

**FACTORS AFFECTING THE GROWTH AND SURVIVAL OF
PROBIOTIC IN MILK**

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CHAPTER ONE

INTRODUCTION

In the late nineteenth century, microbiologists identified microflora in the gastrointestinal tract of healthy animals that differed from those found in diseased animals. As further research continued into the isolation and characterization of these microorganisms, it was revealed that ingestion of these bacteria could confer a wide range of therapeutic benefits to humans. These beneficial microflora were termed probiotics. Since then, the popularity of probiotics has been increasing rapidly worldwide (Benkouider, 2004 (a, b); Koutilainen *et al.*, 2006).

The most commonly studied probiotic bacteria include members of the genus *Lactobacillus*, especially, *L. acidophilus* and *Bifidobacterium* spp. (Tannock, 2002). These bacteria were found to prevent diarrhea in children, suppress pathogens in the intestinal tract, alleviate symptoms of lactose indigestion and enhance the population of beneficial bacteria in the human gut (Rafter, 2004; Sanders, 2000, 2003).

The numerous perceived health benefits and the growing awareness about probiotics have caught the attention of the food industry (Saarela *et al.*, 2002; Salminen and Gueimonde, 2004). Food companies are increasingly manufacturing foods with incorporated probiotic bacteria, which fall under the new category of foods called Functional Foods. Probiotic dairy products such as yogurts containing *L. acidophilus* and *Bifidobacterium* spp. constitute a significant amount among the commercially available probiotic foods (Reid *et al.*, 2003). Functional foods have been developed in most food categories and even by conservative estimates, the global market size already exceeds that for organic

foods. In addition to providing consumers options for improving their health and well-being, functional foods such as probiotics in dairy products are an attractive market sector, providing new economic opportunities.

However in recent times, the increasing popularity of probiotic dairy products, especially probiotic yogurt has led to the creation of a multibillion dollar market for probiotic yogurt and other foods where probiotics can be incorporated (Playne, 2002).

As the popularity of yogurt and yogurt drinks grew, various factors related to the growth and survival of probiotics in yogurt and yogurt drinks became more apparent. Although several of the yogurt products contain either *L. acidophilus* or *Bifidobacterium* spp., there are only few reports of yogurt and yogurt drink containing only *Bifidobacterium* spp (Godward *et al.*, 2000).

For probiotics to be effective, scientists have suggested that there be a minimum number of 10^6 - 10^7 cfu of probiotic bacteria /gram of product at the time of consumption (IDF, 1992; Lourens-Hattingh *et al.*, 2000; Lourens-Hattingh and Viljoen, 2001). While some reports have shown probiotic growth and survival numbers to be stable during the shelf life of the product (Dinakar and Mistry, 1994), others have cited a rapid decline in the number of viable probiotic bacteria over the shelf life (Stanton *et al.*, 2003). Studies have shown that a number of factors can affect the growth and the survival of *Bifidobacterium* spp. in dairy products. These factors include strains of probiotic bacteria, pH of milk, presence of lactic and acetic acids, interactions with other microorganisms, storage temperature and manufacturing conditions (Shah, 2000 (a, b); Boylston *et al.*,

2004). As matter of using prebiotic or hydrolyzed milk to enhance the growth and survival of probiotic bacteria and specially bifidobacteria, the result showed a variety of effects depending on the strain of probiotic used and the dairy products used. However, none of these studies has examined the synergistic effect of prebiotic and hydrolyzed milk on the growth and survival of bifidobacteria spp.

The combination of probiotic and prebiotics has been used on several aspects of health benefit, which have also been documented (Gibson and Roberfroid, 1995; Niness, 1999). However, very little attempt has been made to develop a synbiotic product with combination of probiotic and prebiotics (Gibson and Roberfroid, 1995).

Moreover, very little information is available on the synergistic effects of prebiotics and hydrolyzed milk on the growth and survival of bifidobacteria spp., which could lead to new products for the dairy market.

Chapter Two of this thesis deals with the literature review that highlights the use of probiotics in dairy products and the history of fermented dairy products and functional foods, and the mechanisms involved of using probiotic with prebiotics *in-vivo* studies.

Chapter Three emphasizes the evaluation of prebiotics in specific FOS and Inulin concentrations on the growth and survival of *Bifidobacterium lactis* Bb12.

Chapter Four reports the effects of different degrees of hydrolyzed skim milk on the growth and survival of *Bifidobacterium lactis* Bb12.

Chapter Five focuses on the screening and optimization on the growth and survival of *Bifidobacterium lactis* Bb12, *Bifidobacterium bifidum* NCIMB 702203,

Bifidobacterium infantis NCIMB 702205 and *Bifidobacterium breve* NCIMB 702257 and *Lactobacillus casei* strain shirota in the presence of two prebiotics and hydrolyzed skim milk *in-vitro*.

Chapter six examines the sensory analysis for a new product with *Bifidobacterium lactis* Bb12 and evaluated the over all preferences.

Chapter seven focuses on the overall conclusion and recommended future research

Chapter eight lists all references.

CHAPTER TWO

LITERATURE REVIEW

2.1. FERMENTED MILK AND PROBIOTIC BACTERIA:

Fermented milk has been used throughout the history of mankind. Nearly every civilization have developed some type of fermented milk product such as buttermilk, labneh, acidophilus milk and yogurt, which were familiar to many people, although the fermentation process was not defined yet. Yogurt, which came from the Turkish term in the eighth century as “yoghurut” (Pashapour and Lou, 2006) and the spelling changed over time to the present spelling in the eleventh century. Yogurt is defined as “product obtained by the fermentation of milk with cultures of *streptococcus thermophilus* and *lactobacillus delbrueckii* ssp. *bulgaricus*”, however, “yogurt-like products” are made by substituting *L. bulgaricus* by other *Lactobacillus* species for the fermentation of milk or yogurt containing probiotic bacteria (Guarner *et al.*, 2005).

The fermentation process of dairy products and its bacteria have received great attention over the decades after discovering the importance of viable bacteria in food for health benefits. At the beginning of the last century first scientific work had been done by Metchnikoff to investigate the beneficial effects of fermented milk for human health. Numerous scientific studies have been published describing the health benefit associated with the consumption of fermented dairy products (Modler *et al.*, 1990; Shin *et al.*, 2000b),

The lactic acid bacteria (yogurt bacteria; *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) do not inhabit the human and animal intestinal

tract, nor do survive passing through the digestive system (Klaver *et al.*, 1993). Therefore, to get the beneficial health effects associated in the fermented dairy products, there has been and increase interest in the incorporation of the intestinal species *Lactobacillus acidophilus* and *Bifidobacterium* species in the fermented milk products (Reuter, 1990; Shah and Lankaputhra, 1997; Shah, 2001), these include fermented liquid milks, ice cream, yogurt, and cheese and in soya milk and soya yogurt (Modler *et al.*, 1990; Hekmat and McMahon, 1992; Shah, 1997b; Heller, 2001; Champagne *et al.*, 2005).

A probiotic is defined classically as a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract. This definition, however, was initially intended for use with animal feed products. For human nutrition, the following definition has been proposed: Probiotics are viable microorganisms that are beneficial to the host when administered in appropriate quantities (FAO/WHO, 2001).

In the last decade, the use of probiotics in Fermented dairy product and feed product applications has a noticeable interest and develop. These organisms play significant role in lowering the pH of the large intestine through the release of lactic and acetic acid (Asahara *et al.*, 2004), as to Shah (2001) that yogurt containing *L. acidophilus* and *Bifidobacteria* is “AB” yogurt and if *L. casei* incorporate in addition to *L. acidophilus* and *Bifidobacteria* yogurt it will be referred as “ABC” yogurt.

A large number of probiotic strains Isolates from food, especially dairy products, but also from many applications samples for human, and nutrients for animals.

All commercially available isolates of the genera *Lactobacillus* and *Bifidobacterium* were identified and compared with reference strains from official culture collections (ATCC, DSMZ, LMG, CCUG, and others).

As bifidobacteria are used as probiotic cultures in commercial dairy products, the questions arose “which species are used?” (Bonaparte and Reuter, 1997; Dunne *et al.*, 1999).

In general, there are 56 species of *Lactobacillus* and 29 species of *Bifidobacteria*, which used worldwide in dairy products (Shah, 2001). However, the main species of *Lactobacillus* and *Bifidobacteria* had been reported in literature such as; *L. lactis*, *L. casei*, *L. paracasei* and more. *B. longum*, *B. infantis*, *B. breve*, *B. lactis* and *B. bifidum* (Shah, 2001). Recent experience revealed that *B. animalis* had been applied frequently in fermented dairy products due to the fact that this species is somewhat less sensitive against acidification (pH below 4.2) and elevated oxygen tolerance, which is remarkable within the *Bifidobacteria* (Prasad *et al.*, 1998; Masco *et al.*, 2004).

Bifidobacteria are unique in many other respects. Human related sequence of *bifidobacteria* differs from both homo and hetero-fermentative bacteria in that they metabolize carbohydrates by a special enzyme called Fructose-6-Phosphate Phosphoketolase (F6PPK), which also metabolize Fructooligosaccharides (FOS) which are polymers of fructose and cannot be metabolized by either human digestive enzymes or most undesirable gut microorganisms.

The use of *Bifidobacteria* in fermented milk shows that it has very slow properties and it requires specific growth factor, known as bifidogenic factors, these are

carbohydrates such as N-acetyl-D- glucosamine and lactulose found in human milk (Liepke *et al.*, 2002) and processed milk products, respectively. “Unlike yogurt bacteria, probiotic bacteria grow very slow” (Shah, 2001). Moreover, it has shown that breast-fed infants have higher numbers of *Bifidobacterium* than bottle-fed ones (Heinig and Dewey, 1996; Coppa *et al.*, 2006)

The use of bifidobacteria in fermented milk has many benefits to human and animals by several mechanisms (Deguchi *et al.*, 1985; Heinig and Dewey, 1996; Rowland *et al.*, 1998; Heenan *et al.*, 2004). Therefore, it has been very important to determine the efficacy of the product containing probiotics, such as the acceptance of the product by the consumers and the survival of probiotic microorganisms during its production. In general, the food industry has applied the recommended level of 10^6 CFU/g at the time of consumption for *Lactobacillus acidophilus*, *bifidobacteria* and other probiotic bacteria (Shah, 2001; Boylston *et al.*, 2004).

Although milk contain the essential nutrients for the growth of bifidobacteria, but the level of amino acids and peptides insufficient to provide the ideal condition for rapid their growth and survival (Klaver *et al.*, 1993; Gomes *et al.*, 1998). In consequences bifidobacteria have been used only to limited extent in commercial fermented milk (Gomes and Malcata, 1999; Heller, 2001) in spite that fermented milk is the preferred carrier for bifidobacteria.

2.2. FUNCTIONAL FOOD AND PROBIOTIC BACTERIA

The growing understanding of the relationship between diet and health increased the demand for food with specific benefit beyond their basic nutrition such as

improving the health and well being of human. This food is called Functional Food. However, Functional food has defined as one, which provides a specific health benefit over and above its normal nutritional status (Gibson and Rastall, 2004). Moreover, the functional foods must remain as foods (not capsules, etc.) and they must also reveal their effects in amount that can usually be expected to be consumed in the diet.

It has been suggested that food will use as functional when it has shown beneficial effect on one or more target in the body and that beside their nutritional effects such as well-being and health of the host (Isolauri *et al.*, 2002).

The old generation of functional foods indicates of using supplements to the food to increase their nutrition and health effects such as vitamins and micronutrient. However, in the new concept of functional foods there was more interest in the gastrointestinal interactions (Salminen *et al.*, 1998a), that led for more interest in the dominated organisms in the gastrointestinal tract (indigenous microflora) which found to beneficially effect human health, which known as probiotic bacteria.

Therefore, the use of probiotic micro-flora was one of the most promising areas for the development of functional foods in the recent studies (Gibson and Roberfroid, 1999) because of what probiotics has established a great benefit to human health.

Bifidobacteria were the most dominated organisms in the gastrointestinal tract and their viability and metabolic activity have shown very beneficial effects on the health of the gastrointestinal tract (Gibson and Roberfroid, 1995) and that always

related to the presence of a suitable environment and nutrients, which are very important for the viability and activity, for bifidobacteria to use it in the bowel as carbon and energy source, these compound were referred to as “bifidogenic factors” (O’Sullivan, 1996).

At present, probiotics products and especially probiotics dairy foods are marketed successfully all over the world because of their acceptance of consumer and the awareness about their positive aspect for the health benefits.

2.3. HEALTH BENEFITS FOR HUMAN:

2.3.1 HEALTH BENEFITS:

Bacteria have a reputation for causing disease, but a growing body of scientific evidence suggests that you can treat and even prevent some illnesses with foods and supplements containing certain kinds of live bacteria. For example, Metchnikoff related the longevity of Bulgarians to the present of *Lactobacillus bulgaricus* in the souring milk “yogurt”. Moreover, in the Northern Europeans consume a lot of these beneficial microorganisms in the dairy products, because of their tradition of eating foods fermented with bacteria, such as yogurt and cheese, these beneficial bacteria have named later as probiotics..

Probiotic microflora displays numerous health benefits beyond providing basic nutritional value. The health benefit reported of probiotics is the improvement in gut health and the prevention of intestinal infections and stimulating the immune system (Kailasapathy and Chin, 2000; Salminen and Gueimonde, 2004). Infection prevention is increasingly preferred over using the traditional action by

chemotherapy with antibiotics, that rise the concern over development of antibiotic resistance has placed probiotics at the fore.

The principal mechanism for this action is known as competitive colonization or competitive inhibition (Alander *et al.*, 1999; Chen *et al.*, 1999; Naidu *et al.*, 1999; Leahy *et al.*, 2005). This is described as the creation of probiotic bacteria in the human intestine, which acts as a vital barrier to invasion by pathogens in the gastrointestinal tract of the human host.

Over 90% of the total cells in the body are present as bacteria in the colon, getting 10^{12} CFU (Colony Format Unite) for every gram of large intestinal contents (Bourlioux *et al.*, 2003; Anuradha and Rajeshwari, 2005). Under natural conditions, a protective gut microflora develops and there is no need for a bacterial supplement (Shanta-Retelny, 2005) but the changing food habits and lifestyle force us to take processed food, which affects our access to, and colonization, by probiotics. Moreover, we also consume antibacterial substances ranging from vinegar to antibiotics.

To reside in the gut, When ingested, probiotic bacteria are resistant to gastric acidity and bile salts (Rinkinen *et al*, 2003; Minellia *et al.*, 2004) and therefore pass through the upper gastrointestinal tract and have the ability to adhere to the intestinal mucosa.

Further, the probiotics' secretion of by products such as lactic acid and acetic acid lower the pH in the intestine and producing hydrogen peroxide inhibiting the growth of pathogens and helped to speed pathogens through the intestines (Laroia and Martin, 1991; Mishra and Lambert, 1996).

The enhancement of the immune system is another reported health benefit of probiotics (Saarela *et al.*, 2000; Perdigon *et al.*, 2003), as it appears that this effect by balancing control of pro-inflammatory and anti-inflammatory cytokines (Pessi *et al.*, 2000, Shah, 2001) and therefore probiotics are considered as immune-stimulatory agent and an important tools to prevent intestinal inflammation, stop gut mucosal dysfunction and decrease hypersensitivity (Isolauri, 2001).

Recently, there is a relatively large volume of scientific literature basis supporting the use of probiotics for diarrhoea has started to become established. Probiotics have been shown effectiveness in the prevention of several types of diarrhea, including antibiotic-associated diarrhea, bacterial and viral diarrhea (including travelers' diarrhea), as well as that caused by lactose intolerance (Rolle, 2000). It is thought that this action is due to the secretion of antimicrobial polypeptides known as bacteriocins and that by; reduction in gastrointestinal pH through stimulation of lactic acid-producing bacteria; a direct antagonistic action on gastrointestinal pathogens; competition with pathogens for binding and receptor sites; improved immune function; and competition for limited nutrients (Collins and Gibson, 1999). It seems that the effect of probiotics on travellers' diarrhea depends on the bacterial strain used and the destination of the travelers (De Roos and Katan, 2000).

Observational data suggest that consumption of fermented dairy products is associated with a lower prevalence of colon cancer, which is suggested that probiotics are capable of decreasing the risk of cancer (Mital and Garg, 1995).

The Mechanisms described by Inhibition of carcinogens and pro-carcinogens, Inhibition of bacteria capable of converting pro-carcinogens to carcinogens (McIntosh, 1996).

Moreover, probiotic and especially bifidobacteria has shown to increase the α - and β -galactosidase activities in the faecal samples after feeding with the fermented milk containing probiotics, which is considered to be an important probiotic quality, as it supports lactose digestion in the intestine and compensates for lactase-deficiency (Shah, 2001; De Vrese *et al.*, 2001; Lourens-Hattingh and Viljoen, 2001).

Playne (2002) suggested that Health benefits imparted by probiotic bacteria are strain-specific and not species- or genus- specific. For example the strains *Lactobacillus rhamnosus* GG (Valio), *Lactobacillus paracasei* Shirota (Yakult), and *Bifidobacterium Lactis* Bb12 (Chr. Hansen), *L. acidophilus* La5 (Chr. Hansen) have the strongest human health efficacy data, against some or all of: lactose intolerance; rotaviral diarrhea; antibiotic-associated diarrhea and some other bacterial diarrheas and infections. *B. animalis* found to stimulate the immune response *in vivo* (Sanders, 2000; Perdigon *et al.*, 2003). Moreover, it has improve in control study the useful of *Bifidobacterium lactis* strain Bb12 in prevention of acute diarrhea in infants (Chouraqui *et al.*, 2004; Larsen *et al.*, 2006; Mohan *et al.*, 2006) and same by *Lactobacillus* GG (Jain *et al.*, 2004). *Bifidobacterium lactis* Bb12 well known in their ability to improve the growth of children when supplement in their formula (Nopchinda *et al.*, 2002). Candida can also be suppressed or controlled by viable *L. acidophilus*. It is also thought that

non-viable forms exert such control but to a lesser extent (Ouwenhand and Salminen, 1999).

2. 3.2. GASTROINTESTINAL MICROFLORA BALANCE AND PROBIOTIC

Probiotics, naturally found in the mouth, lower intestine and vagina of healthy individuals, help defend the body against invading pathogenic bacteria. Due to the dominance of common antibiotic treatment (which kills the beneficial organism as well as the harmful bacteria), many people are lacking healthy intestinal flora. The composition of the intestinal flora is relatively stable in healthy human beings between harmful and beneficial or natural bacteria.

Among the beneficial bacteria are *Lactobacillus* spp. and *Bifidobacterium* spp. which play a useful role in the production of vitamins, organic acids and anti-microbial factors to inhibit pathogens.

Any interactions of this balance in the gut microflora lead to make the harmful bacteria dominate the intestinal flora, which effect produce essential nutrients and increase the level of damaging substances, including carcinogens, putrefactive products and toxins (Mitsuoka, 1996; Salminen and Gueimonde, 2004).

However, this balance could affect by the age, diet, travel and stress, which may lead to disease and can occur when toxins are secreted by pathogens in the intestinal mucosal barrier and other bacteria.

Therefore, to maintain a well-balanced microflora in the gastrointestinal tract it has suggested introducing live bacteria or stimulating growth of beneficial

bacterial population groups which prevent harmful effects and promote beneficial actions of the intestinal microflora (Salminen *et al.*, 1996; shah, 2000a).

Consuming probiotics with dairy foods buffers stomach acid and increases the likelihood that the bacteria will survive into the intestine. Dairy products containing probiotics also provide a number of essential nutrients including calcium and protein (Stanton *et al.*, 2001)

2.4. HISTORY OF PROBIOTICS AND ITS DEVELOPMENT

Fermented milks are one of the oldest medical sciences, which date back to 2500BC; the consumption of yogurt has believed to help with maintenance of overall health (Chopra and Doiphode, 2002). A scientific explanation of the beneficial effects of lactic acid bacteria present in fermented milk was first provided in 1907 by the Nobel Prize winning Russian physiologist, Eli Metchnikoff, who stated “The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes” (Holzapfel and Schillinger, 2002).

2.4.1. HISTORY AND DEFINITION OF PROBIOTIC

2.4.1.1. HISTORY AND DEVELOPMENT OF PROBIOTICS:

Early scientists recommended fermented milk for its nutritional and medicinal properties, so the use of live flora to enhance the human health is over thousands of years.

In 1900 and before Metchnikoff, and for the first time bifidobacteria, members of the lactic acid bacteria group have been isolated from the Intestinal layer of breast-fed infants and found that they are the predominant component of the intestinal microflora (O'Sullivan *et al.*, 1992; Ishibashi and Shimamura, 1993; Harmsen- Hermie *et al.*, 2000; Fooks and Gibson, 2002).

While work in the earlier of the last century concentrated on the use of fermented milk with probiotics to take care of intestinal infections, recent studies have focused on the survival of these bacteria in the gastrointestinal tract and the carrier food to have their beneficial effect on the host (Lourens-Hattingh and Viljoen, 2001; Roy, 2005). Moreover, there has been more interest to use probiotics instead of antibiotics (Isolauri, 2001; Gibson and Rastall, 2004).

However, the use of the term “Probiotic” was in the 1970s and that from the Greek term meaning (for-life). However, there were a lot of scientific studies have been done about the beneficial effect of probiotics, which suggested high use in the fermented market. Interestingly, it is invented new term of food subject start in Japan earlier named as Foods for Specified health Use (FOSHU), which named as Functional Food in Europe and USA.

2.4.1.2 DEFINITION OF PROBIOTICS:

The explanation of probiotics has been growing over time, which used for the first time by Lilly and Stillwell (1965) to describe compounds produced by organisms that stimulated the growth of another. However, Parker (1974) used this term to the substances that applied to the animals feed as supplements in purpose of health improve by contributing to its intestinal microbial balance. This term

'probiotics' was taken by Roy Fuller (1989) and under a continuous work he referred to these substances as life microbes and substances supplements and give his definition as "a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance".

Thereafter, lactic acid and other bacteria, or yeasts applied in a fermented product or found in the upper respiratory tract or the argental tract beside to the intestinal and have a beneficial effect on the health of the host by improving its indigenous microflora balance (Havenaar and Huisin't Veld, 1992) as define "a mono- or mixed culture of live microorganisms which applied to animal or man, affects beneficially the host by improving the properties of the indigenous microflora". These definitions by Fuller and then by Havenaar and Huisin't Veld are still successfully in use.

Applying probiotics to the human studies show more new definitions and the essential requirements have been moderated to suit the future researches for examples; Food and Agriculture Organization/World Health Organization Working Group (FAO/WHO) (2002) recognize probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". But the Joint International Scientific Association for Probiotics and Prebiotics recently adopted this definition (Reid *et al.*, 2003) "Probiotic bacteria are live food supplements which benefit the health of the consumer".

2.5. PROBIOTICS CHARACTERISTICS

Most the probiotics are related to the *Lactobacillus* and *Bifidobacterium* genera (Bezkorovainy *et al.*, 1997; Salminen and von Wright, 1998; Sanders, 2003; Guarner *et al.*, 2005).

However, to consider use of different strain as probiotics it should be a normal inhabitant of a healthy intestinal tract, survive the upper digestive tract and capable of surviving and growing in the intestine (acid and bile resistant), safe for human consumption, production of antimicrobial substances like bacteriocins and has the ability of adherence to human intestinal cell lines and colonization (Guarner and Schaafsma, 1998; Morelli, 2000; Guarner *et al.*, 2005).

