred to the shell freezer

at −50°C for 2 h and lyophilized in a Labconco freeze-dryer at −55°C for 72 h under 0.040 Mbar pressure to produce freeze-dried starters.

5.2.2. Mushroom substrate. Mushroom extract at different concentration 0% (control) and 4% were added into skim milk. The substrates were heated at 85ºC for 10 min and then cooled to 37ºC.

5.2.3. Fermentation and storage. The mushroom substrate were inoculated with 5% (v/v) starter culture and incubated at 37ºC for 10 h. Yoghurt produced were stored at 4ºC for 5 weeks.

5.2.4. Viable cell enumeration. Enumeration of viable cells of *Lactobacillus reuteri* DSM 20016 and *Bifidobacterium breve* were performed through the estimation of colony forming unit on MRS agar plates after incubating at 37ºC for 48 h.

5.2.5. Determination of Ph and titratable acidity. The pH meter (model 410A, Orion, Boston, MA) was used for the measurement of pH. Titratable acidity was determined by titrating 10-mL samples with 0.1 N NaOH with an end point of pH 8.6 under constant stirring. Titratable acidity was recorded as the percentage equivalent of lactic acid.

5.2.6. Enzyme assay.

5.2.6.1. α-Galactosidase assay. α-Galactosidase activity was assayed according to the Food Chemicals Codex (2003) protocol. α-Galactopyranoside activity was tested by adding 1 mL of p-nitrophenyl-α-D-galactopyranoside substrate to 0.5 mL of each sample and transferring all the samples into a shaker water bath at 37ºC for 15 minutes. All reactions were stopped by adding 2.5 mL of borax buffer. A blank for each sample was prepared by sequentially adding 0.5 mL sample, 2.5 mL borax buffer and 1 mL of substrate solution. Spectrophotometer (Model

Genesys 10 Vis, Thermospectronic, Rochester, NY, USA) was used to measure absorbance at 405 nm.

5.2.6.2. β-Galactosidase assay. β-Galactosidase activity was assayed according to the method described by Nagy, Kiss, Szentirmai, & Biro (2001). The reaction mixture was composed of 0.5 mL of enzyme source and 0.5 ml of 15 mM *o*-nitrophenyl-β-D-galactopyranoside in ml of 0.03 M sodium phosphate buffer (pH 6.8). Reactions were maintained on a shaker water bath at 37ºC for 10 minutes, and then 2.0 mL of 0.1 M sodium carbonate was added to stop the reaction. The optical density for each of the samples was measured at 420 nm with spectrophotometer (Model Genesys 10Vis, Thermospectronic, Rochester, NY, USA). Units of activity was expressed as micro-moles of *o*-nitrophenyl released per minute, 1U=1μmol/min (Hughes & Hoover, 1995).

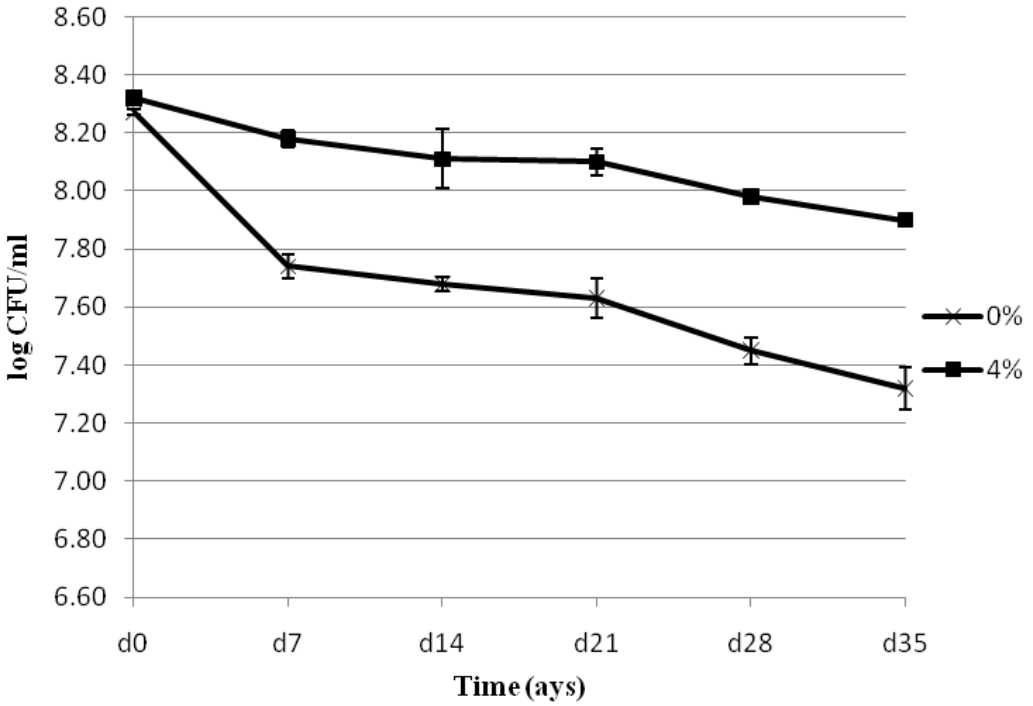
5.2.7 Statistical Analysis. Data analyses were concerned on determining if addition of shiitake mushroom to the yogurt products resulted in significantly affect the viability and enzymes activity of the control group. Statistical analysis of data was performed using the SAS General Linear Model program (1999). Least square means of triplicate samples were calculated and Duncan's multiple range test used to determined significant ($P<0.01$) differences.