Lactic acid bacteria have a good safety record as compared to the probiotics and are rarely involved in disease. The most commonly used of these probiotics are *Lactobacillus* spp., *Bifidobacterium* spp. and *Lactococcus* spp. therefore, have been accorded the GRAS (Generally Recognized As Safe) status (Salminen and von Wright, 1998).

2.5.1. CHARACTERISTICS OF LACTOBACILLUS AND BIFIDOBACTERIA

2.5.1.1. *LACTOBACILLUS* SPP.:

Lactobacilli are characterized as Gram-positive facultative bacteria, they are non-spore forming, non-flagellated rods or coccobacilli (Hammes and Vogel, 1995). They are strictly fermentative, so they have the ability to ferment lactose and other monosaccharides to lactic acid predominantly with the homo-fermenters ones and to lactic acid with carbon dioxide and ethanol for the hetero-fermenters

ones. They are well use in the diet, therefore they claim as probiotics include *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. reuteri*.

However, the most studied one, which widely used as probiotic culture in dairy products is *Lactobacillus acidophilus* (Salminen et al., 1998b; Shah, 2001; Tannock, 2002). *L. acidophilus* belongs to the homofermentative Group of lactobacilli. *L. acidophilus* is non-motile, non-flagellated and non-sporing. It is facultative bacteria and Gram-positive rod around 0.6 to 0.9 µm in width and 1.5 to 6.0 µm in length with rounded ends. Cells may appear singularly or in pairs as well as in short chains.

The optimum growth occurs within 35-40°C but it can tolerate temperatures as high as 45°C. The optimum pH for growth is between 5.5-6.

2.5.1.2. *BIFIDOBACTERIUM* SPP.:

Bifidobacteria constitute the major part of the normal intestinal microflora in humans throughout life. They appear in the stools a few days after birth and increase in number thereafter especially in the breast-fed infants (Ishibashi and Shimamura, 1993; Heinig and Dewey, 1996). They are predominant in the large Bowel contributing to 6-36% of the intestinal microflora in adults (Hall, 2006), but a decrease in these levels of bifidobacteria is showing with age. They are all non-motile, non-sporulating, Gram-positive rods with varying appearance including short curved rods, club shaped rods and bifurcated Y-shaped rods. Most strains

are strictly anaerobic; some bifidobacteria can tolerate oxygen (Shimamura *et al.*, 1992; Shah, 1997a; Nebra and Blanch, 1999).

There are many species of bifidobacteria such as; *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. denticolens*, *B. dentium*, *B. gallicum*, *B. infantis*, *B. inopinatum*, *B. lactis*, *B. longum*, *B. pseudocatenulatum*. (Gomes and Malcata, 1999)

Bifidobacteria produce acetic and lactic acids under the fermentation process where two moles of glucose result of three moles of acetate and two moles of lactate. They can ferment galactose, lactose and fructose because of possessing Fructose 6 phosphate phosphoketolase (F6PPK) (Shah, 1997).

Bifidobacteria counted on the probiotics group as it has a beneficial effect on the host health, include synthesis of vitamins (Shah, 2001),

The optimum pH for growth is 6-7; with almost no growth at pH 4.5-5.0 and below or at pH 8 and above, but *Bifidobacteria Lactis Bb12* has shown ability to growth until pH 4.2. The optimum temperature for growth is 37-41°C with little growth below 25°C and above 46°C.

2.6. REGULATIONS AND SAFETY OF PROBIOTICS IN MANUFACTURE:

Since the early of last century beneficial effects of probiotics have been proved and well used in the dairy Industry, which suggested of daily intake to get the beneficial effect on the host. That directs the research and Industry organizations to suggest special regulations for the use of probiotics in food industry to obtain the desired therapeutic effects.

Probiotics are sensitive because they die after exposure to low pH in the human stomach. Therefore, the high-count number of probiotics recommended in the products at a minimum count of 10^6 CFU/g at the expiry date (Kurmann and Rasic, 1991; Gomes and Malcata, 1999). However, it has been suggested that the daily intake should be at least 10^8 CFU (Salminen *et al.*, 1998b; Lourens-Hattingh and Viljoen, 2001; Playne, 2002).

The more use of probiotics in the worldwide as dairy products the more suggested the need of principles and regulations as standard for the minimum count of viable probiotics bacteria in the dairy and fermented milk products to get the beneficial effects. The National Yogurt Association (NYA) of the United States suggested that any yogurt with live culture and use for health benefit recognize as containing significant amounts of live and active cultures. The seal is a voluntary identification available to all manufacturers of refrigerated yogurt products contain at least 10^8 cfu per gram at the time of manufacture, and at least 10^7 cfu per gram in frozen yogurt contains at the time of manufacture Exactly in the same way, the International Standard of IDF requires 10^7 CFU of *L. acidophilus* in products containing Lactobacillus bacteria and 10^6 CFU /g of bifidobacteria in fermented milks containing bifidobacteria at the time of sale (IDF, 1992). In Japan, the Fermented Milks and Lactic Acid Beverages Association have already established a standard that requires $\geq 10^7$ CFU mL⁻¹ to be present in dairy products that claims to contain bifidobacteria (Ishibashi and Shimamura, 1993; Martinez-Villaluenga *et al.*, 2006). Likewise, The Swiss Food Regulation requires a minimum of 10^6 CFU of viable bifidobacteria in those

products (Ishibashi and Shimamura, 1993; Bibiloni *et al.*, 2001). Some countries like Australia and New Zealand are still not introduce regulations for probiotics use. However, the Australian and New Zealand Food Standards Code (ANZFA) doesn't put any minimum count of probiotics products, but its mentioned the important of viable organisms in the manufactured of fermented milk products and that should be at least 10^6 CFU/g and pH of 4.5 at the end of manufactured of yogurt (ANZFA, 2003).

Bifidobacteria and *lactobacillus* strain have a long history as safe organisms in the dairy manufactured and they recognized as "GRAS" (Donohue and Salminen, 1996). There are no guidelines for the safety of probiotics exists so far. However there is responsibility of probiotic for four types of side effects; Systemic infections, deleterious metabolic activities, Disproportionate immune stimulation and gene transfer (Marteau *et al.*, 2002). Some potential infections of different species of probiotics has reported such as; *Streptococcus* likely to cause opportunistic infections (Salminen *et al.*, 1998b). The current studies have shown that this probiotic effect is link to a strain specific. Most the case of safety considerations were related to products because of certain species of *Streptococcus*, *Enterococcus* and *bacillus*, which are opportunistic pathogens (Donohue and Salminen, 1996; Spinosa *et al.*, 2000).

The guidelines for the Evaluation of probiotics in Food by FAO/WHO working group in 2002 suggested the use of valid methodology such as phenotypic and genetic tests, which will help to establish the species currently used and evaluation of probiotics.

Therefore, the introduction of regulations and safety for probiotic productions by food authorities are essential in the manufacturers to guarantee a specific count and species of probiotic bacteria in their products.

2.7. PREBIOTICS AND THEIR BENEFICIAL EFFECT ON HUMAN HEALTH.

Since 1980s the awareness of the healthier food and drink market has been increased in all over the wide world, which named as Functional Foods (Roberfroid, 2002). The popularity of these foods reflects nutritional guidelines recommended increase the dietary fibre intake. The uses of insoluble fibre ingredients (Gibson, 2004), such as bran, have been used in product such as breakfast cereals, bread and pasta, but the acceptability of these materials is limited in different systems, which decreased their incorporated into foods. Soluble fibre ingredients such as oligosaccharides are currently more interest in formulating of healthy foods because they are more acceptable. Moreover, some of them can be used as thickness in the food system, add viscosity or gel (Dreher, 1999).

The main reason of prebiotics supplementation to human diet is to beneficially enhance the gut microflora (Kolida *et al.*, 2002), which is *Bifidobacterium spp.* the most dominant and important flora in the breast-fed and healthy infants.

The beneficial effects of the presence of bifidobacteria in the gastrointestinal tract are dependent on their viability and metabolic activity. Their growth is dependent on the presence of complex carbohydrates known as oligosaccharides. Some oligosaccharides, because of their chemical structure, are resistant to digestive

enzymes and therefore pass into the large intestine, these considered as prebiotics which are defined as “non-digestible food that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995).

Therefore, prebiotics are used as bifidogenic factors in diet applications, especially because of their ability not to degrade in the stomach and small intestine (Crociani *et al.*, 1994).

Inulin and oligofructose are recognized as safe ingredient supplements to food without limitation but the European Commission confirmed that oligofructose (FOS) and inulin could be used in foods targeted to infants older than six months of age at a concentration of 0.8 g/day (Rao, 2002).

Kaplan and Hutzins (2000) have shown the ability of a selection of twenty-eight lactic acid bacteria and bifidobacteria to ferment inulin and oligofructose on MRS agar

Gibson (2004) stated that for a dietary substrate to be classified as a prebiotic, it has to meet at least three requirements; (1) the substrate must not be hydrolysed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial bacteria in the colon such as the bifidobacteria and (3) fermentation of the substrate should induce beneficial luminal/systemic effects within the host.

A range of dietary compounds has suggested as prebiotic, most of the selected prebiotics were on their health benefits on host. Gibson *et al.* (1995) presented the popularity of Inulin, Fructo-oligosaccharides (FOS) and Galcto-oligosaccharides (GOS) as health benefit substracts.

In human studies, a suggestion of added in bread a 7g/day of FOS intake for human affects the dominant bifidobacteria comparing intake bread without FOS. Therefore, data clearly proved that the use of FOS exerted a profound effect upon bifidobacteria (Gibson, 2004).

The only prebiotics for which sufficient data have been generated to allow an evaluation of their possible classification as functional food ingredients are the inulin-type fructans, which include native inulin, enzymatically hydrolyzed inulin or oligofructose, and synthetic fructooligosaccharides (Roberfroid and Delzenne, 1998; Roberfroid *et al.*, 1998).

2.7.1 INULIN:

Inulin is natural food ingredient, which found in many vegetables and obtained industrially from chicory roots (Van Loo, 1995). Usually GF_n is the symbolized use for inulin as polysaccharides mixture, where G is the glucose moiety, F is the fructose moiety and (n) equals the number of fructose moieties linked by β (2→1) linkages (Figure 2.1). This bonding renders it resistant to hydrolysis, both in the stomach and the intestine (Robinson, 1995). The degree of polymerization (DP) of inulin typically ranges from 2 to 60.

Usually 10% of the inulin extracted from chicory roots is sucrose and fructose, and 30% is oligosaccharide (DP=10).

The production processes of inulin naturally from chicory roots is by diffusion in hot water, followed by refining and spray drying.

The nutritional properties of inulin as a supplement to the healthy foods is obtained when inulin is not broken down in the upper digestive systems of human

and should be given energy value of 6.28 kJ/g (Roberfroid, 1999a), fermented by the intestinal flora causing increase in the biomass, producing of short chain fatty acids and decrease in the pH, and significant increase of bifidobacteria in the colon and inhibits the growth of less beneficial bacteria, (Roberfroid, 1999b). So using these ingredients in food allows improving the nutrition value of the products, by reducing the calorie content and increasing the bifidus-promoting capacities.

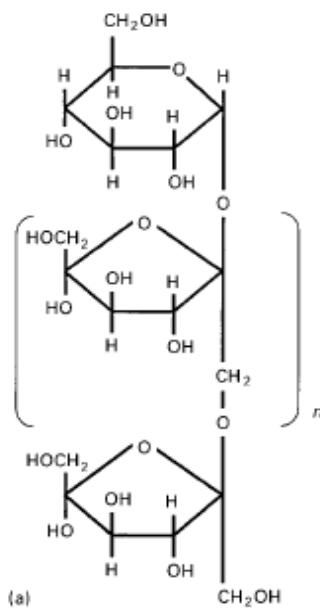


Fig 2.1 Structure of polysaccharides: Fructooligosaccharides: e.g. inulin, n=2-60; oligofructose, n=2-20.

Inulin has natural taste, colourless and minimal influence on the natural characteristics of the products.

Inulin is highly soluble fiber ingredient, which gives its more importance in the dairy industries such as milk drink, yogurt, and cheeses. Combining inulin with sweetness improves the taste of products giving more sugar-like sweetness. At

concentration of 40-45% inulin form a fatty tasting solution, which make it highly used in the free-fat products to give the smooth creamy texture and taste; Inulin has successfully applied in fat-reduced table spread, cheese products, meat products, frozen desserts, fillings, sauces and meat replacers. (Frank, 1997; Frank and Coussemant, 2001).

Using the food as vehicles for inulin considering in its ability to keep the inulin save to the intestine where the bifidobacteria inhabit, but yogurt has shown to be the best choice for that purpose. However, bio-yogurt “with inulin” showed problem in transferring the short chain length of the inulin because of the possibility to interfere too much with the inter-micellar bonding of the casein, that why it is important to watch the optimum level of casein to give maximum gel to natural yogurt with inulin. Moreover, the different effect of the yogurt culture or *Bifidobacterium spp.* on the different levels of oligosaccharides which generate undesirable acids when utilize some of the inulin and the effect of the inulin on the gel strength, viscosity and flavour of the natural yogurt.

2.7.2 OLIGOSACCHARIDES:

Oligosaccharides of various types found in common foods such as fruit, vegetables, milk and honey (Van Loo, 1995).

Oligosaccharides often have between 2-10 polymers of sugar. It is often contain low levels of monosaccharide and di- or polysaccharides.

They have various properties depended on their molecular- weight; low molecular- weight can used in food freezing temperature and the high once use for thickness and increase body and mouth feel.

Roberfroid (1999a) shows that the increase use of these ingredients back to their reduce energy value, potential anticariogenic and bifidogenic properties.

Grittenden and Playne (1996) have selected food grade oligosaccharides and listed them: Fructo-oligosaccharides (FOS), Galacto-oligosaccharides (GOS), Lactulose, Lactosucrose, Malto-oligosaccharides, Isomalto-oligosaccharides, soybean- oligosaccharides and xylo-oligosaccharides in the commercial productions. Of these food grade oligosaccharides there are some of them, which have the bifidogenic effect and health promoting properties; such as Fructo-oligosaccharides and Galacto-oligosaccharides.

2.7.2.1 GALACTOOLIGOSACCHARIDES:

Galacto-oligosaccharides (GOS) are found in the human milk and proved to have the bifidogenic promoting effect for the breast-fed infants (McVeagh and Miller, 1997; Rubaltelli *et al.*, 1998; Rycroft *et al.*, 2001).

GOS is primary production from Cow's Whey-derived lactose (Yanahira *et al.*, 1995). The lactose solution is converted to GOS by the action of β -galctosidase, which has transgalactosylation activity (Figure 2.2). GOS have a DP of 2 to 10 units, which are able to pass through the upper gut without hydrolysed by digestive enzymes, to reach the large intestine and beneficially affects the host by stimulating the growth and/or activity of target flora only.

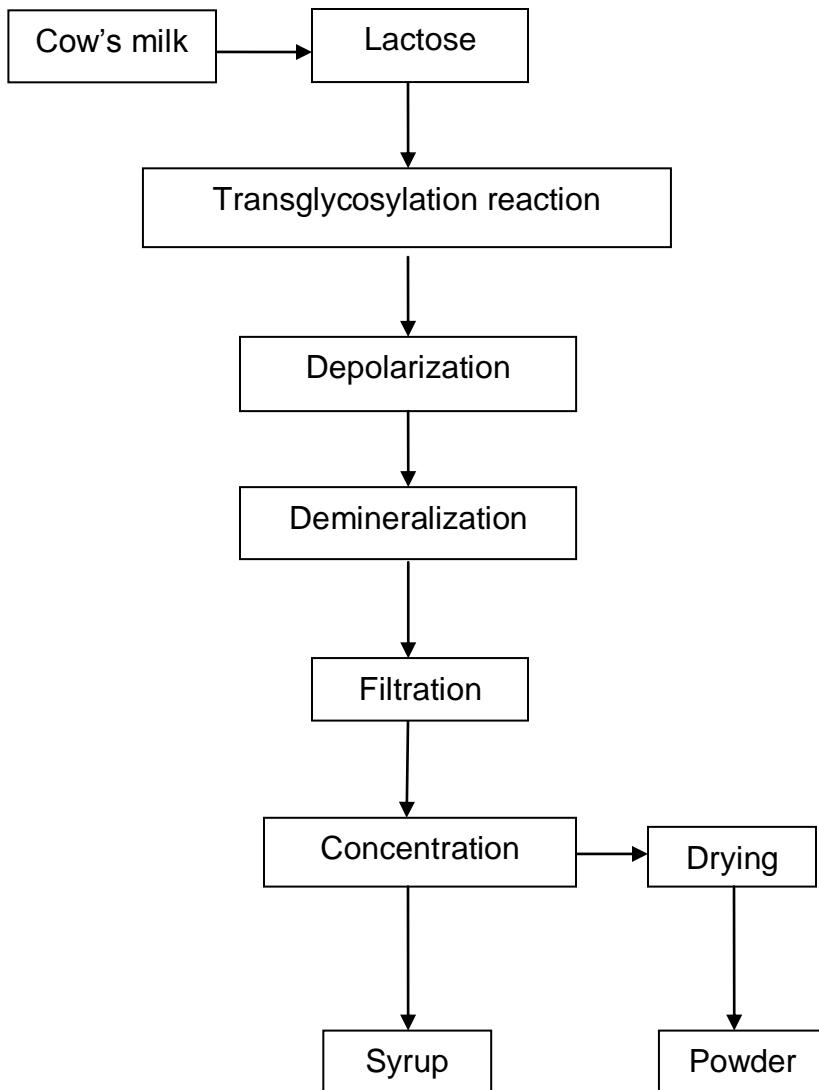


Fig2.2 Production of galactooligosaccharide from cows' milk (adapted from Tungland (2003))

GOS found in the breast milk at level of 1g/L (Chaturvedi *et al.*, 1997). Therefore, it is considered as bifidogenic factors in fermented milk products and baby foods.

GOS consist of number of β 1-6 linked galactosyl residues bound to a terminal glucose unit via α 1-4 linkage (Kunz and Rudloff, 2006), that make GOS utilized by some probiotics (stimulating their growth) depend on their enzymes and especially Lactobacillus group (Huebner *et al.*, 2007). GOS can be used in food

contributing to both thickness and mouth-feel, sweeteners as its sweetness is 35% of sucrose and source for fiber. They have calorie value of 1.7Kcal/g (Watanuki *et al.*, 1996).

2.7.2.2 FRUCTOOLIGOSACCHARIDES:

Fructo-oligosaccharides (FOS) and Inulin are the most classified as bifidogenic oligosaccharides in production (Slavin, 1999).

Fructooligosaccharides (FOS) contains between 2- 4 of β (2 \rightarrow 1) linked fructose units linked to the terminal α -D-glucose residue. The natural way of FOS production is by enzyme hydrolysis of Inulin (Figure 2.3). Therefore, there is no difference from the chemical view between inulin and FOS, but the DP for FOS is between 2-10 units with an average of 4 (Kaur and Gupta, 2002).

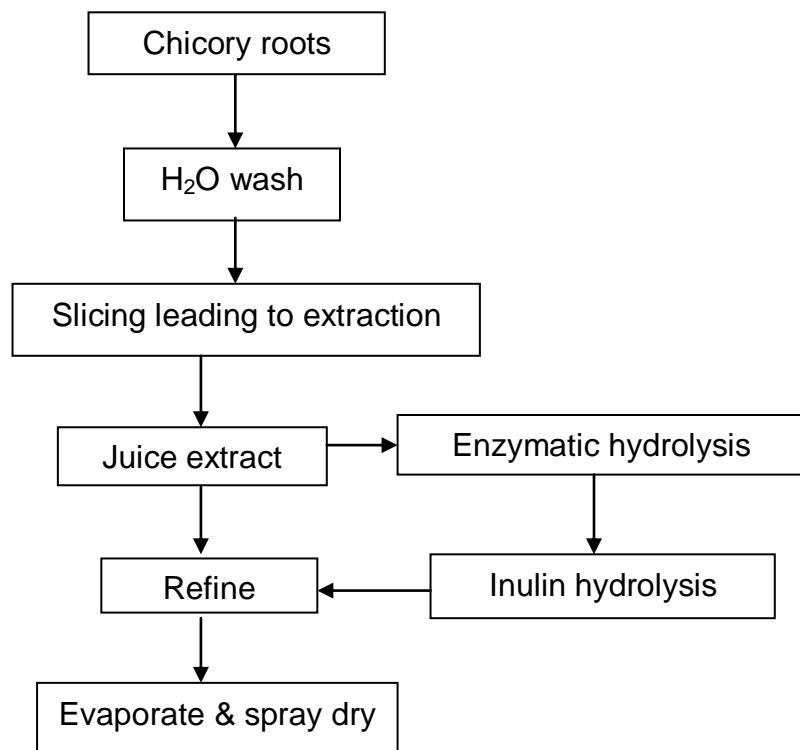


Fig2.3 Inulin and oligofructose manufacturing process (adapted from Tungland (2003))

Oligofructose is more soluble than sucrose and Inulin, provides 30-50% of the sweetness of table sugar. It is considered to be low-calorie ingredients which around 1.5 Kcal/g.

Inulin and FOS are widely found in nature and manufactured by companies such as Orafti, Sensus and Cosucra.

A lot of scientific studies *in vivo* have shown that FOS (RAFTILOSE® P95) and Inulin (RAFTILINE® ST) from ORAFTI have bifidogenic effect on host; When consumed at a dose of 5g/day for oligofructose and \leq 8 g/day for inulin, they significantly modify the composition of the intestinal (faecal) flora, selectively increasing the numbers of Bifidobacteria and reducing the harmful bacteria (Gibson and Wang, 1994; Reddy *et al.* 1997; Roberfroid *et al.* 1998; Rao, 1999; Tuohy *et al.* 2000; Menne *et al.* 2000) (Figure 2.4).

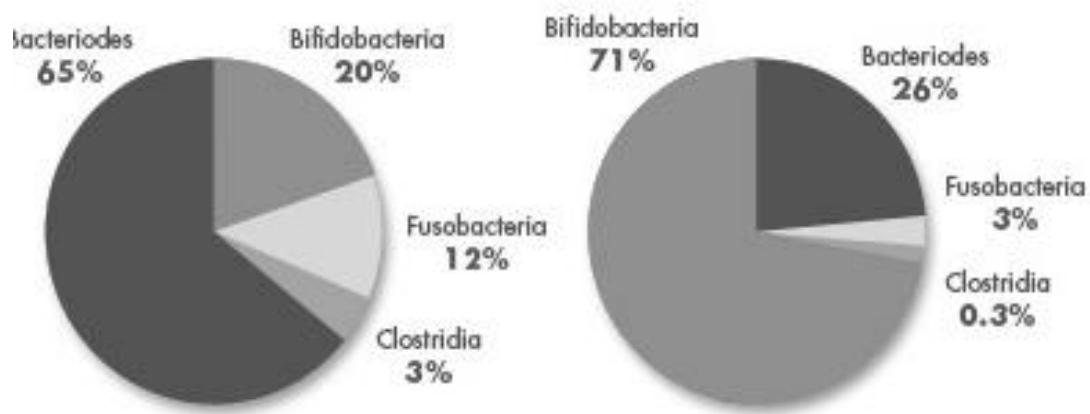


Fig2.4 Increase in Bifidobacteria after intake of 15g/day of inulin (Adapted from Gibson *et al.*, 1995).

Food for Specified Health Use (FOSHU) is unique system in Japan for approving ingredients/foods with a functional claim (Verschuren, 2002; Shortt, 2003). Prebiotic containing foods are most common in this category because the most FOSHU food designed for the gastrointestinal health benefits.

The survival of probiotics including *L. acidophilus*, *L. Casei* and *Bifidobacterium spp.* was improved by adding of FOS (P95, from ORAFTI) at 1.5% w/v to yogurt containing these microorganisms (Capela *et al.*, 2006).

Inulin and oligofructose have found to improve 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 *et al.*, 2001). Use oligofructose in the diet by 15g/day produce substantial changes in the intestinal microflora and increase the faecal bifidobacteria count and reduce the potential pathogens such as *Clostridium*, *E. coli* (Gibson *et al.*, 1995).

It has found that symbiotic supplements of Oligofructose and *L.rhamnosus* and *B. animalis* subsp. *Lactis* improved the immune responses and stopped colon tumors (Roller *et al.*, 2004; Watzl *et al.*, 2005).

Prebiotics have shown resistance to gastrointestinal infection because of their stimulatory effect on *Bifidobacterium* spp., which produces several anti-pathogenic mechanisms (Gibson *et al.*, 2005).

Although prebiotics can help to increase the beneficial bacteria in the gastrointestinal tract, a general increase in the beneficial bacterial population

may however not necessarily contribute to increased health effects, as it is strain related.

2.8. FACTORS AFFECTING THE GROWTH AND SURVIVAL OF LACTOBACILLUS AND BIFIDOBACTERIUM IN MILK PRODUCTS

The consumption of probiotic bacteria within food products is the most popular way to re-establish the gastrointestinal microflora balance. The literature had stated that probiotic products have to present no less of 10^6 cfu in ml of probiotic bacteria at the time of consumption to get the beneficial health on the host (Rybka and Kailasapathy, 1995; Dave and Shah, 1997a; Lourens-Hattingh and Viljoen, 2001; Adhikari *et al.*, 2003).

Dairy products is one of the most common carrier have been used as probiotic food products (Lourens-Hattingh and Viljoen, 2001; TianHong and XiangChen, 2004). Therefore, it is of interest to study some factors that affect the growth and survival of probiotic bacteria while in transit in dairy products to human use.

Many factors have been reported to affect the growth and survival of probiotic bacteria in dairy products, including acid and hydrogen peroxide produced by yogurt bacteria, oxygen content in the product and oxygen permeation through the package and the storage temperature (Shah, 2000a; Talwalkar and Kailasapathy, 2004; Bolduca *et al.*, 2006). Dave and Shah (1997c) suggested of using ascorbic acid in dairy products as scavenger to reduce the oxygen content and redox potential of dairy products to enhance the viability of probiotic bacteria. The survivability and viability of probiotic bacteria were one of the most interested elements in the probiotic world studies and that because *L. acidophilus* and

Bifidobacterium spp. are considered sensitive in dairy products, but this sensitivity was different in different strains of *L. acidophilus* (Nighswonger et al., 1996; Gilliland et al., 2002). However, it has been found that Bifidobacteria are not as acid tolerant as *L. acidophilus* and have been reported to show weak growth in milk and require an anaerobic environment and the addition of bifidogenic factors to achieve the desired levels of growth (Kailasapathy and Chin, 2000; Lourens-Hattingh and Viljoen, 2001; Matto et al., 2006b; Donkor et al., 2007).

Vernazza et al. (2006) has stated “Most bifidobacteria are poorly resistant to strongly acidic conditions with the exception of *Bifidobacterium lactis* Bb12”.

L. acidophilus and *Bifidobacterium* spp. were the most important highlighted strains in dairy products, which were subjected to growth and survivability tests, including exposure to low pH (Palframan et al., 2002; Cotter and Hill, 2003; Vernazza et al., 2006), high sucrose concentration and low storage temperature or high growth temperature (Ostlie et al., 2005; Mortazavia et al., 2007). Growth and survival of probiotic bacteria was found to be strain dependent (Ventling and Mistry, 1993; Godward et al., 2000; Crittenden et al., 2001a; Phillips et al., 2006). The growth and viability of probiotic bacteria is also found to be affected by the culture conditions, the phase of manufacture that has been added to the dairy products, production of hydrogen peroxide due to bacterial metabolism, and the concentrations of lactic and acetic acids (Shah, 2000a; Saarela et al., 2004; Kongo et al., 2006).

The interaction among probiotic species and yogurt starter cultures is also considered important in determining their growth and survival status in dairy

products. Various inhibitory interactions were found among these bacteria (Dave and Shah, 1997b; Joseph *et al.* 1998; Vinderola *et al.*, 2002). However, it has been found that co-inoculation of bifidobacteria with yogurt starter culture during yogurt production tended to suppress the growth and reduce the survival rate of bifidobacteria (Samona and Robinson, 1994; Tharmaraj and Shah, 2003; Donkor *et al.*, 2007).

Growth and survival of probiotic bacteria has also been found to be affected by the chemical and microbiological composition of milk, milk solids content, and availability of nutrients (Kneifel *et al.*, 1993; Kailasapathy and Supraidi, 1996; Shah, 2000b).

Loh and Maznah (1999) have found that using different milk has different effect on the growth and survival of probiotic bacteria. In the same subject Liepke *et al.* (2002) suggested that human milk have high stimulate nutrient for the growth and survival of bifidobacteria. The term bifidus factors has been used to refer to these nutrients, which has been found high in human milk and less in cow milk (Roy *et al.*, 1990; Shah, 2000a).

The growth and survival of *L. acidophilus* and bifidobacteria can also be influenced by the fat content of the milk, where it has been found that full fat yogurt has inhibitor properties for *B. bifidum* in compare with the reduced-fat yogurt (Vinderola *et al.*, 2000).

Moreover, the incubation temperature, fermentation time, storage temperature, and adding casein hydrolysates have been shown effect on the growth and survival of probiotic in dairy products (Kneifel *et al.*, 1993; Lourens-Hattingh and

Viljoen, 2001). Since bifidobacteria strictly anaerobic, so the dissolved oxygen levels in the product have also been cited to be important factors affecting the survival of probiotic bacteria in dairy products (Klaver *et al.*, 1993).

Hughes and Hoover (1995) suggested possibility to enhance the survivability of bifidobacteria in skim milk by keep the pH same with no change in low temperature storage 3-4°C.

2.9. EFFECT OF DIFFERENT NUTRIENTS ON BIFIDOBACTERIA GROWTH AND SURVIVAL:

Maintaining the growth and viability of bifidobacteria in dairy products has been a major challenge to the fermented dairy producers because of the inability of bifidobacteria to grow in milk. Therefore, there were a lot of suggestion to use a lot of nutrients to enhance the growth and survival of different strain of probiotic strains. This nutrient has termed as Bifidogenic Factors and defined by Modler (1994) as following “Compound that survive direct metabolism by the host, and reach the large bowel or cecum for preferential metabolism by bifidobacteria”.

It has found that the growth and viability of probiotic *in vitro* were dependent on prebiotics as well as strain. However, using prebiotics with probiotic in milk has found to enhance the growth and survival of probiotic bacteria in fermented dairy products (Desai *et al.*, 2004). *Bifidobacterium animalis* showed good stability in yogurt in present of FOS and it was better than *B. longum* in yogurt contain FOS which remained above 10^6 CFU/g (Akalin *et al.*, 2004; Akalin *et al.*, 2007).

Zhao *et al.* (2006) have found that casein hydrolysates shorten the fermentation time of yogurts and increase the probiotic count but the total number in the

storage period has decreased, which related by the hydrolysates. FOS and lactulose were used in human diets to support the growth of bifidobacteria (Modler *et al.*, 1990; Modler, 1994). Where it found that such prebiotics have potential to increase bifidobacteria level in a symbiotic yogurt products during the shelf life of the dairy products (Crittenden *et al.*, 2001a). Using the prebiotics and probiotics in combination coined as symbiosis (Roberfroid, 1998). Additions of Raffinose family oligosaccharides (RFOs) have beneficial effects on the survival of *Bifidobacterium Lactis* Bb12 and *L. acidophilus* in dairy products (Martinez-Villaluenga *et al.*, 2006).

Dave and Shah in (1997b) and Guler- Akin and Akin in (2007) suggested adding cysteine to enhance the viability of probiotic bacteria in bio-yogurt. Others found that using of number of growth-promoting substances can improve the viability of bifidobacteria such as cysteine, acid hydrolysates, and tryptone (Dave and Shah, 1998).

Bifidobacteria require carbonate or bicarbonate (or CO₂ gas) as carbon sources and to help tolerant oxygen present, but it can't use fatty acids or organic acid as carbon source. Where it has established that cysteine or cystine is an essential nitrogen sources (Shah, 1997b).

Bouhallab *et al.* (1993) found that using tryptic digest of caseinomacropeptide enhanced the growth bifidobacteria in skim milk. Janer *et al.* (2004b) have used supplementation of 2% Caseinomacropeptide (CMP) or whey protein in milk to enhance *B. lactis* growth. Klaver *et al.*, (1993) and Dave and Shah (1998) stated

that the amino acids and peptides of acid hydrolysis of casein improved the viability of probiotic bacteria.

A previous study by Poch and Bezkorovainy in (1988) has indicated that only a limited number of bifidobacteria will grow optimally in synthetic medium contain vitamins, nitrogen and carbon, but bovine casein hydrolysed and yeast extract have a promoting activity on bifidobacteria, and they have reported that κ -casein is the main growth promoter for bifidobacteria in bovine milk (Azuma *et al.*, 1984). In the same subject Saxelin *et al.* (1999) Indicate that the addition of glucose, yeast extract or milk protein fractions enhanced the growth of the most probiotic strains and co-combined with yogurt culture enhanced the survival of all the probiotic strains.

The idea of mix probiotic, which is with low proteolytic activity with high proteolytic organisms such as Lactic Acid Bacteria (LAB) or *S. thermophiles*, will enhance the growth and survival of these probiotic bacteria (Klaver *et al.*, 1993; Shihata and Shah, 2000; Champagne *et al.*, 2005). In other hand, the addition of casein hydrolysates was suggested to recover the slow proteolytic activity of some probiotic strains and enhance their growth and survival in dairy products (Lourens-Hattingh and Viljoen, 2001; Warminska-Radyko *et al.*, 2002).

In vitro study Ustunol and Gandhi (2001) have shown the great enhancement occur of using honey on the growth and survival of *Bifidobacterium bifidum* in Honey-Sweetened skim milk.

2.10. BIOCHEMISTRY STUDY OF THE FERMENTATION PROCESS OF BIFIDOBACTERIA IN MILK:

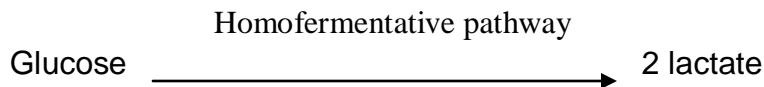
The common result of the bacterial fermentation process is lactate. It is referred to the bacteria, which produce lactate in their fermentation as Lactic Acid Bacteria (LAB). Some of these organisms require very complex nutrition, which is related to their environments such as plant, milk and intestinal of animals or humans. Lactic Acid Bacteria are strictly fermentative and some are oxygen tolerant, such as *Streptococcus*. Others are obligate anaerobes such as *Bifidobacteria* spp. but some can tolerate oxygen in the presence of carbon dioxide (CO_2) (Shah, 1997). Bifidobacteria naturally inhabit the gut of animals and humans and their sensitivity to oxygen is strain related and the less sensitive strain appears to possess weak catalase activity that removes H_2O_2 . Hydrogen peroxide inactivates Fructos-6-phosphate phosphoketolase F6PPK (Shah, 1997), a key enzyme of bifidobacteria in carbohydrate metabolism (Fandi *et al.*, 2001; Marks, 2004).

Bifidobacteria do not produce CO_2 , Butyric or propionic acid (Modler *et al.*, 1990). Their optimum growth temperature is 37°C to 43°C , and the optimum pH is 6.5 to 7.0 (Shah, 1997). Bifidobacteria also produce thiamine, riboflavin, and vitamin B and K (Deguchi *et al.*, 1985; Morishita *et al.*, 1999; Hou *et al.*, 2000; Sybesmaa *et al.*, 2004).

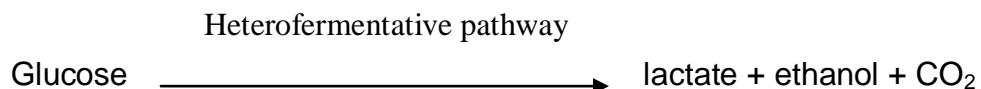
These genera are also unique by producing the lactic acid in the form L (+)- lactic acid that is easier to metabolize by infants in comparison with the D (-)- lactic acid form which is produced by *L. acidophilus* and *L. bulgaricus* (Marks, 2004).

There are three pathways suggested for the fermentation process of LAB of the carbohydrates to lactate.

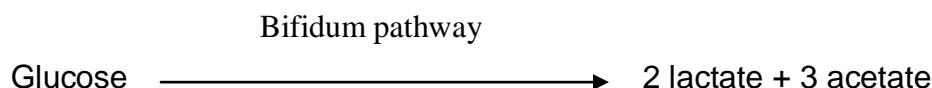
The homofermentative pathway, which yields 2 mol of lactate per mol of glucose:



The heterofermentative pathway, which yields 1 mol of each lactate, ethanol, and CO_2 per mol of glucose:



The bifidum pathway, which yields acetate and lactate in ratio of 3 to 2 respectively:



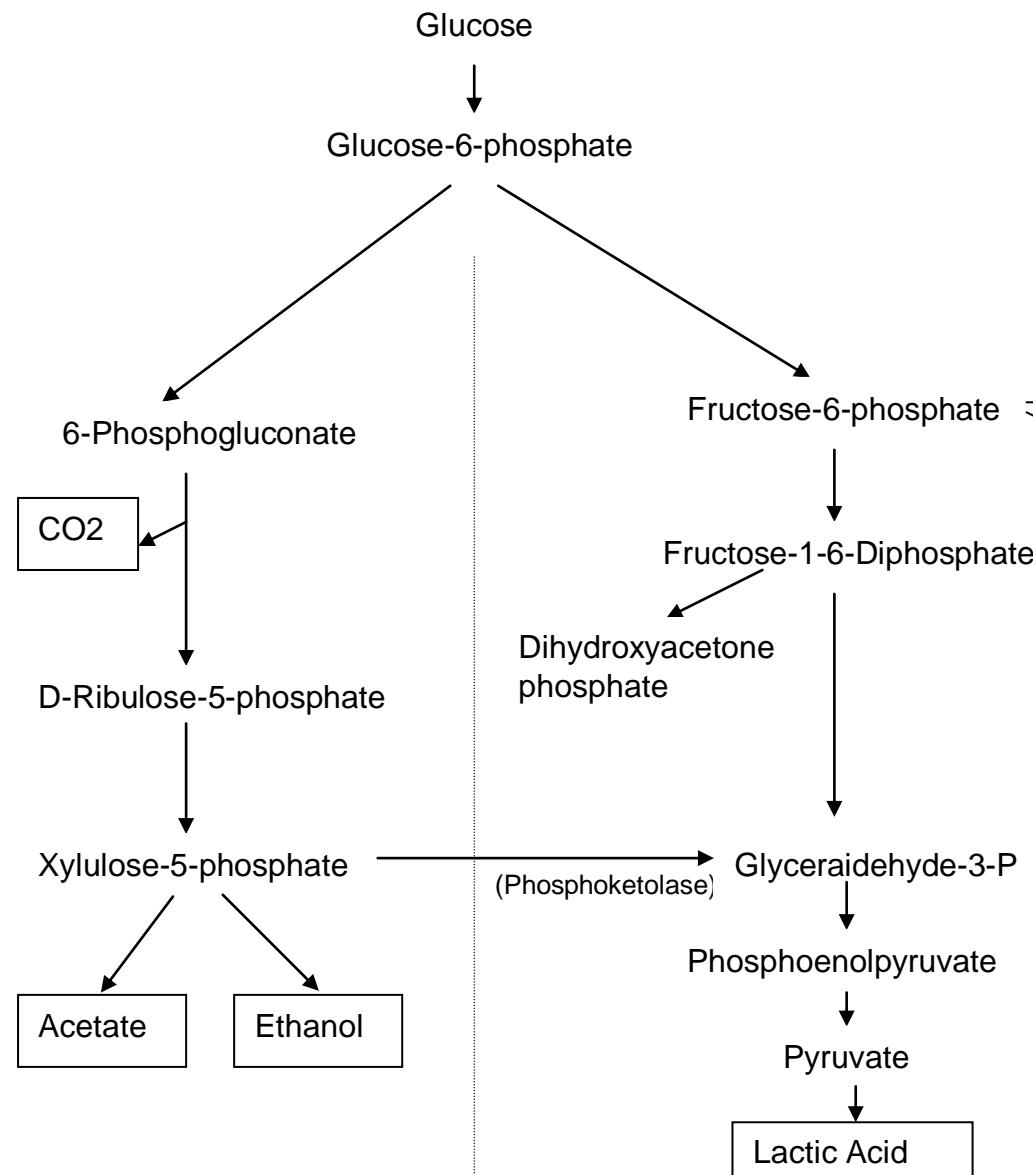
Bifidobacteria metabolized carbohydrate as main carbon source is through fructose-6-phosphate shunt by using Fructos-6-phosphate phosphoketolase (F6PPK), which is distinguishing bifidobacteria from lactobacilli. This pathway produces L (+) lactic acid and acetic acid in ratio 3:2, some species produce formic acid and ethanol as well.

Figure (2.5) show differs in the process of carbohydrates fermentation among homo-, heterofermentative bacteria and *Bifidobacteria* spp.

Bifidobacteria utilize lactose, galactose and fructose beside glucose, and has the ability to metabolize oligosaccharides beside the simple sugars such as Inulin, FOS etc. This ability is strain related, for example it has found that *Bifidobacterium Lactis* Bb12 possessed the enzymes required to utilize some

kind of sugar such as raffinose family and lactose which are unable to metabolism by other strain of bifidobacteria (Martínez-Villaluenga and Gomez, 2007). These enzymes which were found in most strains of bifidobacteria and not found in the lactic acid bacteria (Desjardins *et al.*, 1990), *Bifidobacterium Lactis* Bb12 were found to possess the highest activity of such like enzymes which found with low activity in other strains, these enzymes such as β -glucosidase, α -glucosidase, D-glucosaminidase and β -galactosidase, which are very important in the fermentation process with bifidobacteria (Semjonovs *et al.*, 2004; Martínez-Villaluenga and Gomez, 2007). *Bifidobacterium Lactis* Bb12 has the ability to grow faster in milk because of possessing the highest activity of leucine aminopeptidase to help the hydrolysis of milk proteins, which stimulate their growth (Desjardins *et al.*, 1990; Martínez-Villaluenga and Gomez, 2007).

**HETROFERMENTATIVE
BACTERIA**



**HOMOFERMENTATIVE
BACTERIA**

**FRUCTOSE-6-PHOSPHATE PHOSPHOKETOLASE
(F6PPK) BIFIDOBACTERIA**

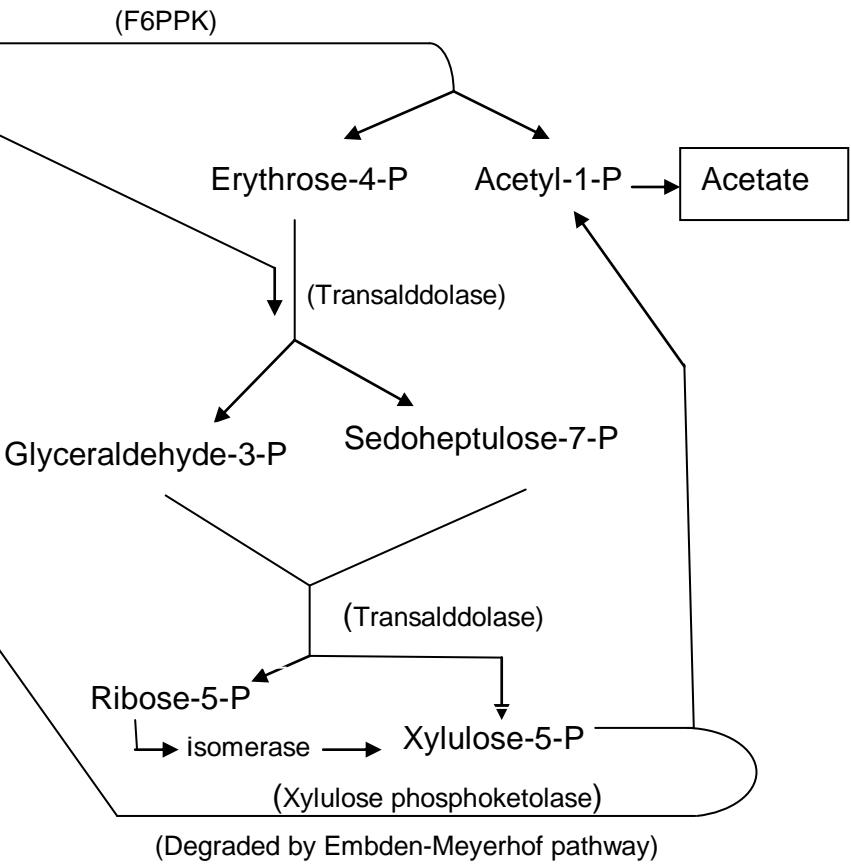
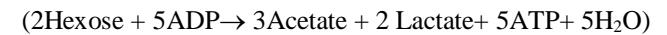


Fig 2.5 Brief summary of the metabolic pathways and products of hetro- and homofermentative bacteria and bifidobacteria
(Adapted from Modler et al., 1990)

Janer *et al.* (2004a) has found the ability of *Bifidobacterium Lactis* Bb12 to cleave fructooligosaccharides (FOS), which has the β (2-1) linkage related to possess β -fructofuranosidase.

The high activity of N-Acetyl-D-glucosaminidase were found in *B. bifidum* strain and lower in *B. infants* and *B. breve*, these bacteria isolated from human source, which help to utilize N-Acetylc-D-glucosamine sugars which has found only in human milk (Desjardins *et al.*, 1990; Ward *et al.*, 2006). Sakai *et al.* (1987) indicated that Bifidobacteria breve grow very good in soymilk because it has α -D-galactosidase which help to utilize α -D- galactosyl oligosaccharides in soy milk.

Al-Tamimi *et al.* (2006) indicated enhance the fermentation and growth of bifidobacteria by using of lower degree polymers (DP of 1-2 and less than 8) of oligosaccharides such as arabinose in the first 24 hours.

2.11. AIMS AND OBJECTIVES

This study had two aims. The first was to screen the effect of different prebiotics, various concentrations, and different degrees of hydrolyzed skim milk on the growth and survival of bifidobacteria spp., *in vitro*.

The second aim was to develop a new product by using a mixture of prebiotics and hydrolyzed skim milk with optimum health benefits by promoting better growth and survival and be acceptable to the consumers with high number of *Bifidobacterium lactis* Bb12.