5.3. Results and Discussion

5.3.1. Storage study. For the storage study yogurt samples processed using shiitake mushroom extract at different concentration levels (control, 1, 2, and 4%) were stored in the refrigerator at 4ºC and samples were pulled out at 0, 7, 14, 21, 28, and 35 d to determine the microbiological, and chemical quality.

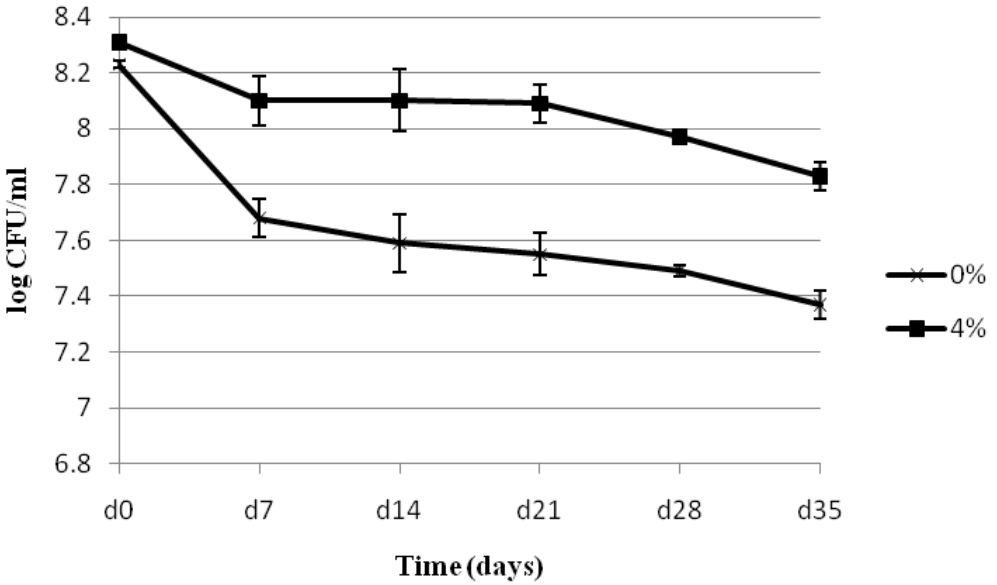
5.3.2. Determination of viability during refrigerating storage. Figures 5.1 and 5.2 show the viability of Lactobacillus reuteri DSM 20016 and Bifidobacterium breve in yogurt

samples prepared using different concentration of shiitake mushroom extract (SME) during 5th week of storage at 4°C. At initial time of storage, the mean bacterial population among the yogurt samples ranged from 8.15-8.62 log CFU/ml (Table 5.1 and Figures 5.1-5.2). The bacterial samples population in all the samples ranged between 7.72-8.20 log cfu/ml at first week storage period, showing a significant decreased (P<0.05) from the initial time among samples without SME (control) compared to other samples with SME (Figures 5.1-5.2). The bacterial population at 2nd and 3rd week showed stability within strains in yogurts with 4% of SME and in the same time decreased in the population stored without SME (0.20-0.30 log cfu/ml) (see Figures 5.1-5.2).



Error bars were taken of triplicate samples as standard deviation.

Figure 5.1. Viability of *Lactobacillus reuteri* DSM 20016 grown in yogurt containing 0 or 4% concentrations of shiitake mushroom extract after 35 days of refrigerated storage at 4°C



Error bars were taken of three samples as standard deviation.

Figure 5.2. Viability of *Bifidobacterium breve* grown in yogurt containing 0 or 4% concentrations of shiitake mushroom extract after 35 days of refrigerated storage at 4°C

The bacterial population for all samples at 4th weeks storage period, reduced subsequently. At 5th week storage period, the bacterial population for control (no SME added) and 4% of SME for *Bifidobacterium breve* and *Lactobacillus reuteri* DSM 20016 was 7.37, 7.95, 7.40 and 8.00 log CFU/ml respectively compared to their initial bacterial population of 8.23, 8.31, 8.24 and 8.33 log cfu/ml (Table 5.1).

Table 5.1

Determination of pH of Lactobacillus reuteri DSM 20016 and Bifidobacterium breve growth in yogurt with 0 and 4% of SME over 35 days refrigerated storage at 4ºC.

	***Lactobacillus reuteri* DSM 20016**		***Bifidobacterium breve***	
	Shiitake mushroom extract		**Shiitake mushroom extract**	
pH value	0%	4%	0%	4%
Initial	4.29±0.03	4.21±.02	4.39±.03	4.23±.03
Final	4.01±0.01	3.6±0.1	4.0±.03	3.6±.03

Note. Samples were taken in triplicate.

Generally, the bacterial population at 5th week storage period when compared to initial time, showed a significant decreased (P<0.05) in the control samples, while samples with 4% of SME showed stability in strains viability when stored at 4°C. The results suggested that prebiotic especially 4% SME in yogurt can stimulate the viability of *L. reuteri* DSM 20016 and *B. breve* in the fermented products and possibly in the gastrointestinal tract. The results indicated that the addition of 4% concentration of SME showed growth promoting effect of *Bifidobacterium spp*. and *Lactobacillus reuteri* strains in pasteurized skim milk compared to control (containing no prebiotics). Shin et al. (2000) and Akalin et al. (2004) indicated similar results where a maximum effect was observed when they used 5% of FOS or Inulin. This research indicated that growth, survival and activity of *L. reuteri spp* and *B. breve* at 4% concentration of SME in skim milk was beneficial, and these strains improved in the presence of SME compared to the control without prebiotics.

5.3.3. Change in pH of yogurt. Table 5.2 shows the initial pH and pH values after 35 days of storage. Figures 5.3 and 5.4 show the pH change over the period of fermentation for skim milk containing shiitake mushroom extract (SME) and in pasteurized skim milk cultured with *Lactobacillus reuteri* DSM 20016 and *Bifidobacterium breve*. The fermentation was carried under anaerobic conditions at 42ºC for all samples for 10 h, and then transferred to the refrigerator at 4ºC for 35 days. The results provided in Table 5.2 and Figures 5.3 and 5.4 are the mean of the triplicate pH measurements for SME samples. As indicated in Table 2, the samples with different prebiotic concentrations required different fermentation times. Data displayed in Figures 5.3 and 5.4 show the pH change over the fermentation period. As displayed in Figures 5.3 and 5.4, in the absence of SME, the decrease of in pH indicated an increase in bacterial growth and reached its maximum in about 35 days. For successful growth, bacteria require

appropriate sources of carbon, nitrogen and sulfur together with certain inorganic ions (Forrest & Walker, 1971; Payne, 1976).

Table 5.2 shows that the fermentation period decreased by increase in the concentration of the SME in the skim milk. The greatest effect was observed with the 4% SME in skim milk as it reached the lower pH in 35 days which decreased significantly ($P<0.05$) as compared to the control.

Table 5.2

Population of Lactobacillus reuteri DSM 20016 and Bifidobacterium breve grown in yogurt with 0 and 4% of SME over 35 days refrigerated storage at 4ºC

pH value	***Lactobacillus reuteri* DSM 20016**		***Bifidobacterium breve***	
	Shiitake mushroom extract		**Shiitake mushroom extract**	
	0%	4%	0%	4%
Initial	8.24±0.02	8.33±.01	8.23±.0	8.31±.01
Final	7.4±0.05	8.0±0.03	7.37±.01	7.95±.05

Note. Samples were taken in triplicate.

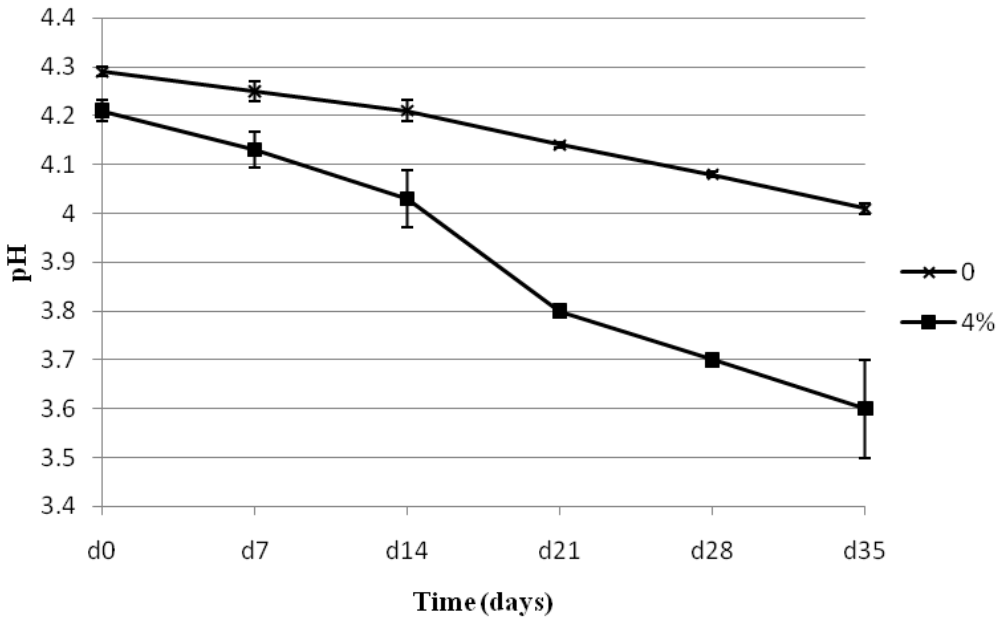
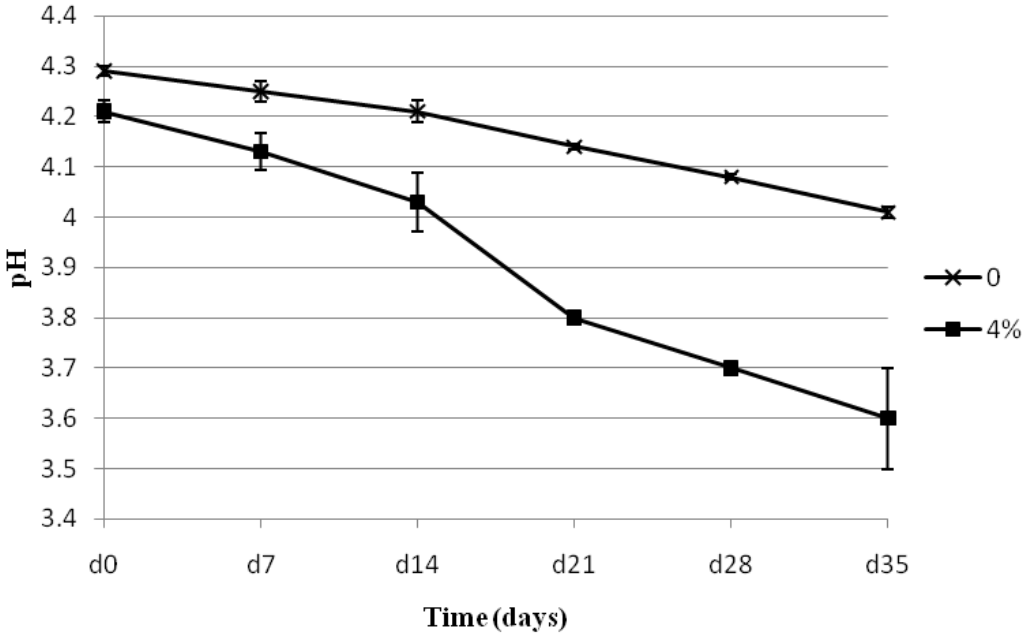