Thus, the specific objectives of this project were to:

1. Evaluate the effect of varying concentrations of Inulin on the growth and survival of *Bifidobacterium lactis* Bb12,
2. Evaluate the effect of Fructooligosaccharides (FOS) in different percentage on the growth and survival of *Bifidobacterium lactis* Bb12,
3. Study the effect of different Degrees of Hydrolysis (DH) on skim milk hydrolyzed with trypsin on the growth and survival of *Bifidobacterium lactis* Bb12,
4. Evaluate the combination of the best environment results obtained from objectives 1, 2, and 3 on the growth and survival of *Bifidobacterium lactis* Bb12. and defined as the optimal product mixed design.
5. Evaluate the effect of the Optimum Product Mix Design Skim Milk (MDSM) on the growth and survival of the following strains of probiotics: *Bifidobacterium bifidum* NCIMB 702203, *Bifidobacterium infantis* NCIMB 702205 and *Bifidobacterium breve* NCIMB 702257 and *Lactobacillus casei* strain Shirota,
6. Examine the sensory characteristics of a new fermented probiotic yogurt drink using the MDSM formulation.

CHAPTER THREE

EFFECT OF PREBIOTICS (FOS & INULIN) ON PROBIOTIC GROWTH AND SURVIVAL IN SKIM MILK

3.1. INTRODUCTION:

The health benefits and bifidogenic effect of prebiotics have been recognized from the last century (Shimoyama *et al.*, 1984; Bouhnik *et al.*, 1994; Gibson *et al.*, 1995; Djouzi and Andrieux, 1997; Roberfroid *et al.*, 1998; Sanders, 2003; Martinez-Villaluenga and 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 and Roberfroid, 1995).

A tactic that combines both probiotics and prebiotics has been termed *synbiotic*. *Synbiosis* is defined as “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 and Roberfroid, 1995).

In recent years, there has been more focus on a combination of pre-and probiotics in a single product (Hammes and Hertel, 2002; Bielecka *et al.*, 2002).

Fructooligosaccharide and Inulin are the premium prebiotics used for the purpose of stimulating the growth and/or activity of beneficial bacteria in the large intestine. Therefore, this research is to study the effect of prebiotic (Inulin and/or

FOS) on the growth, survival and/or activity of Probiotics in dairy products (the carrier).

3.2. SYNERGSITIC INTERACTIONS AMONG INULIN, FRUCTOOLIGOSACCHARIDES (FOS) AND PROBIOTICS.

The synbiotic concept has been widely used by European dairy drink and yogurt manufacturers (Niness, 1999). It reflects the synergistic relationship between the beneficial bacteria and their selective substrates to stimulate their growth when they survive passing through the stomach to the large intestinal to establish their predominance (Rao, 2002).

Inulin and Fructooligosaccharides have their stimulating effect because of their ability to be fermented by bifidobacteria and Lactic acid bacteria *in vivo* (Gibson et al., 1995) and *in vitro* (Kaplan and Hutkins, 2000, Perrin *et al.*, 2001). However, recent studies have determined that the ability of bifidobacteria to metabolize fructo-oligosaccharides and inulin is a species-dependent feature and only to a small extent a strain-dependent one related to their enzyme content (Bielecka *et al.*, 2002, Bielecka and Biedrzycka, 2004). For example β -fructofuranosidase from *B. adolescentis* G1 has a unique substrate specificity to fructooligomers rather than inulin (Muramatsu *et al.*, 1994, Van der Meulen *et al.*, 2004), and the same applies for *B. bifidum* strain (Hartemink & Rombouts, 1997). On the other hand, strains of *B. longum* and *B. animalis* were able to hydrolyse the wide range of oligosaccharides with a high degree of polymerization (DP) including FOS with degree of polymiraztion (DP= 2-4) and inulin (DP> 8) (Van Laere *et al.*, 1997, Bruno *et al.*, 2002).

The highest viable number of bifidobacteria ($3.59\text{--}2.25 \cdot 10^7$ CFU/g) was obtained in the product containing *B. animalis* and FOS and was greatest with hi-amylose corn starch (hi-maize) (Bruno *et al.*, 2002). Viability of *B. longum* in yogurt containing FOS remained above 10^6 CFU/g for up to 21 days (Akalin *et al.*, 2004). However, *Bifidobacterium* is significantly less tolerant to low temperature storage in milk than *L. acidophilus* (Hughes and Hoover, 1995). *Bifidobacterium lactis* reclassified as a subspecies of *Bifidobacterium animalis* (Meile *et al.*, 1997). Commercial probiotic strain *Bifidobacterium animalis* ssp. *lactis* Bb12 were classified with a distinctive tolerance to heat and oxygen (Simpson *et al.*, 2005) and was isolated from over fifteen dairy products in Europe as the main *Bifidobacterium* strains (Iwana *et al.*, 1993, Meile *et al.*, 1997, Klein *et al.*, 1998, Gueimonde *et al.*, 2004). *B. lactis* possesses the required enzyme (β -glucosidase) (Martinez-villaluenga and Gomez, 2007) and β -fructofuranosidase (Janer *et al.*, 2004a) to utilize oligosaccharides (Gopal *et al.*, 2003) and to cleave the related β -(2,6) linked fructans containing substrate (Semjonoves, *et al.*, 2004) in MRS media and Fermented Milk which enhanced its growth and metabolic activity.

Literature has shown that the growth, activity and survival of Bifidobacteria *in vitro* depend on the strain of *bifidobacterium* (Bruno *et al.*, 2002, Biedrzycka and Bielecka, 2004). It appears that the synergistic effect of prebiotic, hydrolyzed milk and strain specific properties of *bifidobacterium* have not been investigated yet.

3.3 AIM

The study investigates the effect of prebiotics, specifically inulin and fructooligosaccharides (FOS) in various concentrations on the growth and survival of *Bifidobacterium Lactis* Bb12 in skim milk.

3.4 OBJECTIVE

- Prepare different concentration of Fructooligosaccharides and Inulin to apply it with *Bifidobacterium Lactis* Bb12
- Study and investigate the effect of Fructooligosaccharide FOS in different concentrations on the growth and survival of *Bifidobacterium Lactis* Bb12
- Examine the effect of different concentrations of Inulin on the growth and survival of *Bifidobacterium Lactis* Bb12
- Identify the best concentration of each of FOS and Inulin to improve the growth and survival of *Bifidobacterium Lactis* Bb12

3.5 MATERIAL AND METHODS:

This study divides in two parts; the first part examined the growth of the *Bifidobacterium Lactis* Bb12 and the other one examined the survival of *Bifidobacterium Lactis* Bb12 in skim milk with selected prebiotics.

3.5.1. PREPARATION OF FOS, INULIN IN THE SKIM MILK:

FOS and Inulin were obtained from ORAFTI active food ingredient, Belgium labeled as Beneo GR for Inulin (inulin ≥ 90%, glucose+ fructose ≤ 4%, sucrose ≤ 8% and the pH of 5-7) and Beneo P95 for FOS (oligofructose ≥ 95%, glucose+ fructose+ sucrose ≤ 5% and the pH of 4.5-6.5).

UHT Skim milk was obtained locally. Seven sterile bottles, with a capacity of 200 ml for each, were filled with 150 ml of skim milk in each. A different concentration of the selected prebiotics was added to each bottle as follows: three with 1, 3, and 5% FOS; three with inulin 1, 3, and 5% and one used as control without FOS and/or Inulin. Triplicate samples were made of those bottles. The skim milk with either Fructooligosaccharides (FSM), with Inulin (ISM) or with no supplements (Control) were pasteurized at 70°C for 15 min (Shin *et al.*, 2000a), and thereafter let it to cool to 37°C to be ready for the incubation with the *Bifidobacterium Lactis* Bb12.

3.5.2. PREPARATION OF *Bifidobacterium Lactis* Bb12 CULTURE:

An overnight (14-15h) culture was prepared from *Bifidobacterium Lactis* Bb12 freeze dry culture (obtained from Chr. Hansen, UK) in skim milk. This overnight *Bifidobacterium Lactis* Bb12 culture was used as an inoculum culture with concentration of 5% v/v (Vijaya *et al.*, 2002). of the prepared culture of *Bifidobacterium Lactis* Bb12 was added to each one of the prepared bottles in section (3.3.1) After the addition of the *Bifidobacterium lactis* Bb12 to the prebiotic skim milk bottles and the Control and before the incubation process, four sterile test tubes were filled with 10ml from each bottle. The bottles and the test tubes were incubated in anaerobic conditions in anaerobic cabinet designed for anaerobic growth at 37°C until reach the pH 4.2± 0.03, since lower pH will slow down or totally stop and become harmful on *Bifidobacterium Lactis* Bb12 growth (Zisu and Shah, 2003, Grosso and Favaro-Trindade, 2004).

3.5.3. MEASUREMENT OF THE GROWTH AND SURVIVAL OF *Bifidobacterium Lactis* Bb12:

The total counts of *Bifidobacterium lactis* Bb12 were taken at time Zero, which was just after the addition of the *Bifidobacterium lactis* Bb12 culture to the prebiotic skim milk. Afterwards, 1 mL sample of each bottle was obtained and diluted in MRD (Maximum Recovery Dilution, Oxoid, Basingstoke, United Kingdom), to get 10⁻⁴ dilution, then plated onto MRS – agar (Oxoid, Basingstoke, United Kingdom) supplied with 5% v/v L-cysteine-hydrochloride (Sigma Chemical Co., UK) plate (Shah, 2000a) and incubated anaerobically at 37°C for 72 hr.

Another sample was obtained for the total count at pH 4.2 ± 0.03 where the fermentation was stopped by immersing the bottles and the test tubes in ice-water until the temperature reached $4^{\circ}\text{C}\pm1$ at which it were refrigerated later for the survival study.

Samples for pH analysis indicated in three different experiments, that the noticeable change of the pH start after 16 hr of incubations for the *Bifidobacterium Lactis* Bb12 in the FOS and Inulin skim milk separately. Therefore, the pH measurements were taken at the beginning of the incubation and hourly after the first 16 h of the total incubation period for each bottle until the pH 4.2 ± 0.03 was reached using the pH meter (JENWAY, model 3510 pH meter, UK). Thereafter, all of the bottles and the test tubes were taken out of the anaerobic cabinet and immersed in ice and cold water to stop the fermentation process and to get cooled to $4^{\circ}\text{C}\pm1$, there where after stored under refrigeration for 28 days to apply the survival study. Change in pH and total count of *Bifidobacterium Lactis* Bb12 as (cfu/ml) over the storage period were measured by taking samples every seven days over the 28 days storage from each test tube and in triplicate to examined the survival of *Bifidobacterium Lactis* Bb12 in FSM and ISM separately and compare it with the controls.

3.5.4 STATISTICALL ANALYSIS:

Bifidobacterium lactis Bb12 growth was measured as pH changes over the fermentation period for each of the prebiotic supplements and for each concentration and were repeated three times, this data were analyzed by applying one-way analysis of variance (ANOVA, Minitab version 12). $P\leq0.05$ was

considered statistically significant. The change in the survival study of *Bifidobacterium lactis* Bb12 in each sample with different concentrations of the different prebiotics were determined using the total count of the colonies as cfu/ml and repeated three times, the data were analyzed by applying one-way analysis of variance (ANOVA, Minitab version 12). $P \leq 0.05$ was considered statistically significant.

3.6 RESULTS AND DISCUSSION:

3.6.1 Growth rate of *Bifidobacterium lactis* Bb12 in FSM and ISM environment:

Figures 3.1 and 3.2 show the pH change over the period of fermentation for skim milk containing Fructooligosaccharide (FSM) and Inulin (ISM) in pasteurized skim milk and cultured with *Bifidobacterium lactis* Bb12. Table 3.1 show the time required to reach pH 4.2 ± 0.03 during the fermentation.

Table 3.1 Time (h) to reach pH 4.2 ± 0.03 as a result of *Bifidobacterium lactis* Bb12 growth in skim milk without prebiotic (Control), with FOS 1%, with FOS 3%, with FOS 5%, with Inulin 1%, with Inulin 3%, and with Inulin 5%. Samples were taken in Triplicate.

Fermentation time of <i>Bifidobacterium Lactis</i> Bb12 (h)						
Control	FSM			ISM		
	1%	3%	5%	1%	3%	5%
30	30	28	27	33	28	33

The fermentation was carried under anaerobic conditions at 37°C for all samples and the time of fermentation was stopped when a pH 4.2±0.03 was reached. The results provided in Table 3.1 and Figure 3.1 and 3.2 are the mean of the triplicate pH measurements for FSM and ISM samples. As indicated in Table 3.1, the milk with different prebiotic concentrations required different fermentation times to reach pH 4.2±0.03. Data displayed in Figure 3.1 and 3.2 showed pH change over the fermentation period of ISM and FSM.

Table 3.1 showed that the fermentation period decreased by increasing the concentration of the FOS in the skim milk (FSM). The greatest effect was observed with the 5% FOS in skim milk as it reached the required pH in 27 hours which is decreased significantly ($P<0.05$) by 3 hours as compared to the control. But 5% Inulin concentration required greater fermentation time and was higher significantly ($P<0.05$) by 3 hours as compared to the control and 6 hours as compared to 5% FOS. However, the inulin showed good results with 3% concentration in ISM as the time of fermentation was decreased to 28 hours, which was reduced by 2 hours as compared to the control.

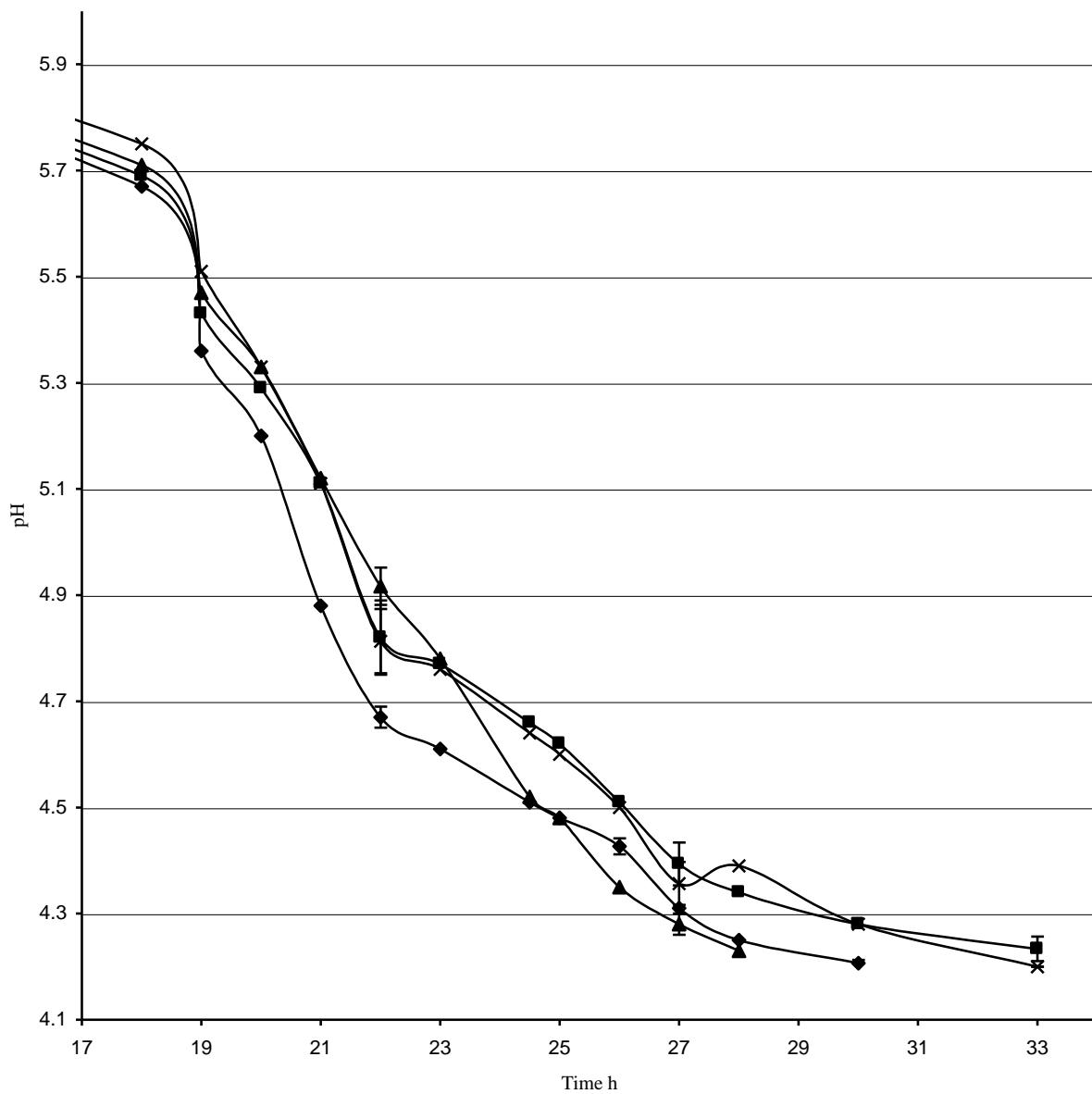


Fig 3.1 the pH change and development of *Bifidobacterium lactis* Bb12 growth to reach the end of fermentation at pH 4.2 ± 0.03 in skim milk without prebiotic (Control) (\blacklozenge), with Inulin 1% (\blacksquare), with Inulin 3% (\blacktriangle), and with Inulin 5% (\times). Error bars were taken of three samples as standard deviation.

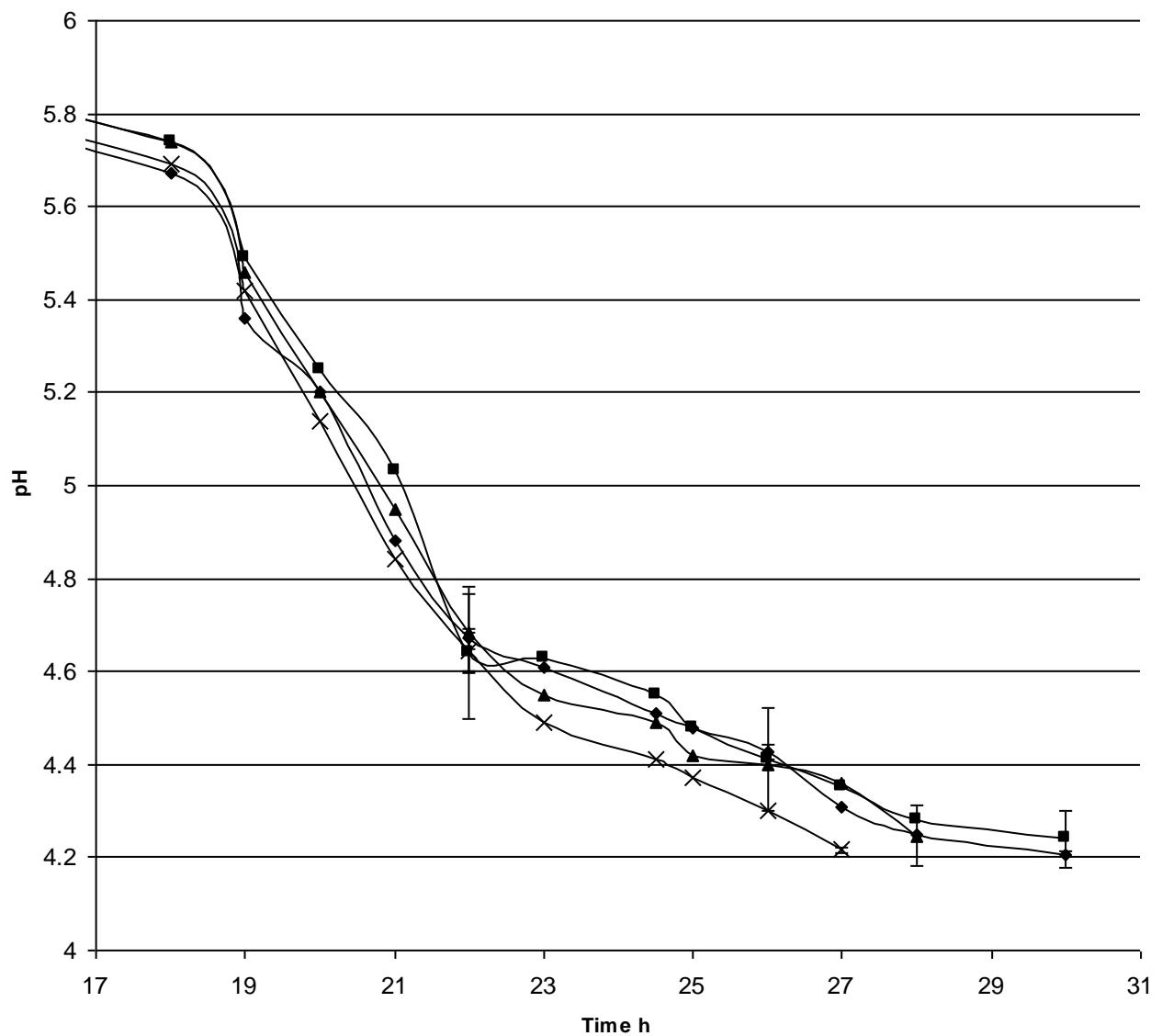


Fig 3.2 the pH change and development of *Bifidobacterium lactis* Bb12 growth to reach the end of fermentation at pH 4.2 ± 0.03 in skim milk without prebiotic (Control) (\blacklozenge), with FOS 1% (\blacksquare), with FOS 3% (\blacktriangle), with FOS 5% (\times). Error bars were taken of three samples as standard deviation.

The ability of *Bifidobacterium lactis* Bb12 to hydrolyze β -(2-1) linkage of oligosaccharides is well established in the literature. This is due to the fact that *Bifidobacterium Lactis* Bb12 contains specific enzymes that are able to hydrolyze oligosaccharides (Klein *et al.*, 1998). Moreover, *Bifidobacterium Lactis* Bb12 has been shown to posses the required enzymes (β -glucosidase) (Martinez-villaluenga and Gomez, 2007) and β -fructofuranosidase (Janer *et al.*, 2004a) to utilize oligosaccharides (variety of fructooligosaccharides) and to cleave the related β -(2,6) linked fructans containing substrate (Semjonoves, *et al.*, 2004) in MRS media and fermented milk that enhances its growth and metabolic activity. However, the low Degree Polymerisation (DP) of FOS helps the *Bifidobacterium lactis* Bb12 to better utilize it as a nutrient as compared with high DP of inulin. This yields through its fermentation pathway 3 moles of acetic acid and 2 moles of lactic acid per 2 moles of glucose, which results in the ratio (3:2) of acetic: lactic acid (Scardovi and Trovatelli, 1965). It seems that the lower the DP the greater the decrease in the pH in a shorter time span as shown FSM with compared to the ISM. The 1% FSM did not affect the fermentation time as compared to the control and the 3% of FSM and ISM showed similar fermentation times with the control. On the other hand, the concentration of 1 and 5% of ISM increased the fermentation time as compared to the control. The total count of *Bifidobacterium Lactis* Bb12 on MRS-agar with 5% v/v L-cysteine-hydrochloride showed that the \log_{10} is 9.88 and 8.78 (cfu/ml) for the 5% FSM and 3% ISM respectively, at the end of fermentation. Even though, the fermentation time of 5% FSM and 3% ISM were similar with only a one hour difference, the

colony count of 5% FSM was more and significantly ($P<0.05$) by 1 \log_{10} cfu/ml and therefore, validates further the use of FOS in skim milk to increase the total count and reduce the time of fermentation.