Figure 5.3. The pH change of *Lactobacillus reuteri* DSM 20016 in yogurt with different concentrations of shiitake mushroom extract stored at 4ºC during 35 days



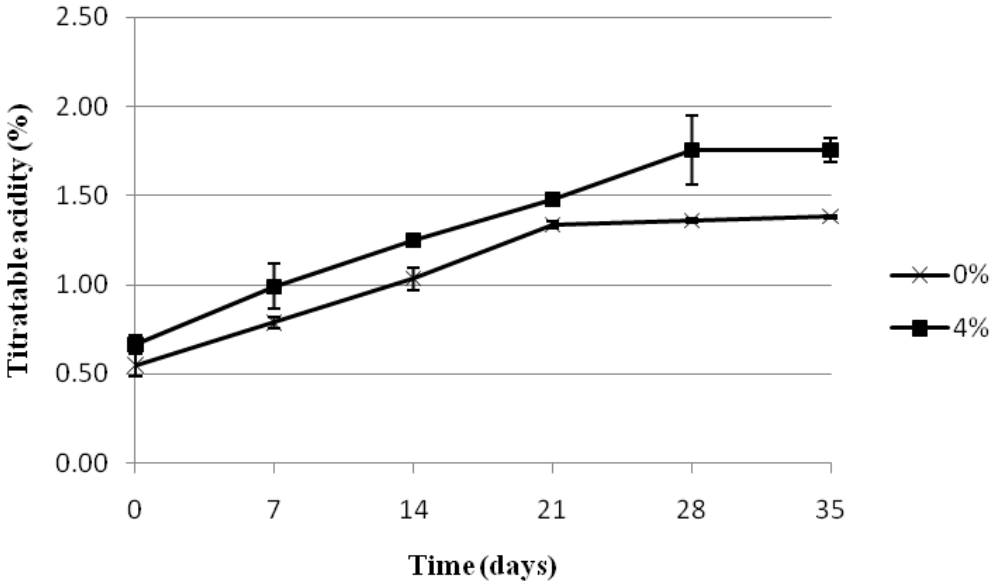
Error bars were taken of three samples as standard deviation.

Figure 5.4. The pH change of *Bifidobacterium breve* in skim yogurt with different concentrations of shiitake mushroom extract stored at 4ºC during 35 days

5.3.4. Titratable acidity. To study the effect of addition of shiitake mushroom extract on the bacterial growth of *Lactobacillus reuteri* and *Bifidobacterium breve*, a time course study of acid production was conducted at 42ºC in skim milk culture media for 10 h, with and without the addition of the mushroom extract, then the samples were transferred to the refrigerator at 4ºC for 35 days. According to Smith, Hillier, and Lees (1975), acidity values can be used as a measure of the lactic acid bacteria growth in milk. Reading was taken over 35 days at intervals of 7 days.