3.6.2 Survival of *Bifidobacterium lactis* Bb12 in FSM and ISM environment:

Table (3.2) and Figures (3.3 and 3.4) show the survival rate and the total count of *Bifidobacterium lactis* Bb12 in FSM and ISM over 28 days of refrigerated storage at $4^{\circ}\text{C}\pm1$. The changes in the total counts of *Bifidobacterium lactis* Bb12 as \log_{10} CFU/ml as shown in Figures (3.3 and 3.4) and in Table (3.2) were the reference for the survival rate over 28 days as considered the shelf life of dairy products (Desai *et al.*, 2004).

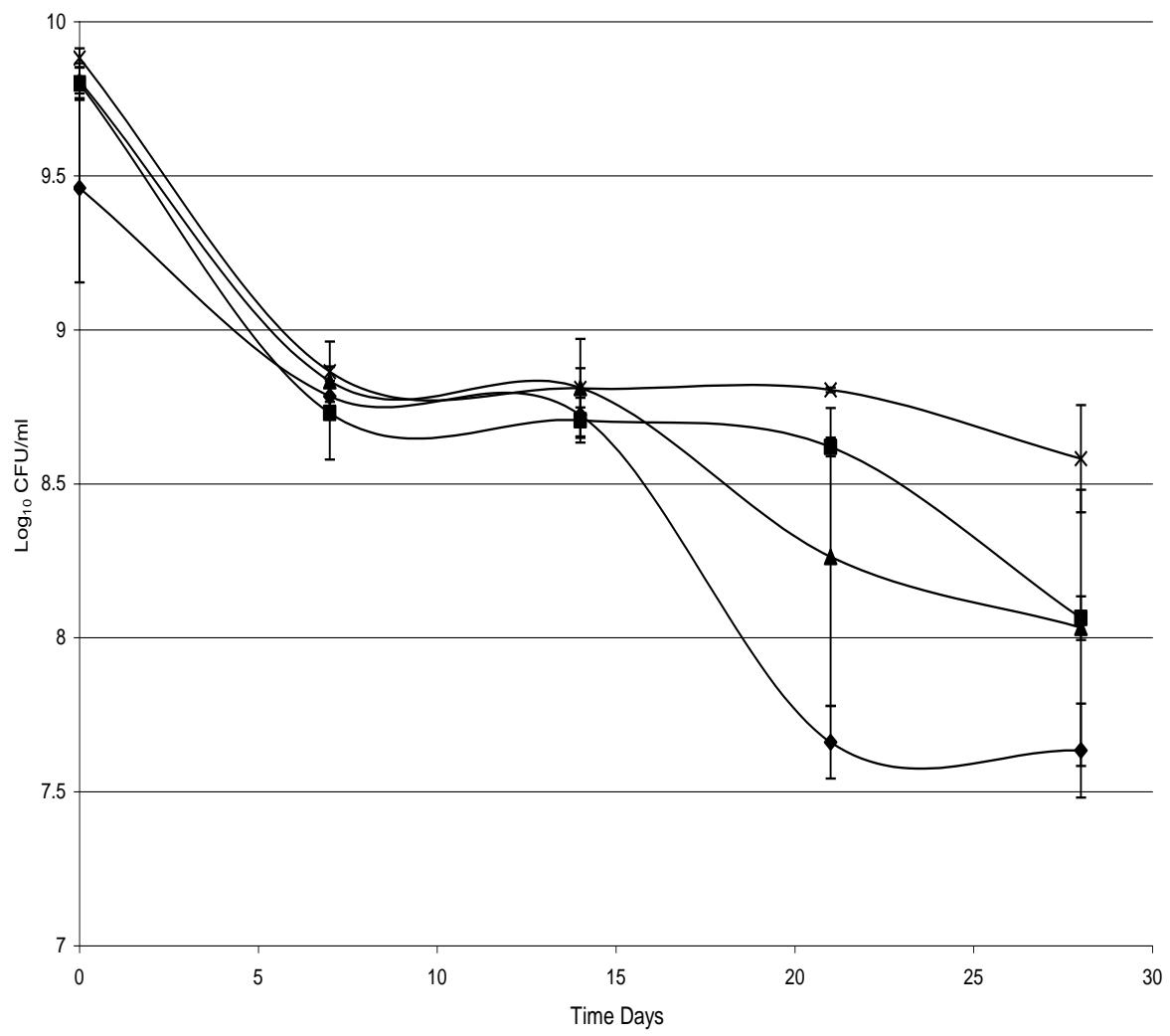


Fig 3.3 the \log_{10} of the viable count of *Bifidobacterium lactis* Bb12 in skim milk without prebiotics (control) (♦), with FOS 1% (■), with FOS 3% (▲), with FOS 5% (×), over 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$. Error bars indicate standard deviation for three samples.

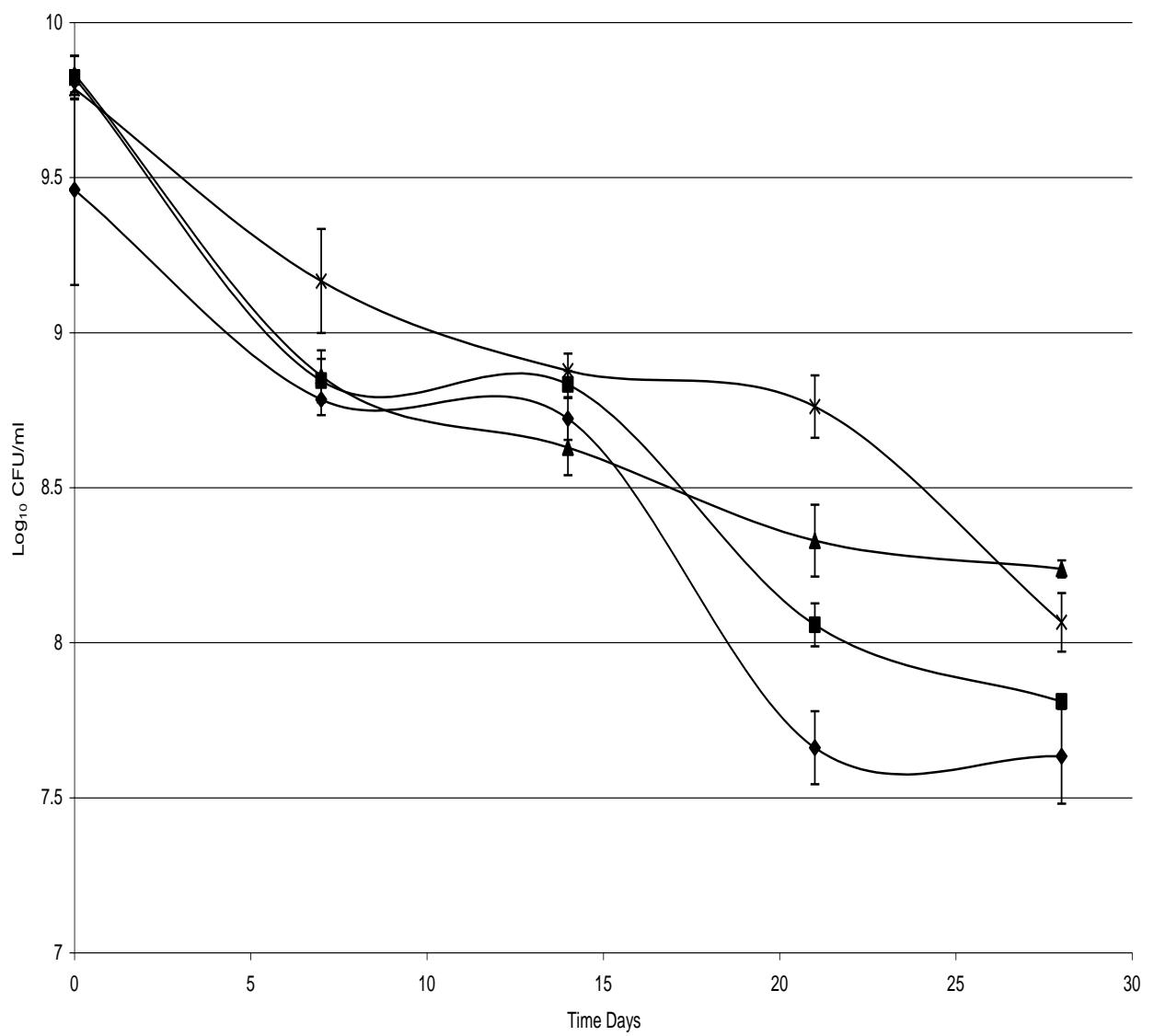


Fig 3.4 the \log_{10} of the viable count of *Bifidobacterium lactis* Bb12 in skim milk without prebiotics (control) (◆), with Inulin 1% (■), with Inulin 3% (▲), and with Inulin 5% (✗) over 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$. Error bars indicate standard deviation for three samples.

Table 3.2 Total count of different *Bifidobacterium lactis* Bb12 and their control in the and with 1,3 and 5% of FSM and ISM over 28 days refrigerated storage at 4°C ± 1.

Time Days	Total count of <i>Bifidobacterium lactis</i> Bb12 Log10 (CFU/ml)						
	Control	In the FSM			In the ISM		
		1%	3%	5%	1%	3%	5%
End of Fermentation at pH 4.2 ±0.03	9.46	9.79	9.80	9.88	9.82	9.83	9.78
After 28 days refrigerated storage at 4°C±1	7.63	8.06	8.03	8.58	7.81	8.23	8.06

Moreover, Table (3.3) shows the viability of *Bifidobacterium Lactis* Bb12 over the 28 days of refrigerated storage at 4°C ±1.

The viability was measured in as published by (Shin *et al.*, 2000a):

$$\% \text{Viability} = (\text{cfu/mL at 4 weeks of storage} / \text{initial cfu/mL}) \times 100$$

Table 3.3 viability % of *Bifidobacterium lactis* Bb12 in 1, 3 and 5% concentration of FSM and ISM after 28 days refrigerated storage at 4°C ± 1.

Viability of <i>Bifidobacterium. Lactis</i> Bb12 (%)						
Control	FSM			ISM		
	1%	3%	5%	1%	3%	5%
1.48	1.91	1.67	5.02	0.98	2.49	1.91

For the loss of the total count of the bacteria over the storage period Figure (3.5) shows these changes as a \log_{10} cfu/ml loss of the *Bifidobacterium lactis* Bb12 in the study environment with adding different concentrations of FOS and Inulin.

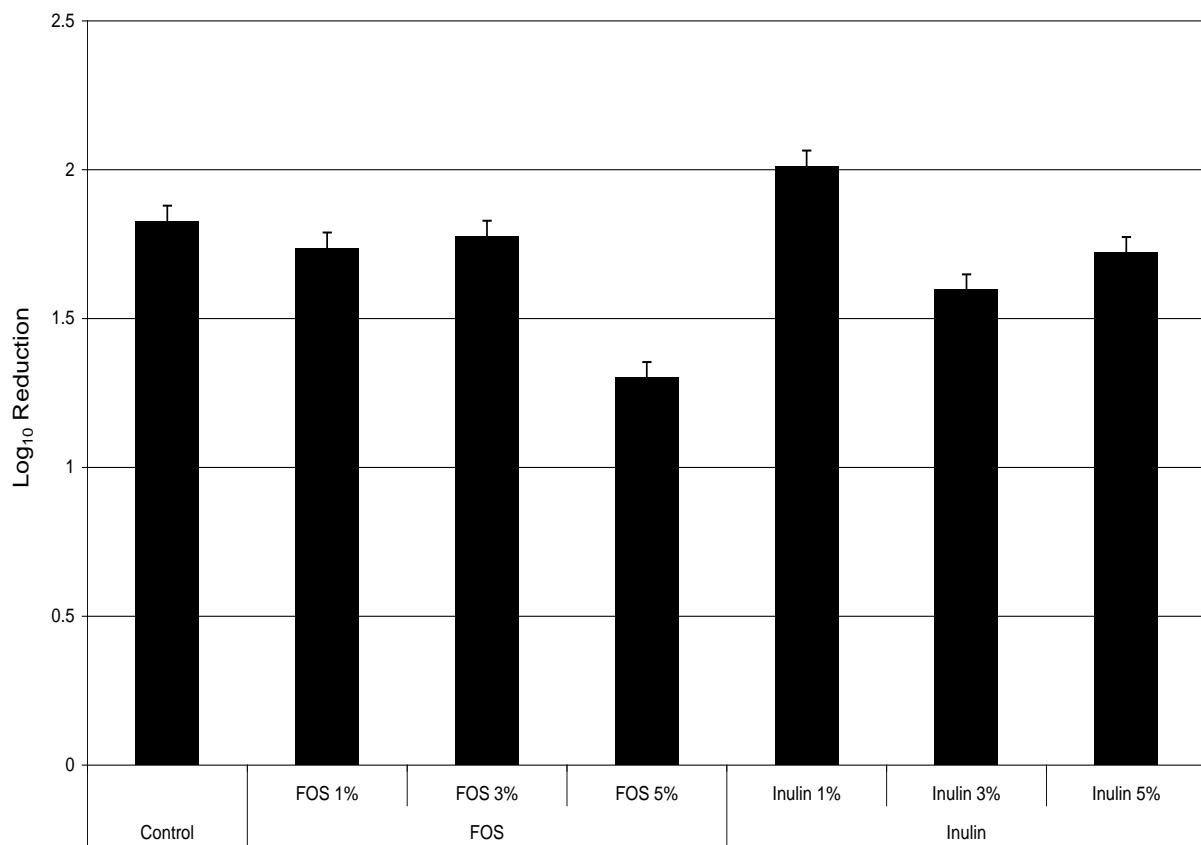


Fig 3.5 the mean Logarithmic reduction of three samples of *Bifidobacterium lactis* Bb12 populations in skim milk without prebiotics (Control) and with FOS (1, 3, 5 %) or Inulin (1, 3, 5 %) after refrigerated for 28 days at $4^{\circ}\text{C} \pm 1$.

All the *Bifidobacterium Lactis* Bb12 total count as \log_{10} cfu/ml has shown decrease after 28 days of refrigerated storage at $4^{\circ}\text{C} \pm 1$. However, these decrease were various between the different samples and that related to the

different concentration has been used in this samples. For example; the 5% FSM showed a good stability and less drop of the \log_{10} total count of *Bifidobacterium Lactis* Bb12 over the first two weeks and keep the figure around 8.8 CFU/mL, to keep stable on this figure over the third week period, thereafter it was dropped slightly of the initial total count of the first week, then steady still until it dropped to 8.5 CFU/mL in the fourth week by showing a total of 1.3 mean logrthmetic reduction over the storage period for 28 day at $4^{\circ}\text{C}\pm1$, the addition of 5% FOS has improve the survival of *Bifidobacterium lactis* Bb12 significantly ($P<0.05$) and give the best figure in compare to the other concentrations and the control. However, the 5% Inulin has showed very good stability in the first two weeks and that followed by dramatically dropping after that to reach 8.06 cfu/ml at the end of the forth week, but the adding of 3% Inulin has showed bad decrease in the first two weeks and then kept steady and gave better survivability with 8.23 cfu/ml at the forth week, the using of 1% Inulin was the only concentration which showed bad drop and reach to less than 7 cfu/ml at the end of the froth week which was close to the control, which had a significant ($P<0.05$) drop and had 1.83 mean logrthmetic reduction at the end of the forth week. The use 5% Inulin or the 1% FOS had showed the same result and they were both dropped dramatically to reach 8.06 cfu/ml with mean logrthmetic reduction of 1.72 and 1.73 for the 5% inulin and the 1% FOS respectively.

The use of 3% FOS has showed steady decrease and reached 8.0 \log_{10} cfu/ml at the end of the fourth week for a mean logarithmic reduction of 1.77.

All previous results showed better survivability level for the *Bifidobacterium lactis* Bb12 in comparison to the control.

However, the 3% ISM was the best of the inulin concentrations to use as stimulating supplement to enhance the survival of *Bifidobacterium lactis* Bb12 with a mean logarithmic reduction of 1.6. The use of 5% FSM had the greatest effect on *Bifidobacterium lactis* Bb12 survival with a mean log reduction of 1.3 at the end of the storage period, Figure (3.5).

The mean logarithmic reduction of *Bifidobacterium lactis* Bb12 at the end of the refrigerated storage was similar in the 1%, 3% concentration of FOS and 5% of Inulin but the 1% Inulin dropped significantly ($P<0.05$) in compared to the control. Therefore, the 5% of FOS in FSM showed the best survival rate of *Bifidobacterium lactis* Bb12 (Akalin et al., 2004) and gave a significant ($P<0.05$) viability with 5.02% Table (3.3) which is much better than the control with just 1.48%. On other hand, the using of 3% Inulin concentration in ISM showed a good viability with 2.49% which is less than the using of 5% FOS, but better than all the rest of concentrations of both Inulin and FOS.

This suggested that prebiotic and especially FOS at 5% in skim milk can stimulate the survival of *Bifidobacterium Lactis* Bb12 in the fermented products and possibly in the gastrointestinal tract.

The results indicated that the addition of 5% concentration of FOS and Inulin shows growth promoting effect of *Bifidobacterium* spp. strains in pasteurized skim milk comparing to control containing no prebiotics. Previous research by (Shin et al., 2000a) indicated similar results where a maximum effect was

observed at 5%. Furthermore, this research indicated that higher concentrations were not beneficial and therefore, a maximum of 5% concentration of FOS or Inulin was used in this research.

The growth, survival and activity of the *Bifidobacterium Lactis* Bb12 in skim milk were improved in the presence of FOS and Inulin compared to the control without prebiotics. However, the FSM shows better promoting effect compared to the ISM milk in the growth and survival. Moreover, it shows that using 5% of FOS in FSM increased the growth and promoted the survival of *Bifidobacterium Lactis* Bb12 (Akalin *et al.*, 2004) significantly ($p<0.05$). The use of 3% inulin in the ISM milk was better than the control but less than the FSM milk.

This study shows that after controlling other factors affecting the growth and survival of *Bifidobacterium Lactis* Bb12 such as acid production during fermentation, oxygen content in the product and oxygen permeation through the packaging material, and the antimicrobial substrates produced by lactic acid bacteria (Shah, 2000), and the storage temperature (Mortazavian *et al.*, 2007), we can have a significant effect on the growth and survival of *Bifidobacterium Lactis* Bb12 in dairy products by adding FOS 5% w/v or Inulin 3% w/v.

CHAPTER FOUR

**THE EFFECT OF
HYDROLYSIS SKIM
MILK EFFECTS ON THE
GROWTH AND
SURVIVAL OF
BIFIDOBACTERIUM
LACTIS**

4.1 INTRODUCTION

Milk is an important part of the diet and has been used for centuries to provide critical nutrients for both neonates and adults (Yamauchi, 1992; McCann *et al.*, 2006). Protein casein is the most important element in milk (Varnam and Sutherland, 1994), where casein constitutes over 80% of the total protein in milk and the 20% remaining are predominately whey proteins.

These caseins are present in milk as macromolecular aggregates or micelles of diameter 30- 300 nm. They belong to four genetic families; α_{s1} - (38%), α_{s2} - (10%), β - (13%) and κ - caseins (36%), these percentages refer to bovine milk; in human milk there are no α_{s1} -caseins (Varnam and Sutherland, 1994; Meisel, 1998; Liepke *et al.*, 2002).

Schanbacher *et al.*, 1997 found that whey proteins such as lactoferrin, lactoperoxidase and lysozyme; all show clear antibacterial activities, and reported that bovine lactoferrin from mature milk increase the growth of bifidobacteria *in vitro* and *in vivo* (Petschow *et al.*, 1999; Berkhout *et al.*, 2003; Kim *et al.*, 2005).

Milk proteins can be hydrolysed into numerous peptide fragments by enzymatic proteolysis and serve as source of bioactive peptides (Lahov and Regelson, 1996; Clear and Swaisgood, 2000; Korhonen *et al.*, 2001; Meisel, 2004). These peptides have been found to be active against a broad range of pathogenic organisms e.g. *Escherichia*, *Helicobacter*, *Listeria*, *Salmonella* and *Staphylococcus*, yeasts and filamentous fungi (Haque and Chand, 2006).

Research has also indicated that another protective mechanism against pathogenic bacteria is the presence of probiotic in sufficient number. Therefore, a minimum of 10^6 CFU/g of probiotics was recommended for daily consumption of fermented milk products to get the health benefit in the gastrointestinal tract (Maldonado *et al.*, 2003; Gibson and Rastall, 2004).

Probiotics and especially *Bifidobacteria spp.* grow very slow in milk and often show a loss in viability at the end of shelf life of the products (Dave and Shah, 1997a; Gomes *et al.*, 1998; Vinderola *et al.*, 2000) and it could be due to the lack of proteolytic activity of bifidobacteria in milk (Klaver *et al.*, 1993).

However, since 1977 Kehagias *et al.* demonstrated that a soluble peptide fraction from acid hydrolysed bovine casein stimulated the growth of *Bifidobacterium bifidum*. Recent studies, have suggested that adding peptides and amino acids (as nitrogen source) may increase the viability and the growth of probiotic organisms, in milk products (Dave and Shah, 1998; Shihata and Shah, 2000; Sodini *et al.*, 2002; Lucas *et al.*, 2004). It has been shown by Lucas *et al.*, (2004) that the addition of more than 4 g/L of hydrolysates supplement can have a detrimental effect on the growth and survival of Bifidobactria in the presence of lactic acid bacteria and this is mainly due to slow growth rate of Bifidobaceria as compared to lactic acid bateria in milk (Dave and Shah, 1998).

Milk does not contain sufficient free amino acids and peptides to stimulate the growth of bifidobacteria (Gomes *et al.*, 1998; Shihata and Shah, 2000; Zisu and Shah, 2003), due to the lack of proteolytic activity of probiotic bacteria especially *Bifidobacterium spp.* which limits their growth and survival (Klaver *et al.*, 1993;

Dave and Shah, 1997b; Shihata and Shah, 2000). All of above factors cause major difficulties for market development of probiotic products containing bifidobacteria (Brasheras and Gilliland, 1995).

4.2 AIM

The aim of this work was to evaluate the effect of different degrees of skim milk protein hydrolysis by trypsin on the growth and survival of *Bifidobacterium lactis* Bb12.

4.3 OBJECTIVE

- To select a suitable enzyme : substrate ratio for trypsin and skim milk protein
- Produce skim milk with different Degree of Hydrolysis (DH) using trypsin
- Study the effect of the different DH on the growth and survival of *Bifidobacterium lactis* Bb12 in comparison to the control.
- Determine the best DH to use for enhancing the growth and survival of *Bifidobacterium lactis* Bb12.

4.4 MATERIAL AND METHODS:

4.4.1 PREPARATION OF TRYPSIN AND SKIM MILK HYDROLYSIS:

Trypsin was obtained from Sigma Chemical Co., UK. Skim milk (UHT) was obtained locally; three sterile bottles, each one with a capacity of 1000 ml (1 L) were used for the hydrolysis process. One of the bottles was used to prepare the 5 minute hydrolysis skim milk sample; another was used to prepare the 10 minute hydrolysis skim milk sample, and one used as a Control (without hydrolysis).

Ten minute hydrolysis of skim milk by trypsin was the maximum time used in this study. Previous studies showed that the hydrolysis of skim milk over 15 minutes at an enzyme: substrate ratio 1:20 000 led to more than 5% Degree Hydrolysis (DH); hence hydrolysis for longer time was restricted because it was found that bitter peptides with flavour defects present beyond 5% DH (Vijaya *et al.*, 2002). However, the same study indicated that using 10 or 15 minute hydrolysis skim milk by Neutrerase or Proteinase to promote the growth of *Bifidobacterium bifidus* showed similar result and any increase in the DH didn't show a stimulatory response. The authors concluded that further degradation of milk protein was not essential as nitrogen source for use by *Bifidobacterium lactis* ssp. (Gomes *et al.*, 1998, Lucas *et al.*, 2004).

4.4.2 MILK HYDROLYSIS:

A ratio 1:20 000 E: S (Enzyme: Substrate) was used to modify the skim milk proteins by trypsin and this was calculated based on the protein content in the skim milk samples which was 33.4- 34 g/ L.

Hydrolysis was performed with a total volume of 588 ml skim milk (calculated to the total protein)

(This just to show how the calculation was done:

1:20000 we have 34g protein in 100 ml milk which is 0.034g/1 ml which is 34 mg/ ml

$20000/34 = 588$ ml of milk

1 mg enzyme : 588 ml milk = 1:20000)

and were performed in 1000 ml bottles which allowed stirring during the hydrolysis process. Afterward, 1 ml of the trypsin solution in distilled water with concentration of (1 mg/ mL) was added to each 588 ml of skim milk in each bottle except the control to make the 1:20 000 ratio (Enzyme:Substrate) (Vijaya *et al.*, 2002). The hydrolysis was performed in a water bath at 37°C. One of the bottles was under the hydrolysis procedure for 5 minutes and the other bottle for 10 minutes. At the end of the hydrolysis process all bottles were immersed in boiling water for 10 min to inactivate the enzyme reaction in the skim milk (McCann, *et al.*, 2005). All the samples for the growth and survival measurement were taken for further analysis.

The degree of hydrolysis DH was determined using Trinitrobenzenesulphonic acid (TNBS) method by reading spectrophotmeter at 420nm (Polychroniadou, 1988). In this study, the dilution was performed at 1:25 of the supernatant in distilled water for assaying instead of the 1:50 dilution as indicated in the original method (Adler-Nissen, 1997). However, the rest of the experiment procedure

was the same. This change was suggested after three experiments, which showed that this dilution ratio yielded better results. Standard curve for TNBS assay were done using standard glycine (Sigma Chemical Co, United Kingdom), solutions ranging from 5-50 mM (Adler-Nissen, 1997).

4.4.3 PREPARATION OF *Bifidobacterium lactis* Bb12 CULTURE:

See section (3.5.2)

4.4.4 MEASUREMENT OF THE GROWTH AND SURVIVAL OF *Bifidobacterium lactis* Bb12:

See section (3.5.3)

4.4.5 STATISTICALL ANALYSIS:

Bifidobacterium lactis Bb12 growth were assayed as pH changes over the fermentation period for each of the Hydrolysis time used and were repeated three times, this data were analyzed by applying one-way analysis of variance (ANOVA, Minitab 12). The change in the survival study of *Bifidobacterium lactis* Bb12 in each sample with time hydrolysis were determined using the total count of the colonies as cfu/ml and repeated three times, the data were analyzed by applying one-way analysis of variance (ANOVA, Minitab 12). $P \leq 0.05$ was considered statistically significant.

4.5 RESULTS AND DISCUSSION

4.5.1 Growth of *Bifidobacterium Lactis* Bb12 in Hydrolysed skim milk (HSM):

Proteins in skim milk were modified using enzymatic hydrolysis by Trypsin at 1:20 000 Enzyme: Substrate ratio. This hydrolysis were followed by analysis of the degree of Hydrolysis DH using the TNBS methods (Figure 4.1), where samples were withdrawn from the milk hydrolysis in 0, 5, 10, 30 and 50 min and measured using spectrophotometer at 420 nm. The same method was used to measure the absorbance for appropriate concentrations of glycine standard solutions to obtain the standard graph for TNBS assay (Figure 4.2), to allow conversion of assay values to equivalent mM of soluble amino nitrogen.

This method used to measure the DH of skim milk using the formula;

$$DH\% = X / C \times 11.55$$

DH ; Degree of Hydrolysis %,

X ; mM equivalent of glycine,

C ; initial protein concentration in skim milk g/L,

11.55 ; conversion factor derived from Avogadro number taken into consideration the average molecular weight of the proteins.

Figure (4.1) show the TNBS Assay of 1:20 000 ratio of Trypsin to skim milk over 1 h hydrolysis at 37°C

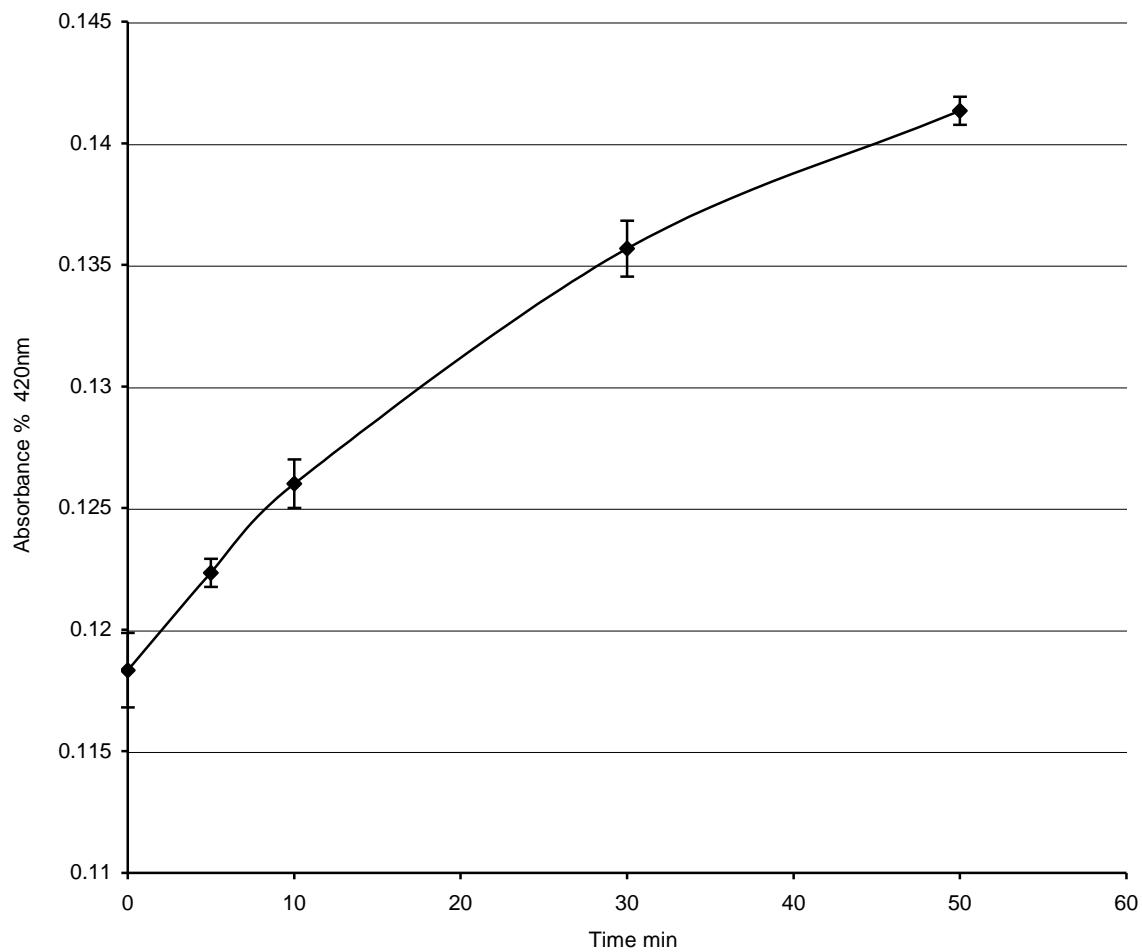


Fig 4.1 Curve for TNBS assays of 1:20 000 ratio of Trypsin to skim milk over 1 h hydrolysis at 37°C, values taken as average of triplicate assays with error bars presented. Error bars indicate standard deviation of three samples.

Figure (4.2) shows the TNBS assay of 12% TCA (Trichloroacetic acid) soluble nitrogen, as mM glycine at 420 nm.

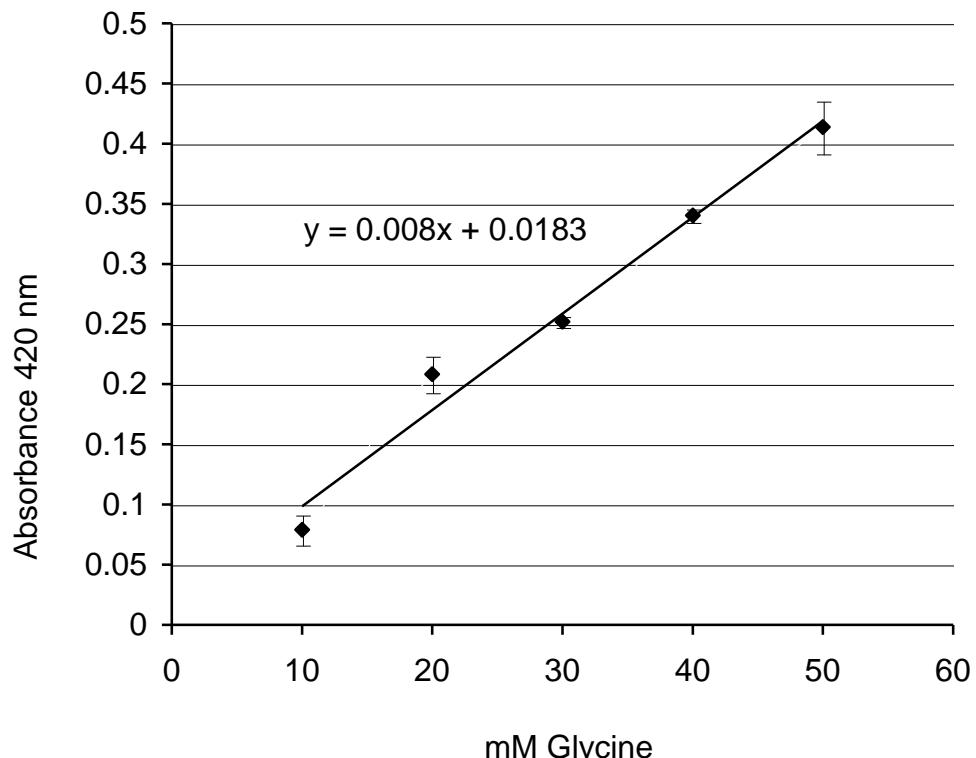


Fig 4.2 Standard curve for TNBS assay of 12 % TCA (trichloroacetic acid) soluble nitrogen, as mM glycine, at 420 nm, values taken as average in triplicate assays with error bars presented. Error bars indicate standard deviation for three samples.

The DH of 4.55% for the 5 min hydrolysis and 4.71% at 10 min hydrolysis of the skim milk and this DH is acceptable for food production. Higher DH values

beyond 5% are known to produce bitter peptide in enzymatic-hydrolysed milk (Vijaya *et al.*, 2002).

Figure (4.3) shows faster fermentation time as indicated by the pH decrease and the growth promoting effect on *Bifidobacterium lactis* Bb12 by increasing the time of hydrolysis using trypsin from 5 to 10 minutes.

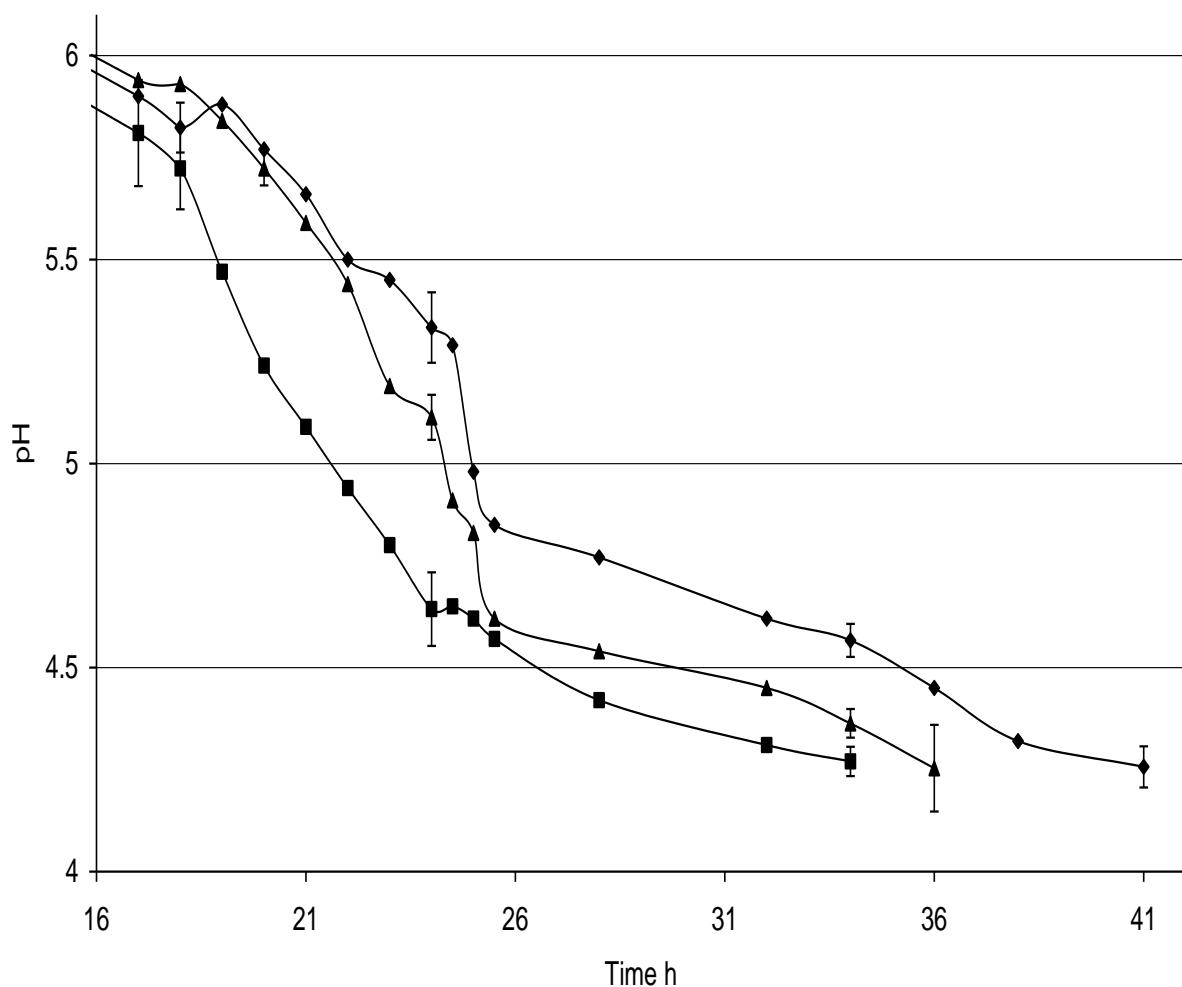


Fig 4.3 The pH change and development of *Bifidobacterium lactis* Bb12 growth to reach the fermentation point at pH 4.2 ± 0.03 in skim milk without hydrolysis (Control) (♦), and 10 min Hydrolysis skim milk (■) and in 5 min hydrolysis skim milk (▲). Error bars were taken as standard deviation for three samples.

Table (4.1) illustrates the fermentation time in hours of bifidobacteria in 5 minute and DH<5-milk hydrolysis. This study showed that both the 5 and DH<5milk hydrolysis showed better fermentation time as compared to the control sample.

Table 4.1 the fermentation time (hours) of *Bifidobacterium lactis* Bb12 in skim milk without hydrolysis (Control), 10 min Hydrolysis skim milk and in 5 min hydrolysis skim milk to reach the pH 4.2±0.03.

Fermentation time of <i>Bifidobacterium Lactis</i> Bb12 (hours)		
Control	5 min Hydrolysis	10 min Hydrolysis
41	36	34

A significant ($P<0.05$) stimulating shows of *Bifidobacterium lactis* Bb12 growth Figure (4.3) and (Table 4.1) in the hydrolysed skim milk in comparison to the control, it shows that the time used to reach the desired pH 4.2±0.03 in the 10 min hydrolysis skim milk (HSM) were reduced by 7h and 2h compared to the control and 5 min hydrolysis skim milk (HSM), respectively.

However, the total counts of the *Bifidobacterium lactis* Bb12 Figure (4.3) were higher in the 10 min HSM at \log_{10} cfu/mL 9.63, at the end of the fermentation (pH 4.2±0.03), which is significant ($P<0.05$) compared to 8.96 and 9.43 in the control and 5 min HSM, respectively. The improvement in growth of *Bifidobacterium Lactis* Bb12 with higher total count in less time can be related to the presence of free amino acids or bioactive peptides in the HSM which become directly

accessible source of nitrogen for cell growth and acidification increase of *Bifidobacterium Lactis* Bb12 environment (Desjardins *et al.*, 1990, Gomes *et al.*, 1998, Poch and Bezkorovainy, 1988, Proulx *et al.*, 1994, Oliveira *et al.*, 2001, Janer *et al.*, 2004b). In addition to the release of peptides by using trypsin (Poch and Bezkorovainy, 1991, Lorenzen and Meisel, 2005) from β -, κ -casein, α -lactoalbumin and β -lactoglobulin which are found in low degree hydrolysed milk (Lucas *et al.*, 2004), the improved growth could also be due to the presence of disulfide/sulphydryl from the cysteine residues, and from human milk by other enzymes such as pepsin and chymosin (Azuma *et al.*, 1984). Moreover, opioid peptides (Daniel *et al.*, 1990) which have been shown to have a stimulating effect on bifidobacteria growth (Poch and Bezkorovainy, 1991, Shah, 2000b) reported that the trypsin had minimal activity on α - and β - casein and full activity with κ -casein. Another possible mechanism is the presence of caseinophosphopeptide-rich fraction (CPPs) released in the trypsin treated milk, which is considered a stimulator to the growth of bifidobacteria (Shah, 2000b; Lorenzen and Meisel, 2005). However, the hydrolysis of whey proteins α -lactoalbumin and β -lactoglobulin using trypsin produced at least seven major functional peptides in less than 15 min hydrolysis (Mota *et al.*, 2006), which are classified as bioactive peptides and shown to stimulate the growth of *Bifidobacterium Lactis* Bb12 (Ibrahim and Bezkorovainy, 1994; Shah, 2000b; Gauthier and Pouliot, 2003; Antunes *et al.*, 2005).

However, both types of enzymes and the DH affected the amount of peptides produced in the hydrolysates (Proulx *et al.*, 1992; Gomes *et al.*, 1998). It was

reported that the poor growth and acidification of bifidobacteria in milk was caused by lack of essential amino acids in free form or in peptides (Roy *et al.*, 1990; Gomes *et al.*, 1998; Sodini *et al.*, 2002),

4.5.2 Survival of *Bifidobacterium lactis* Bb12 in HSM:

To achieve the health benefits of consumption of bifidobacteria drink, it is expected to have 10^6 CFU/g at the time of consumption (Dave and Shah, 1998; Sodini *et al.*, 2002; Lucas *et al.*, 2004), and this information raised the awareness in the industry of studying bifidogenic factors to stimulate the survival and viability of bifidobacteria in fermented milk.

However, survival of probiotic bacteria was improved by using hydrolysates in milk (Zhao *et al.*, 2006). These hydrolysates were found to be affected by the enzyme and conditions of hydrolysis process (Gomes *et al.*, 1998) that found that the tryptic casein fractions have a greater stimulating effect on the growth of bifidobacteria when compared to the addition of amino acids (Proulx *et al.*, 1994).

The survival rate of *Bifidobacterium Lactis* Bb12 in hydrolysis skim milk by trypsin was measured as \log_{10} cfu/mL over 28 days at $4^\circ\text{C}\pm1$, as shown in triplicate (Figure 4.4).