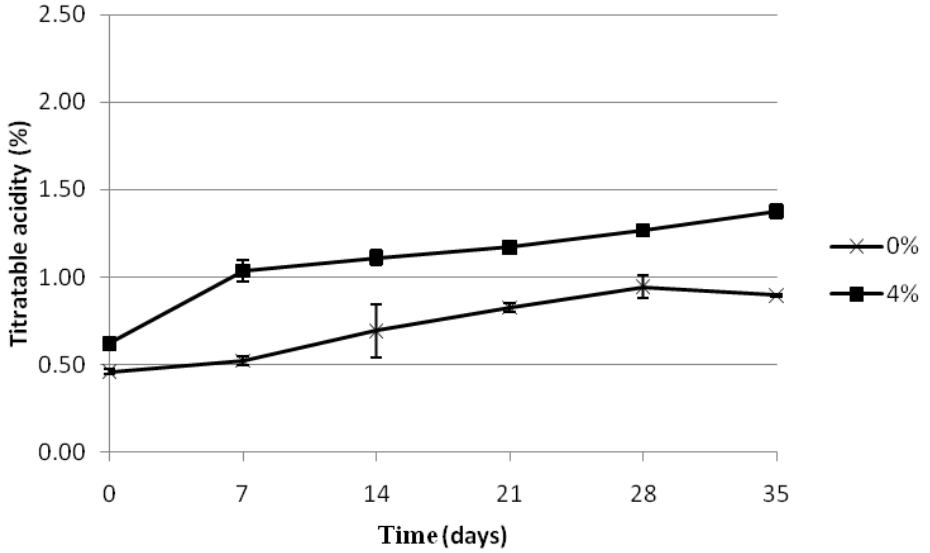
Samples containing 4% of SME showed a significant amount of lactic acid in both strains (1.75 and 1.40% respectively), while in contrast control samples that contained no SME had minimal amounts of lactic acid production (Figures 5.5 and 5.6). The largest amounts of lactic acid production were observed in strain *L.reuteri* DSM 20016. The statistical analysis showed that there was a significant difference in lactic acid production between *L. reuteri* DSM 20016

and *B. breve* (P<0.05) and also between samples containing SME and the samples containing no SME. This was well correlated with pH, by increasing the storage time.



Error bars were taken of triplicate samples as standard deviation.

Figure 5.5. Stimulatory effect of shiitake mushroom extract on lactic acid production of *Lactobacillus reuteri* DSM 20016 in yogurt after 35 days stored at 4ºC

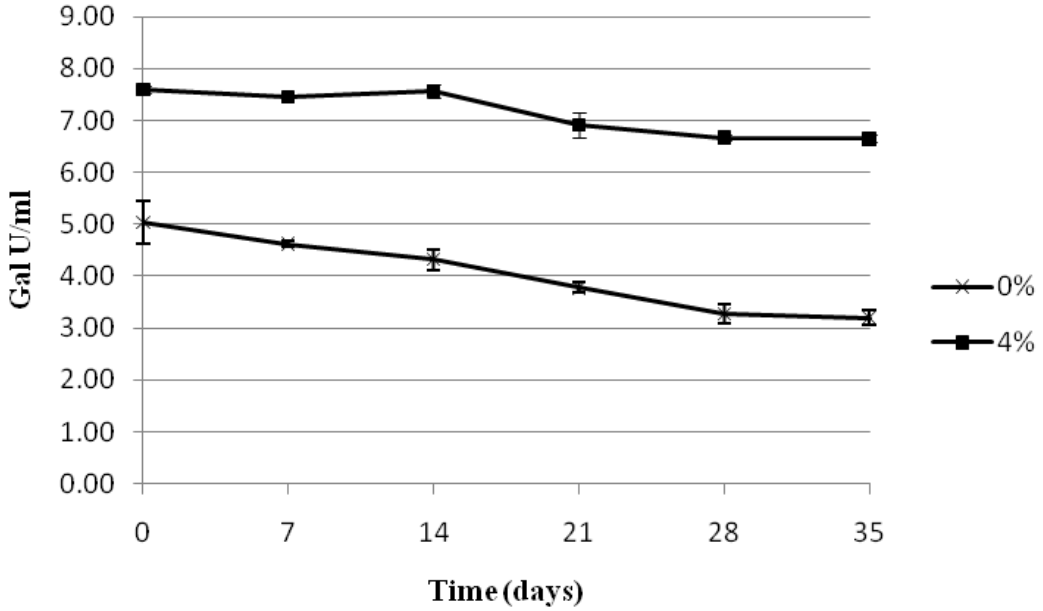


Error bars were taken of triplicate samples as standard deviation.

Figure 5.6. Stimulatory effect of shiitake mushroom extract on lactic acid production of *Bifidobacterium breve* in yogurt after 35 days stored at 4ºC

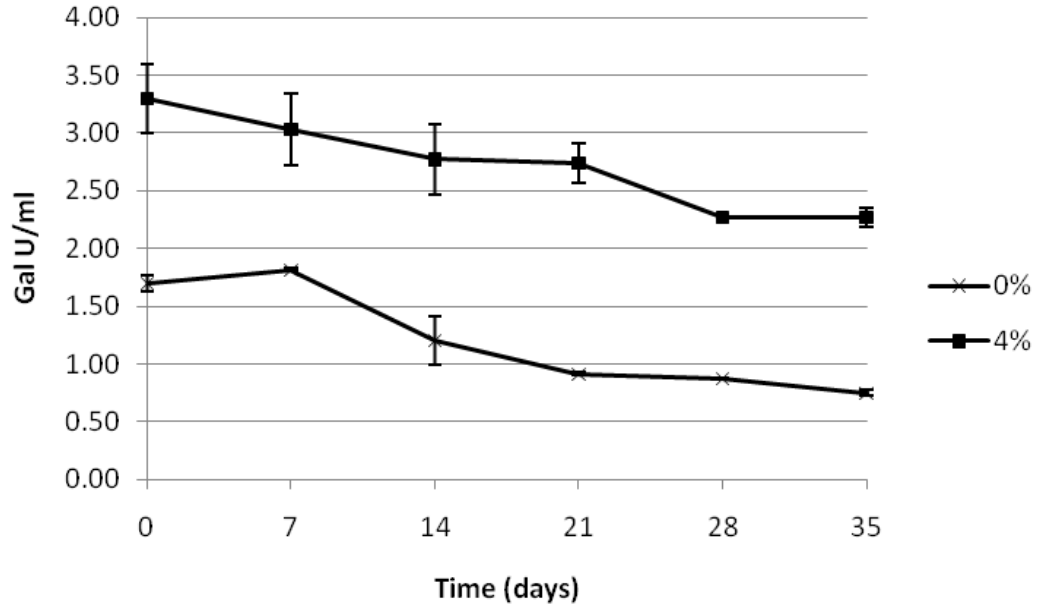
5.3.5. Enzyme activity. Enzyme activities were measured by determining the rate of hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside and o-nitrophenyl-β-D-galactopyranoside, respectively. β and α -galactosidase activities varied considerably between the strains used in this study. The differences in the enzyme activity, among *Bifidobacterium* and *Lactobacillus,* were essential. Bifidobacteria exhibited unique characteristics in its survival in yogurt mixed with mushroom extract. β-Galactosidase and α-galactosidase activities were detected in all strains. β-Galactosidase activity of *Lactobacillus spp*., was much greater than that of *Bifidobacterium spp*. During refrigerated storage, there was a significant reduction in enzyme activity of all strains depending on the amount of mushroom extract added to the sample. Samples treated with 4% of SME showed higher amount of enzyme activity and stability during incubating at 4ºC.

5.3.5.1. α-Galactosidase assay. Results showed that *L. reuteri* strains DSM20016 grown in yogurt mixed with 4% of SME was higher than the control (5.0 and 3.0, Gal U/ml, respectively) (Figure 5.7). *L. reuteri* DSM 20016 exhibited significantly higher ($P < 0.05$) *α*-galactosidase activity than *Bifidobacterium breve* since first week, when 4% of SME was used. *L. reuteri* DSM grown in yogurt without SME dropped on the first week and continued dropping until the end of the experiment (from 4.0 to 2.8 Gal U/ml). Refrigerated storage samples of *L. reuteri* DSM grown on yogurt with 0% of SME showed significantly reduced α-galactosidase activity ~1.2 Gal U/ml. However, the *L. reuteri* DSM samples grown on yogurt with 4% SME demonstrated stable α-galactosidase activity over 35 days period (Figure 5.7), at 4^{th} week of storage at 4ºC, α-galactosidas activity decreased over time in samples without SME samples (Figure 5.7). *Bifidobacterium breve* in yogurt with and without SME had α-galactosidase activity in the range of (3.75 and 3.50 Gal U/ml respectively) at initial time (Figure 5.8).



Error bars were taken of triplicate samples as standard deviation.

Figure 5.7. α-Galactosidase activity (Gal U/ml) of *L. reuteri* DSM 20016 in yogurt containing 0 or 4% concentration of shiitake mushroom extract



Error bars were taken of triplicate samples as standard deviation.

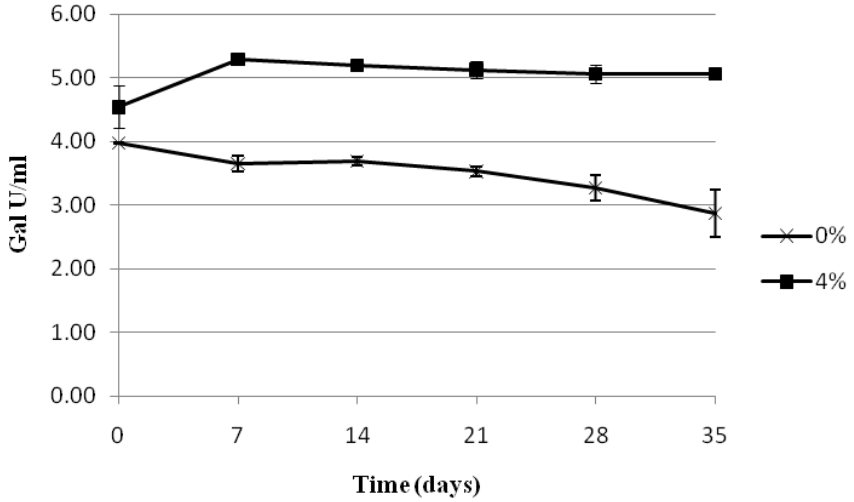
Figure 5.8. α-Galactosidase activity (Gal U/ml) of *Bifidobacteriu breve* in yogurt containing 0 or 4% concentration of shiitake mushroom extract

Generally, all strains used in this study showed stability and significant production of α-galactosidase activity until the fifth-week when we used 4% of SME compared to the control. These results agree with Alazzeh et al. (2009) when they found that α-galactosidase activity in *L. reuteri* (0.067-0.603 Gal U/ml) was higher than that produced by bifidobacteria (0.013-0.396 Gal U/ml) and α-galactosidase activity of *L. reuteri* strains DSM20016 and MM2-3 was higher in comparison with the other bifidobacteria they investigated. Garro et al. (2006) also reported that raffinose in a high pH environment leads to an increase of α-galactosidase activity in *B. longum.* These results were expected since α-galactosidase hydrolyzes the α-galactoside bonds in monosaccharide such as raffinose. Shiitake extract rich in carbohydrate shows the highest peak for raffinose (Hassan et al., 2011).