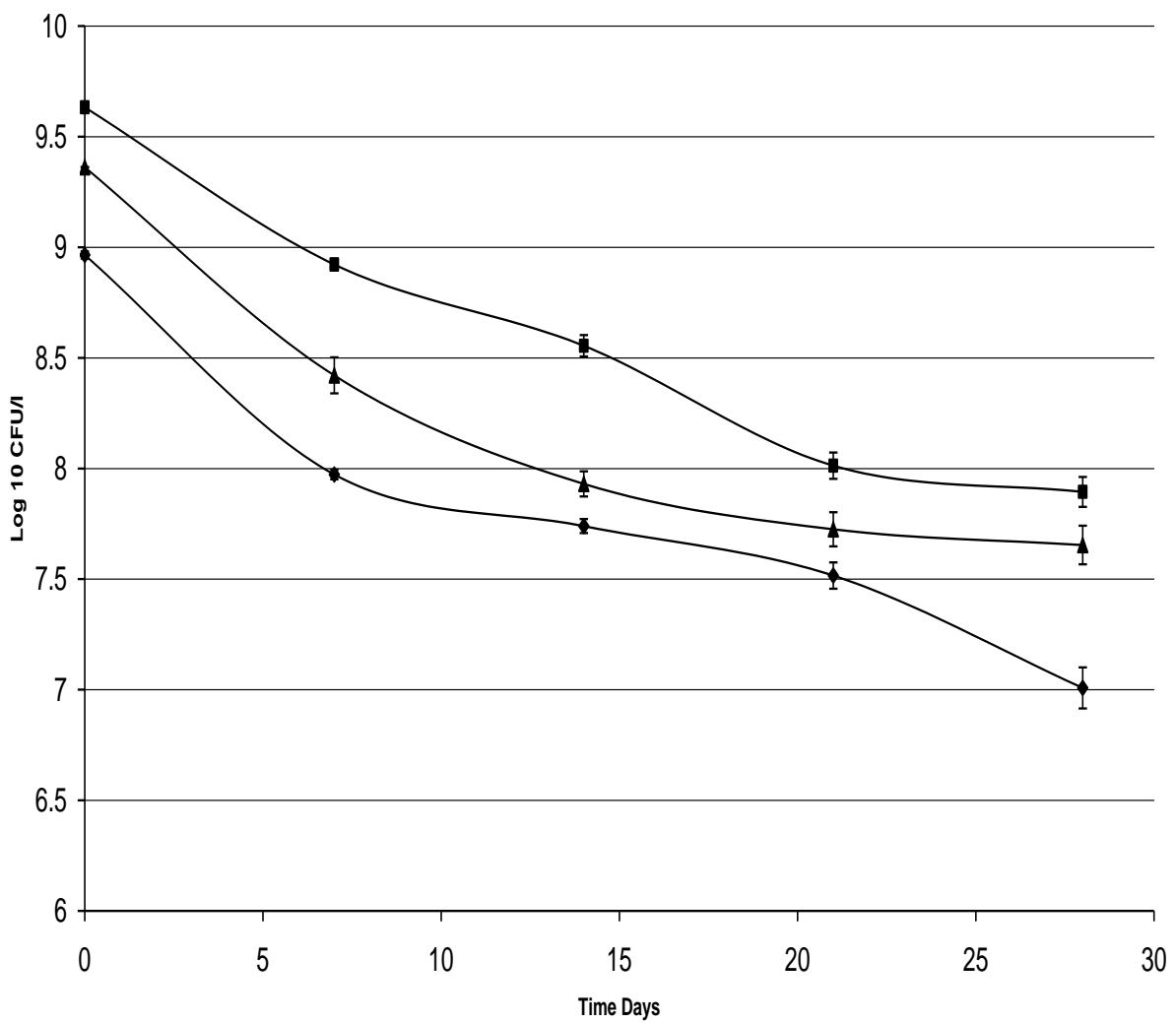


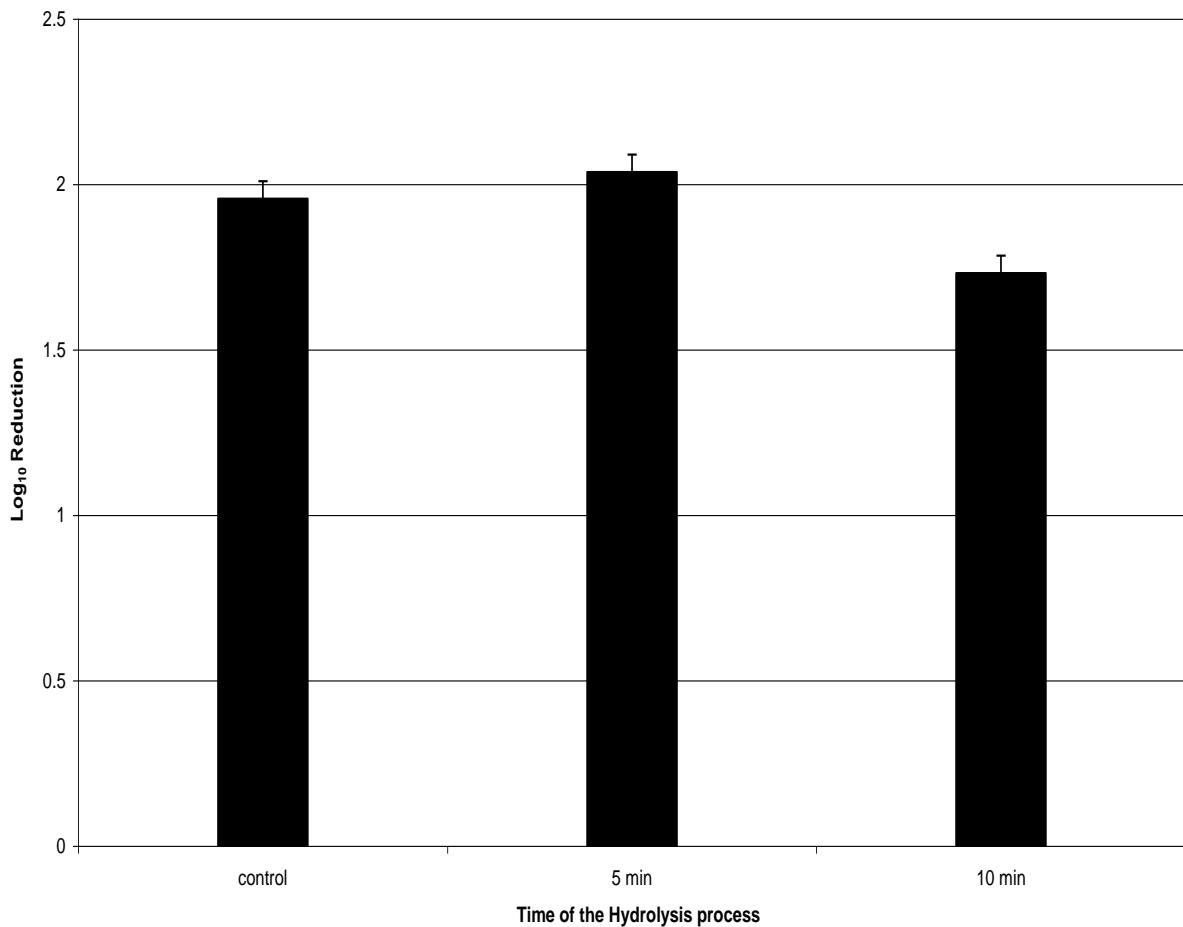
Fig 4.4 the \log_{10} of the viable count of *Bifidobacterium lactis* Bb12 in skim milk without hydrolysis (control) (♦), 10min HSM (■) and 5min HSM (▲) over 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$. Error bars were taken as standard deviation for three samples.

All samples show a decrease in the total count of *Bifidobacterium Lactis* Bb12 over the storage period. However, the HSM shows better effect on the survival compared to control samples. The survival of *Bifidobacterium Lactis* Bb12 in the 10min HSM shows greater effect as it only dropped slightly and steady counts until it dropped to 7.89 by the end of the fourth week with viability of 1.86% (Table 4.2).

Table 4.2 the Viability % of *Bifidobacterium Lactis* Bb12 in non-hydrolysed milk, 10 min HSM and 5 min HSM after 28 days refrigerated storage at 4°C ± 1

Viability of <i>Bifidobacterium Lactis</i> Bb12 (%)		
Control	5 min HSM	10 min HSM
1.11	0.93	1.86

The total reduction of the count was significant ($P<0.05$) in the 5 min HSM sample and control in compare with the 10 minute hydrolysed sample has been shown in Figure (4.5).



. Fig 4.5 the mean logarithmic reduction of three sample of *Bifidobacterium Lactis* Bb12 in skim milk without hydrolysis, with 5 minute hydrolysis and with 10 minute hydrolysis over refrigerated storage for 28 days

An explanation for these findings could be the availability of free amino acids and peptides in the HSM, which provides the nitrogen source for the survival of *Bifidobacterium Lactis* Bb12 over the storage period. A similar observation of improved survival of bifidobacteria by adding hydrolysed milk was reported by (Dave and Shah, 1998; Sodini *et al.*, 2002) who showed that adding casein

hydrolysis improved the survival of bifidobacteria after refrigerated storage. The cysteine release in the HSM by trypsin is one the amino acids expected to have a protective effect on the survival of bifidobacteria and increase the viability of bifidobacteria in fermented milk (Biavati *et al.*, 1992; Dave and Shah, 1997b; Guler-Akın and Akın, 2007).

Whey protein is a source of peptides and sulphur-containing amino acids, which are liberated during the hydrolysis process and heat treatment leading to a reduced redox potential and thus provide a favorable environment for Bifidobacteria viability (Dave and Shah, 1998, Antunes *et al.*, 2005). Furthermore, the ability of whey protein concentrate and hydrolysates to act as a buffer in the fermentation of lactose to lactic and acetic acids may further contribute to the maintenance of sufficiently high numbers of *Bifidobacterium Lactis* Bb12 during refrigerated storage (Kailasapathy and Supraidi, 1996; Antunes *et al.*, 2005; Drgalic *et al.*, 2005; Zuo *et al.*, 2005).

The viability of the *Bifidobacterium Lactis* Bb12 in the 10 min HSM were improved 1.86% in which is significant ($P<0.05$) in compared with the control and 5 min HSM, which is 1.11% and 0.93% respectively.

4.6 CONCLUSIONS:

The growth and survival of *Bifidobacterium Lactis* Bb12 in HSM improved in comparison to the control with no hydrolysis. However, it has been shown that increase the hydrolysis time over 10 minute and get degree of hydrolysed over 5% didn't show improvement in the growth and survival of *Bifidobacterium lactis* Bb12. The sample with DH<5 hydrolysis skim milk showed better effect on the growth and highly improved the survival and viability of *Bifidobacterium Lactis* Bb12 as compared with the 5 minute hydrolysis skim milk or the control. Therefore, the results show improvement in the growth and survival of *Bifidobacterium Lactis* Bb12 in hydrolysis skim milk for 10 minute.

CHAPTER FIVE

SYNERGISTIC EFFECT OF PREBIOTICS AND HYDROLYSED SKIM MILK ON THE GROWTH AND SURVIVAL OF BIFIDOBACTERIA

5.1 INTRODUCTION:

Most probiotic bacteria are mainly used in fermented foods and dairy products (Lourens-Hantting and Viljoen, 2001; Haines, 2005). Probiotics are added to dairy products (as a supplement and/or culture in fermented products), to improve the microflora balance in the large bowel and have to be viable to contribute to the overall health benefits (Elmer *et al.*, 1996). Therefore, the most important requirement for probiotic bacteria is to survive in sufficient numbers and guarantee stability during the production process and storage of the product. Fermented dairy products have been well studied and especially the relationship between probiotic bacteria and the starter cultures of dairy products such as; production of Bacteriocin, antagonism, and synergism (Heller, 2001), but the interactions of probiotics with the food matrix and the starter culture is most important especially when probiotics are used as culture in the fermented product (Mattila-Sandholm *et al.*, 2002). The presence of probiotic bacteria in fermented foods causes interactions with their components through the metabolic process. Thus, the chemical composition of dairy products is very important for the metabolic activity of probiotic bacteria. Essentially, the availability, kind and amount of carbohydrates and the degree of hydrolysis of milk proteins (availability of peptides and amino acids) in the products (Dave and Shah, 1997b), are all important factors in this interaction which is known to have a synergistic effect (Heller, 2001).

The term Synergy (from the Greek syn-ergo, meaning working together) is defined as: 'the interaction or cooperation of two or more organizations,

substances, or other agents to produce a combined effect greater than the sum of their separate effects' (New Oxford Dictionary, 2001).

The interaction between probiotic and yogurt starter culture has been established over decades. For example, the normal yogurt starter *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* work in synergy and affect the growth and survival of probiotics in yogurt products (Driessens *et al.*, 1982, Zourari *et al.*, 1992; Matto *et al.*, 2006a) and this synergistic effect depends on the time that probiotics are added to the product, for example, whether they are present during the fermentation process or added after (Roy *et al.*, 1997, Joseph *et al.*, 1998). One important adaptable thing in this respect is lactic acid production and the reduction in pH during fermentation, which results in inhibiting the growth of probiotic bacteria in dairy products made with normal yogurt culture and probiotics (Lankaputhra and Shah, 1998). However, to enhance the probiotic growth in fermented products, a suggestion of using a selective non-digestible carbohydrate found to promote the proliferation of probiotics, which is defined as prebiotics has been raised (Gibson and Roberfroid, 1995). This combined use of probiotics and prebiotics is termed a synbiotic (Gibson and Roberfroid, 1995), which is defined as "a mixture of pro- and prebiotic, which beneficially affects the host by improving the survival of live microbial dietary supplements in the gastrointestinal tract" (Gibson *et al.*, 2005). In addition, the use of enzymatically hydrolysed milk supplements in the fermentation process to provide the essential peptides and amino acids for the growth and survival of probiotics has been one of the important issues for investigation in the last decade (Kunji *et al.*, 1996;

Korhonen and Pihlanto-Leppala, 2001). The use of prebiotics or protein modified milk was shown to have stimulatory effect on probiotics and especially Bifidobacteria in *in-vitro* and *in-vivo* studies (Kehagias et al., 1977; Kaplan and Hutkins, 2000; Shin et al., 2000a, b; Vijaya et al., 2002; Picot and Lacroix, 2004; Zampa et al., 2004; Aryana et al., 2007; Wang et al., 2007), but the use of the combination of prebiotics and enzymatically hydrolysed skim milk as potential stimulators for the growth and survival of probiotic bacteria has not been studied or reported in the literature. Therefore, this study was conducted to assess the synergistic effect of the presence of combinations of prebiotics and partially hydrolysed skim milk on the growth and survival *in-vitro* of *Bifidobacterium lactis* Bb12 as the culture for fermented milk products.

5.2 AIM:

This study is applied to investigate the synergistic effect of using prebiotics and partially hydrolysed skim milk by trypsin at low ratio (1:20 000) on the growth and survival of *Bifidobacterium lactis* Bb12 and other probiotic species.

5.3 OBJECTIVES:

- Prepare different mixtures of prebiotics and hydrolysed skim milk
- Examine the growth and survival of *Bifidobacterium lactis* Bb12 in the different medium
- Select the medium with the best synergistic effect on the growth and survival of *Bifidobacterium lactis* Bb12.
- Apply the best medium from the last stage to examine its effect on the growth and survival of *B. breve*, *B. bifidum*, *B. infantis*, and *L. casei* strain shirota and investigate the ability of this medium to enhance their growth and survival.

5.4 MATERIAL AND METHODS:

The literature review and the comprehensive studies conducted during this research are outlined in Chapters 3 and 4. The studies conducted indicated that the use of prebiotics and enzymatically hydrolysed milk were the main axis to enhance the growth and survival of bifidobacteria and in particular *Bifidobacterium lactis* Bb12. Therefore, the two treatments were applied to skim milk: First, the skim milk was hydrolysed by trypsin and then adding Fructooligosaccharides (FOS) and Inulin as prebiotic supplements to study the synergistic effect of these treatments on the growth and survival of *Bifidobacterium lactis* Bb12 and to apply the best result to several other probiotic bacteria which are well known and used in fermented dairy products.

5.4.1 PREPARATION OF MODIFIED MILK BY TRYPSIN, FOS AND INULIN:

Trypsin with activity 1550 U/mg (obtained from Sigma Chemical Co., UK) was used for the modification of the milk proteins. Food grade FOS and Inulin (obtained from ORAFTI active food ingredient, Belgium) labeled as Beneo P95 for FOS (oligofructose \geq 95%, glucose+ fructose+ sucrose \leq 5% and pH of 4.5-6.5) and Beneo GR for Inulin (inulin \geq 90%, glucose+ fructose \leq 4%, sucrose \leq 8% and pH of 5-7).

Skim milk was obtained from the local market; two sterile bottles with a volume of 1 L were used for the hydrolysis process using trypsin at 1:20,000 ratio

(enzyme:substrate) at 37°C for DH≤5 hydrolysis to obtain a DH of approximately 4-5% (Chapter 4). Thereafter, each of the 1 liter milk was separated into ten smaller sterile bottles each with a volume of 100 ml. FOS, Inulin, and the hydrolysed skim milk used five of the 100ml bottles for the synergistic effect study, and the other five bottles were used as control samples for the growth and survival studies.

As outlined in Chapters 3 and 4 after obtaining the results of the comprehensive studies conducted, it was found that *Bifidobacterium lactis* Bb12 has the most optimal growth and survival using the concentrations of 5% FOS, 3% Inulin, or using the DH≤5 hydrolysed skim milk by trypsin. However, this study was undertaken to assess other possibilities that may improve the growth and survival of *Bifidobacterium lactis* Bb12, which appear in the following growth media as performed; Three samples of skim milk were hydrolysed for 10 minutes to achieve a degree of hydrolysis DH of approximately 4-5% and the following were added, 5% FOS (10 min Hydrolysis skim milk + 5% FOS) and coded as (A), second one contained 3% inulin (10 min Hydrolysis skim milk + 3% Inulin) and coded as (B), the third one combined all variables together (10 min Hydrolysis skim milk + 5% FOS + 3% Inulin) and coded as (C) and the fourth one was without hydrolysis and contained just (5% FOS +3% Inulin) and coded as (D). Skim milk was used as control and all samples were studied in triplicate for the growth and survival study.

5.4.1.1 ADDING FOS AND INULIN

Two different concentrations of 5% FOS and 3 % Inulin were added to skim milk hydrolysed with trypsin (Chapter 4). The control was skim milk without supplements or hydrolysis and samples were prepared as mentioned above. However, all samples were heat treated at 70 °C for 15 min and then allowed to cool to 37°C (Shin *et al.*, 2000a).

5.4.2 PREPARATION OF *BIFIDOBACTERIUM LACTIS* Bb12 CULTURE:

An overnight culture (14-15h) of *Bifidobacterium lactis* Bb12 (obtained from Chr. Hansen, UK) in skim milk was prepared and was used as the inoculum culture. A concentration of 5% v/v of *Bifidobacterium lactis* Bb12 culture was added to each one of the five bottles for the experiment (Vijaya *et al.*, 2002).

All samples were incubated at 37°C in an anaerobic cabinet until a pH 4.2±0.03 was reached. Thereafter, all samples were cooled in an ice bath to 4°C±1 for the refrigerated storage and survival study.

5.4.3 PREPARATION OF SURVIVAL SAMPLES:

As for the preparation of the samples for the growth study, similar samples were prepared for the survival study in triplicate. Four extra bottles were prepared from each of the five ready bottles for the growth study and inoculated with *Bifidobacterium lactis* Bb12 and incubated with the growth samples anaerobically at 37°C until a pH of 4.2±0.03 was reached, and then cooled to 4°C±1 for the

refrigerated storage temperature. One of the four bottles in triplicate were used every week for the survival study by taking 1ml from each bottle and serial dilutions were done and plated on MRS-agar to study the total count of the *Bifidobacterium lactis* Bb12 over the 28 day storage.

5.4.4 PREPARATION OF *BIFIDOBACTERIUM SPP.* FROM FREEZE-DRIED NCIMB CULTURE:

The organisms used in this study included three strains of Bifidobacteria; *Bifidobacterium bifidum* NCIMB 702203, *Bifidobacterium infantis* NCIMB 702205 and *Bifidobacterium breve* NCIMB 702257 obtained from the National Collection of Industrial, Marine and Food Bacteria (NCIMB Ltd, Aberdeen, UK) and *Lactobacillus casei* strain shirota obtained of Yakult drink product at the local market. The bifidobacteria strains were received as freeze-dried culture and these cultures were revived from NCIMB by culturing the bacteria on MRS agar plate and in MRS broth with 5% L-cysteine hydrochloride, up to 0.5 ml of ready sterilized MRS broth medium with 5%L-cysleine hydrochloride was added to the ampoule and the contents mixed avoiding frothing. The suspension was subcultured onto MRS agar and MRS broth medium with 5% L-cysteine hydrochloride and incubated at 37°C in an anaerobic cabinet for 72 hours. The NCIMB culture was sub-cultured twice before it was used in the experiments. The *L. casei* strain shirota where enumerated on MRS agar with 5% L-cysteine Hydrochloride from Yakult drink and incubated at 37°C in an anaerobic cabinet for 72 hours before it was used as sub-culture for the experiments after the third culture of this bacteria.

An overnight (14-15h) culture was prepared of these bifidobacteria strains and *L. casei* strain shirota in skim milk. This over night culture was used as the inoculum culture and a concentration of 5% v/v (Vijaya *et al.*, 2002) of these strains was added to the synergistic mix to assess the best stimulation on the growth and survival of *B. lactis* Bb12.

5.4.5 STATISTICAL ANALYSIS:

Bifidobacterium lactis Bb12 growth was monitored as pH changes over the fermentation period for each sample with different prebiotic concentration and different time hydrolysis of the hydrolyzed skim milk and were repeated three times, these data were analyzed by applying one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant. The change in the survival study of *Bifidobacterium lactis* Bb12 in each sample with different prebiotic concentration and different time hydrolysis of the hydrolyzed skim milk were determined using the total count of the colonies as cfu/ml and repeated three times, the data were analyzed by applying one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

5.5 RESULT AND DISCUSSIONS:

As a result of the fermentation process, the reduction in pH will be the indicator of acid production in the fermented skim milk over the fermentation period. The use of skim milk reduces the potential of increasing the buffer capacity, which improves the accuracy of the pH reading as that it is an accurate indicator of acid development (Ventling and Mistry, 1992; Lopes *et al.*, 2005). The survival part of probiotics over the storage period was assessed by obtaining the total count of bacteria which was plated on MRS agar medium with 5% L-cysteine Hydrochloride and calculated as \log_{10} cfu/ml (Shah, 2000a). The lack of pH change over the storage period in low temperature may have also enhanced the survivability of bifidobacteria in skim milk (Hull *et al.*, 1984; Hughes and Hoover, 1995).

Dave and Shah (1997a) have indicated that the loss of viability of bifidobacteria has typically more potential in fermented milk than in unfermented milk due to acid injury to the organism. Therefore, incorporation of the bifidobacteria in dairy products suggested the importance of their survivability in the product as they approach the gastrointestinal tract of humans with elevated viable population in order to reap their health benefits (Coussemet, 1999; Oozeer *et al.*, 2006). Recently, there has been interest in the use of prebiotics to enhance the survival of probiotics in food products (Ziemer and Gibson, 1998; Shin *et al.*, 2000; Desai *et al.*, 2004). Peptides and amino acids, which are produced during the hydrolysis of skim milk, have been recently studied for their potential ability to improve the survival and viability of bifidobacteria and their use as growth

promoters (Ervolder *et al.*, 1980; Laroia and Martin, 1991; Gallot *et al.*, 1995; Modler, 1994; Vijaya *et al.*, 2002).

However, from the previous studies conducted (chapters 3, 4) it was found that the best growth and survival of *Bifidobacterium lactis* Bb12 occurred with 5% FOS, 3% Inulin and with DH≤5 hydrolysed skim milk. Thus, these preliminary results warranted further investigation to look at the possible synergistic effect of these compounds and the hydrolysed skim milk on the growth and survival of *Bifidobacterium lactis* Bb12. Various mixtures and concentrations of these compounds and hydrolysed skim milk have been designed as the following; mix A (DH≤5 hydrolysis skim milk+ 5% FOS), B (DH≤5 hydrolysis skim milk+ 3% Inulin), C (DH≤5 hydrolysis skim milk+ 5% FOS+ 3% Inulin) and D (skim milk with no hydrolysis with 5% FOS+3% inulin). The growth of *Bifidobacterium lactis* Bb12 was studied by tracking the change of the pH in the skim milk over the period of fermentation until a pH 4.2 ±0.03 was reached, and then the fermentation process was stopped by immersing the bottles in ice and water. The containers were cooled to a temperature of 4°C±1 and they were then stored at this refrigerated temperature for the survival study.

5.5.1 the synergistic effect of prebiotics and hydrolysed skim milk on the growth of *B. lactis* Bb12

In this study and like the methodology used in chapters 3 and 4, the pH change in the skim milk was the parameter used to assess the growth stimulating effect of prebiotics or hydrolysed skim milk on the growth of *Bifidobacterium lactis* Bb12.

Figure (5.1) shows the change of the pH over the fermentation time to reach the pH of 4.2 ± 0.03 using *Bifidobacterium lactis* Bb12.

There were dramatic decreases in the pH of all samples until a pH of 4.2 ± 0.03 was reached. However, sample (C) shows the greatest decrease to reach the target pH of 4.2 ± 0.03 in just 24 hours (Table 5.1) and reduces the fermentation time by 12 hours as compared with the control. Sample (C) consisted of DH ≤ 5 hydrolysed skim milk supplied with 5% FOS and 3% Inulin together in the same medium. This fermentation time reduction in sample (C) is significant ($P < 0.05$) when compared with each of the treatments above. The reduction of the fermentation time using synergistic media together was 12 hours and that was the best as compared with the reduction of the fermentation time of 3, 2 and 7 hours in the 5% FSM, 3% ISM and the DH ≤ 5 HSM respectively (Chapters 3 and 4) (Table 5.2).