5.3.5.2. β-Galactosidase assay. *L. reuteri* strains had generally higher β-galactosidase activity than bifidobacteria (see Figures 5.9 and 5.10). β-Galactosidase activity obtained with DSM20016 grown in skim milk containing 4% of SME was significantly higher ($P < 0.05$) (6.75 Gal U/ml) than *B. breve* (5.10 Gal U/ml), after the 35 days. β-Galactosidase activity at the initial time of L. reuteri DSM 20016 on 0% and 4% of SME were 5.0 and 7.6 Gal U/ml respectively. On the other hand, *B. breve* grown on skim milk in presence of SME at 0 and 4% concentration gave β-galactosidase activity of 3.3 and 5.2 Gal U/ml respectively after 35 days.

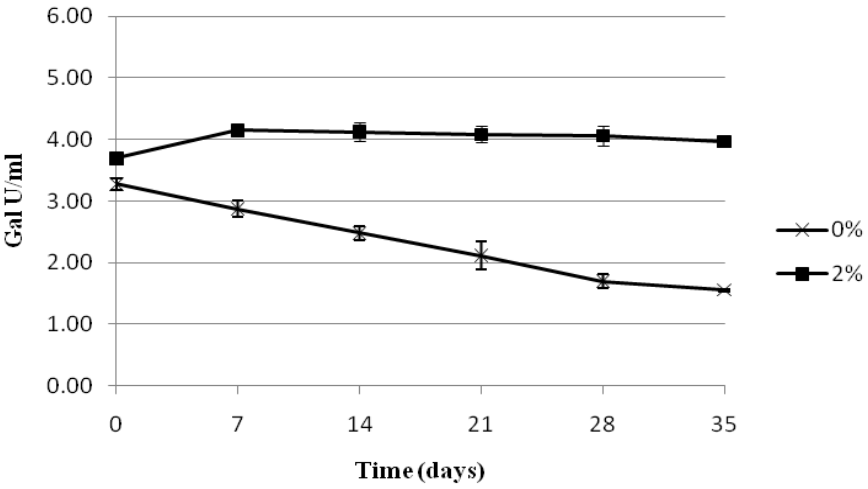
Many scientists have made many attempts to search the possibility of increasing the activity of α- and β-galactosidases in different bacterial strains by using various prebiotics. Dumortier et al. (1994), for example, observed that β-galactosidase activity of *B. bifidum* occurred at higher temperature. Others showed that of α- and β-galactosidases activity changes due to the differences in cell mass, cell growth, or variable number of cell per gram of culture (Occhino et al., 1986; Ramana & Dutta, 1981; Hsu et al., 2005). Hsu et al. (2005) said that

carbohydrate and nitrogen sources, pH of the media, and mineral additive have a significant effect on increasing the β-galactosidase activity in bifidobacteria. Also Ibrahim and O'Sullivan (2000) showed that β-galactosidase activity increased the mutants of *Bifidobacterium spp* when they were exposed to chemical mutagenesis.



Error bars were taken of triplicate samples as standard deviation.

Figure 5.9. β-Galactosidase activity (Gal U/ml) of *L. reuteri* DSM 20016 in yogurt containing 0 or 4% concentration of shiitake mushroom extract



Error bars were taken of triplicate samples as standard deviation.

Figure 5.10. β-Galactosidase activity (Gal U/ml) of *Bifidobacteriu breve* in yogurt containing 0 or 4% concentration of shiitake mushroom extract.

5.4. Conclusion

The viability of *Lactobacillus reuteri* DSM 20016 and *Bifidobacterium breve* in yogurt samples prepared using 4% SME during 35 days of storage at 4ºC compared to samples without SME showed stability in viability (7.9 to 7.8 log CFU/ml respectively). The results indicated that addition of 4% of SME to the samples showed growth prompting effect of *L. reuteri* DSM 20016 and *B. breve* compared to samples without SME. The fermentation period reduced by increase of the concentration of SME in skim milk as it reached the lower pH in 35 days significantly ($P<0.05$) as compared to the control. After 35 days of storage at 4°C samples containg 4% SME showed asignificant amount of acid production in strain, *L. reuteri* DSM and *B. breve* (1.75 and 1.40% respectively), while in contrast samples without SME had minimal amounts of acid production was observed in L. reuteri DSM. This may exert positive effect when shiitake mushroom used as a functional food product to promote the growth of probiotic strains in the human intestinal tract.

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CHAPTER 6

Conclusion and Future Directions

The main purposes of the experiments describes in this dissertation were:

a) To test the different effects of using a range of shiitake mushroom extract (SME) in different percentages, to determine the best concentration of this prebiotics on the growth of *Lactobacillus reuteri* and *Bifidobacterium spp.* When be added to MRS.

b) To find the best shelf life of skim milk by SME on the growth and viability of *Lactobacillus reuteri* (DSM 20016 and CF2-7F) and *Bifidobacterium spp* (*breve* and *adolescentis*)

c) To examine the effect of the prebiotics (SME) and the yogurt on the growth and viability of *Lactobacillus reuteri* DSM 20016 and *Bifidobacterium breve* and develop the best environments yogurt with exact concentrations of SME to have the highest growth and viability rate of Lactobacillus reuteri and *Bifidobacterium spp.* to validate their beneficial effect on growth and survival of other probiotic bacteria.

This study clearly focused on the importance of the growth and viability of probiotics in dairy products. The use of prebiotics has been the focus of many studies over the last decades to enhance the growth and viability of probiotic bacteria. In conclusion this study was able to successfully find novel product and new method that give excellent and accurate CFU of probiotic bacteria. Therefore, this investigation successfully achieved its objectives by adding 4% of SME on MRS media, skim milk and yogurt. In the future more research should be held using more techniques to determine the genetic code that effects the hyper growth and viability of probiotics and also to use HPLC and ion chromatography determined the amino acids produced during the fermentation process.

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Appendix A

Methods to measure carbohydrates in shiitake mushroom

Instrument
-Spectrophotometer
-5 ml pipette
Chemical used:
-Phenol 5% w/v, dissolve 5g of analytical reagents grade phenol in water and dilute to 1 liter
Sulphuric acid (98%) analytical-reagents grade.
Glucose standards solution. (i) Stock solution 1.0 mg/ml. dissolve 1.0 g in water and dilute to volume 1000 ml volumetric flask (ii) working standards solution 0-0.05 mg/ml.
Transfer 0-5 ml of stock solution separately to 100 ml volumetric flask and dilute to volume with water.
Instruction to follow method:
Extract about 0.25 g of ground dried shiitake mushroom with soxhelt apparatus for two hour (i.e until essentially fat free).
Transfer the extracted shiitake mushroom to an evaporating glass dish and dry to constant weight, then heat the sample for 3 hours with 100 ml of water and 10 ml of hydrochloric acid in a 250 ml flask, provided a reflux condenser. Cool, filter and transfer the filtered to a 250 ml volumetric flask and diluted to mark. Pipette 10 ml of this solution to volumetric flask to make 100 ml of solution. Pipette 1 ml of the solution and standard solution separately to test tubes. To each tube add 1 ml of 5% phenol in water and then add 5 ml of concentrated H_2SO_4 rapidly for 10-20 mint water bath 25-30 ^{0}C before readings at 490 nm are taken.

- Blank are prepared substituting distilled water for sugar solution. Plot a curve from the reading of the standard and calculate the amount of CHO in sample expressed as weight % of glucose n sample.
- **%CHO = $\frac{\text{conc. (mg/ml)x 250 ml}}{\text{Sample wt (g) x1000(mg/ml)}} \times \frac{\text{100 ml}}{\text{10 ml}} \times 100\% = \frac{\text{conc.x250}}{\text{sample wt}}$ (as gulocose)**

Source: Chang and Quinnio (1982), p. 87

Appendix B

Calculating the amount of carbohydrates in shiitake mushroom

ID	***absorbance***				
		conc.	***aborb.***	***ppm***	***slope***
0.50	-0.014	0.50	-0.014	0.26	2.24E+02
1.00	-0.011	1.00	-0.011	0.93	
2.00	-0.002	2.00	-0.002	2.95	***intercept***
5.00	0.004	5.00	0.004	4.29	3.39
10.00	0.03	10.00	0.03	10.11	
20.00	0.074	20.00	0.074	19.96	***correl.***
					0.99739
		PPM			
sample	0.031	10.34			

Appendix C

Shiitake mushroom preparation for metal analysis

Synopsis:

a. Microwave Digestion to solubilize all metal.
b. ICP/OES analysis to identify and quantities all metal

a. Microwave Digestion (MW);

1. Weigh approx. 0.1000 gm and record weight
2. Add to digestion tube.
3. Add 7 ml conc. HNO_3 and 3 ml conc. HCL to the digestion tube.
4. Place in MW and program the oven to heat to 200ºC in 15 minutes and then hold oven at 200ºC in 15 minutes.
5. After MW digestion is over let samples cool to below 60ºC
6. Transfer the sample to 50 ml graduate cylinder and build to 50 ml mark with DI water and hold for ICP/OES analysis.
7. A good digestion will be crystal clear.

b. ICP/OES analysis:

1. prepare standard analyte (s) for all metal of interest
2. prepare the concentration curve (for element analysis)to cover the low and high ends of the expected values (range 0.05ppm-500.0 ppm.
3. Follow the ICP/OES manufacture's operation manual to complete the metal analysis.

Appendix D

Shiitake mushroom metal analysis

metal	mg in digested sample	mg/kg in sample
Lithium	0.001	10.4
iron	0.003	33.6
Zink	0.003	34.3
boron	0.006	72.9
Copper	0.007	100
Silicone	0.011	134
Calcium	0.012	167
Sodium	0.051	717
magnesium	0.107	1,423
Phosphorus	0.496	6,607
Sulfur	0.538	7,178
potassium	2.114	28,165
Aluminum	0.004	53