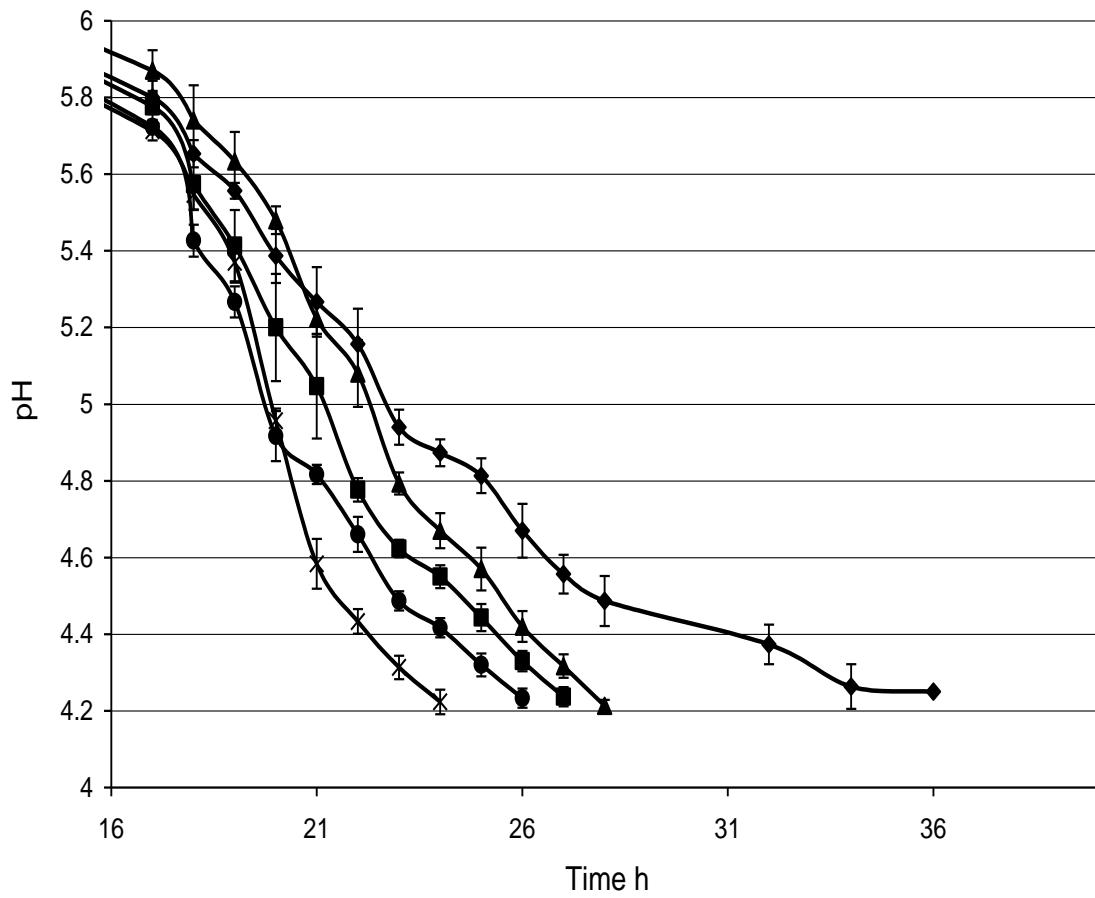


Fig 5.1 the pH change during incubation of *Bifidobacterium lactis* Bb12 at 37°C to reach the fermentation point at pH 4.2±0.03 in skim milk without any addition (Control) (♦), with Hydrolysis for 10 min by trypsin and FOS 5%(A) (■), with Hydrolysis10 min by trypsin and Inulin 3% (B)(▲), with FOS 5% and Inulin 3% (D)(●), with hydrolysis 10 min by trypsin, FOS 5% and Inulin 3% (C) (×). Error bars indicate standard deviation for three samples.

Table 5.1 Fermentation time of *Bifidobacterium lactis* Bb12 in skim milk without any addition (Control), with DH< 5% Hydrolysis by trypsin and FOS 5 %(A), with DH<5% Hydrolysis by trypsin and inulin 3%(B), with FOS 5% and inulin 3%, with DH< 5% hydrolysis by trypsin(C), FOS 5% and inulin 3%(D) to reach pH 4.2 ± 0.03 .

Fermentation time (h) of <i>Bifidobacterium lactis</i> Bb12				
Control	A	B	C	D
36	27	28	24	26

Sample (D) reached the target pH 4.2 ± 0.03 in 26 hours and that supports the notion of using more than one prebiotic which will have better effect on the growth of *Bifidobacterium lactis* Bb12 in skim milk. Furthermore, this result recommends using this medium as it was the second most optimal medium for stimulating the growth of *Bifidobacterium lactis* Bb12, while fermentation times for samples (A) and (B) were 27 and 28 hours, respectively, which were better than the control sample, which took 36 hours to get the target pH of 4.2 ± 0.03 .

Moreover, sample (A), which contained 5% FOS in hydrolysed skim milk for 10 min, showed better reduction in the fermentation time as compared to the sample with just 5% FOS in skim milk without hydrolysis (5% FSM) (Chapter3) and this reduction was significant ($P < 0.05$) as the reduction time of sample (A) was 9 hours while it was just 3 hours in the 5% FSM sample and 7 hours in the DH≤5 HSM (Table 5.2) (Chapters 3, 4).

These results support again the main aim of this work where the synergistic combination of using prebiotics and hydrolysed milk has a pronounced effect on stimulating the growth effect of *Bifidobacterium lactis* Bb12. Moreover, the presence of 5% FOS in DH≤5 hydrolysed skim milk caused the reduction of the fermentation time rather than the use of 5% FOS by itself in the FSM and at least by greater than 2 hours by using the DH≤5 HSM by itself.

However, using inulin in sample (B) with the DH≤5 hydrolysed skim milk was again significantly ($P<0.05$) better than using inulin by itself, by almost 1 hour as compared to the DH≤5 HSM in (Chapter 4). Sample (B) with 3% inulin in DH≤5 hydrolysed skim milk showed better reduction in the fermentation time of the control by 8 hours and it only improved by 2 hours in the 3% ISM (Chapter 3) (Table 5.2), and by 7 hours in the DH≤5 HSM (Chapter 4) (Table 5.2), which again shows the synergistic effect of using prebiotics and hydrolysed skim milk on the growth of *Bifidobacterium lactis* Bb12 and supports the idea of stimulating the growth of Bifidobacteria with the presence of prebiotic, peptides and amino acids in milk (Bouhnik *et al.*, 1994; Dave and Shah, 1998; Bruno *et al.*, 2002; Akalın *et al.* 2004; Bielecka and Biedrzycka, 2004). However, samples (A) and (B) did not greatly affect the growth as the result of the reduction in time as compared with the hydrolysed skim milk for the DH≤5 sample (HSM) by itself was not significant ($P<0.05$) but still better as compared to the control (Table 5.2), and this showed the important effect of the synergistic action of prebiotic and hydrolysed skim milk on the growth of *Bifidobacterium lactis* Bb12 in sample (A) and (B) in comparison with samples 5% FSM and 3% ISM (Chapter 3) and (

Table 5.2). Samples (A) and (B) showed better reduction in the fermentation time of the *Bifidobacterium lactis* in skim milk but that reduction was less than the combination of 5% FOS and 3% inulin in sample (D) which had reduction in fermentation time of 10 hours as compared with the control and by 1 hour and 2 hours in comparison with samples (A) and (B), respectively (Table 5.1). These results support the theory that the synergistic effect of two prebiotics 5% FOS and 3% inulin with *Bifidobacterium lactis* Bb12 was better, and that was also documented in previous studies *in vivo* and *in vitro* (Gibson *et al.*, 1995; Gauthier and Pouliot, 2003; Gibson *et al.*, 2005). Moreover, the DH≤5 hydrolysed skim milk did not enhance the growth of *Bifidobacterium lactis* Bb12 in comparison with sample (C) where the combination of the prebiotic and hydrolysed skim milk showed better effect on the growth of *Bifidobacterium lactis* Bb12.

Sample (c) showed the best stimulating effect on the growth of *Bifidobacterium lactis* Bb12 in the presence of 5% FOS, 3% Inulin in the hydrolysed skim milk for 10 minutes, and it provided the target pH for ending the fermentation at 24 hours which is the best reduction in fermentation time among all samples designed (Table 5.1). The decrease in the pH of skim milk in the fermentation process is well known as a result of the production of lactic and acetic acids which result from the fermentation activity of bifidobacteria of soluble fibers such as FOS and inulin in the milk samples. However, the high activity of *Bifidobacterium lactis* Bb12, which reduced the fermentation time to reach the target pH, may be due to the presence of some peptides in the hydrolysed skim milk, which stimulated the metabolism and growth of *Bifidobacterium lactis* Bb12 (chapter 4). The presence

of peptides in the hydrolysed skim milk helps the bifidobacteria in their metabolism and growth by using the peptides as a nitrogen source, which aids in the fermentation process and provides an efficient source of nitrogen as indicated in sample (C). Comparatively, the slow growth of the control could be due to the fact that bifidobacteria had slower growth and proteolytic activity in normal skim milk (Dave and Shah, 1998; Christensen *et al.*, 1999; Shah, 2001). Moreover, sample (D) has shown reduction in the fermentation time but still less than sample (C) even though it contained 5% FOS and 3% inulin, but the hydrolysed skim milk for DH≤5 in sample (C) stimulated the growth of *Bifidobacterium lactis* Bb12 and showed better reduction in the fermentation time (Table 5.2), which supports the idea that even the proteolytic activity of *Bifidobacterium lactis* Bb12 in hydrolysis skim milk with prebiotics was improved (Dave and Shah, 1998).

Table 5.2 The different effects of using different treatments of skim milk on the reduction of the fermentation time as compared with the control of each sample, the logarithmic reduction as cfu/ml and the viability % of *Bifidobacterium lactis* Bb12 and that in the comparison of using one treatment or synergy of more than one treatment. Where A is (DH<5% skim milk + 5% FOS), B is (DH<5% skim milk + 3% inulin), C is (DH<5% skim milk + 5% FOS + 3% inulin) and D is (5% FOS +3% inulin).

Versus	A vs. FSM (5%)	B vs. ISM with (3%)	A vs. HSM for (10minute)	B vs. HSM for (10minute)	D vs. FSM With (5%)	D vs. ISM With (3%)	C vs. HSM for (10minute)	C vs. FSM with (5%)	C vs. ISM with (3%)
Reduction of the fermentation time (h)	9 vs. 3	8 vs. 2	9 vs. 7	8 vs. 7	10 vs. 3	10 vs. 2	12 vs. 7	12 vs. 3	12 vs. 2
logarithmic cycle loss	1.33 Vs. 1.3	1.56 Vs. 1.59	1.33 Vs. 1.7	1.56 Vs. 1.7	1.88 Vs. 1.3	1.88 Vs. 1.59	0.98 Vs. 1.7	0.98 Vs. 1.3	0.98 Vs. 1.59
Viability %	4.68 VS. 5.02	2.67 Vs. 2.49	4.68 Vs. 1.86	2.67 Vs. 1.86	1.31 Vs. 5.02	1.31 Vs. 2.49	10.44 Vs. 1.86	10.44 Vs. 5.02	10.44 Vs. 2.49

5.5.2 Study the synergistic effect of prebiotics and hydrolysed skim milk on the survival of *Bifidobacterium lactis* Bb12.

After the fermentation process was completed and a pH of 4.2 ± 0.03 was reached, all of the five bottles and their four samples were immersed in an ice bath to reach a temperature of $4^{\circ}\text{C} \pm 1$ followed by refrigeration over 28 days.

In Figure (5.2), the change of the *Bifidobacterium lactis* Bb12 as \log_{10} cfu/ml of all samples (A), (B), (C), (D) and control, over the 28 day refrigerated storage period is outlined.

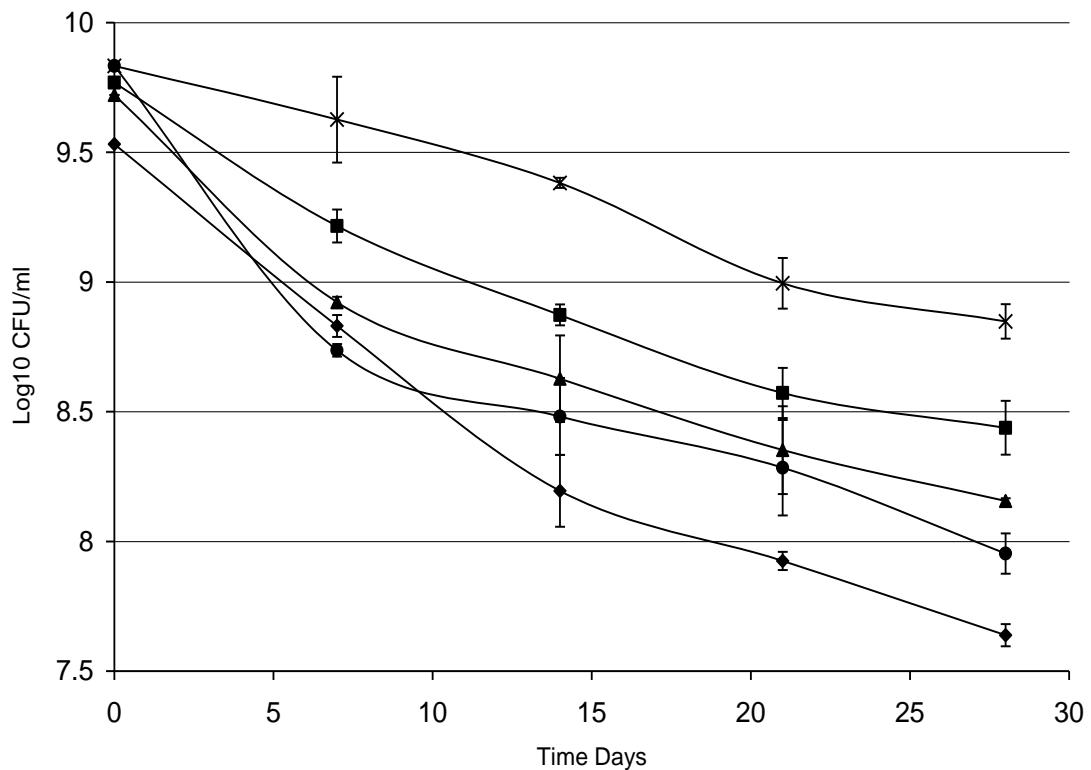


Fig 5.2 the \log_{10} of the viable count of *Bifidobacterium lactis* Bb12 in skim milk without hydrolysis (control) (♦), with hydrolysis for DH ≤ 5 by trypsin and FOS 5% (A)(■), with hydrolysis DH ≤ 5 by trypsin and inulin 3% (B)(▲), with inulin 3% and FOS 5% (D)(●), with hydrolysis DH ≤ 5 by trypsin, FOS 5% and inulin 3% (C)(×) over 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$. Error bars were taken as standard deviation for three samples.

All samples show reduction in the total count of the *Bifidobacterium lactis* Bb12 over the storage period. However, sample (C) demonstrated the best survival rate and kept the total count of *Bifidobacterium lactis* Bb12 at $8.8 \log_{10}$ cfu/ml with less than 1 logarithmic loss over the four weeks refrigerated storage (Figure 5.2, Figure 5.3). However, sample (C) did show a steady decrease over the four weeks and kept the total count of *Bifidobacterium lactis* Bb12 over $9 \log_{10}$ cfu/ml and dropped to just over $8.8 \log_{10}$ cfu/ml at the end of the fourth week, which still indicated the best survival as compared to all other samples.

Samples (A), (B), (D) and control showed a decrease in the total count of *Bifidobacterium lactis* B12, but all samples with treatments either using hydrolysed skim milk for DH<5 % and one of the prebiotics (A, B) or by using a combination of the prebiotics only (D) showed better survival as compared to the control sample (Figure 5.2). However, all samples (A), (B), and (D) which have *Bifidobacterium lactis* Bb12 and either two of the prebiotic or one of them in hydrolysis skim milk for DH≤5 showed better survival and lower logarithmic reduction over the storage period: 1.33, 1.56, 1.88 and 1.89 for samples (A), (B), (D) and control, respectively (Figure 5.3).

Sample (C) showed a significant increase in survival ($P<0.05$) which was achieved by adding 5% FOS and 3% inulin in hydrolysed skim milk for DH< 5 by trypsin, but these results were expected due to the stimulatory effect of each of FOS, inulin or Hydrolysed milk separately on the survival of *Bifidobacterium lactis* Bb12 (Chapters 3, 4). However the synergistic effect of using these components together was not known. Sample (C) showed a significant effect ($P<0.05$) on the

survival of *Bifidobacterium lactis* Bb12 by lowering the total count over the 28 days refrigerated storage by 0.98 log cfu/ml and that was better than the control which has over 1.85 log cfu/ ml loss over the same period (Figure 5.3 and Table 5.2). The viability of *Bifidobacterium lactis* Bb12 in sample (C) was significantly better ($P<0.05$) than the control and the other samples in this study and was at 10.44% which is better than the control by almost 10 times (Table 5.3). These results again corroborate the synergistic effect of 5% FOS and 3% inulin in the DH≤5 hydrolysed skim milk on the survival of *Bifidobacterium lactis* Bb12 over the shelf life of the probiotic product, which is related to the presence of oligosaccharides, peptides and amino acids of the partially hydrolysed skim milk by trypsin, which is well known to have enhancement effect on the survival and viability of Bifidobacteria in milk (Petschow *et al.*, 1999; Alander *et al.*, 2001; Berkhout *et al.*, 2003; Gopal *et al.*, 2003; Semjonoves, *et al.*, 2004; Kim *et al.*, 2005).

Table 5.3 the Viability % of *Bifidobacterium lactis*. Bb12 in skim milk without any addition (Control), with Hydrolysis for DH≤5 by trypsin and FOS 5%(A), with Hydrolysis DH≤5 by trypsin and inulin 3%(B), with FOS 5% and inulin 3%, with hydrolysis 10 min by trypsin(C), FOS 5% and inulin 3%(D) after 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$. Error bars were taken as standard deviation for three samples.

Viability of <i>Bifidobacterium lactis</i> Bb12 % after refrigerated storage for 28 days at $4^{\circ}\text{C} \pm 1$				
Control	A	B	C	D
1.23	4.68	2.67	10.44	1.31

Figure (5.3) show the logarithmic reduction of the total count of *Bifidobacterium lactis* Bb12 over storage period in samples A (DH<5% skim milk + 5% FOS), B (DH<5% skim milk + 3% inulin), C (DH<5% skim milk + 5% FOS + 3% inulin) and D (5% FOS +3% inulin) and the control.

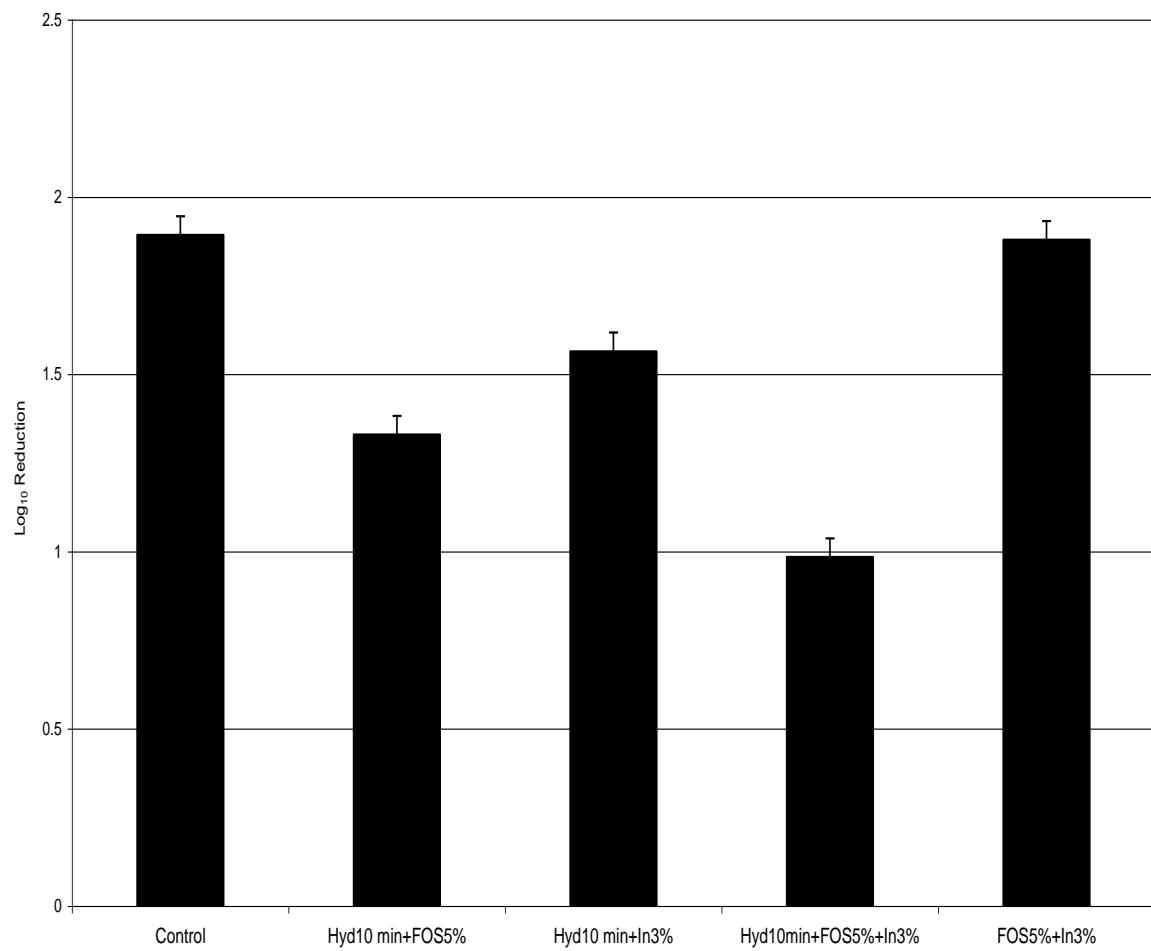


Fig 5.3 the mean logarithmic reduction for three samples of the *Bifidobacterium Lactis* Bb12 as log₁₀ cfu/ml in the different mixtures of 5% FOS and 3% inulin in skim milk with or without hydrolysis for DH≤5 over refrigerated storage for 28 days.

This is further validated by the comparison of the logarithmic loss and viability of *Bifidobacterium lactis* Bb12 in sample (C), which has a combination of prebiotics and hydrolysed skim milk, with those samples which used just one of the prebiotics or the hydrolysed skim milk by itself. Table (5.2) shows the different effect of using a prebiotic and hydrolysed skim milk on the survival as viability and logarithmic loss of *Bifidobacterium lactis* Bb12. Sample (C) had the lower logarithmic loss with about 0.98 log cfu/ml, where it was 1.7, 1.3 and 1.59 in the hydrolysed skim milk, 5% FSM and 3% ISM, respectively. The same comparison with sample (C) showed a very significant increase in viability with 10.44% as compared to 1.86, 5.02 and 2.49 in the hydrolysed skim milk, 5% FSM and 3% ISM, respectively (Table 5.2).

That again illustrates the significant synergy of the combination of these components on the survival of *Bifidobacterium lactis* Bb12.

However, the use of just two treatment options as in samples (A), (B) and (D) showed much better survival as a decrease in logarithmic loss and viability as compared to the control (Figure 5.3 and Tables 5.1 and 5.2) and that is related to the presence of the peptides in the hydrolysed skim milk for improving the survival rate of *Bifidobacterium lactis* Bb12 (Donkor *et al.*, 2007) such as in samples (A), (B) and (D), which showed better logarithmic reduction of 1.33, 1.56 and 1.81 as compared to the use one of the treatments by itself, which showed reduction of 1.3, 1.59 and 1.7 in the 5% FSM and 3% ISM and DH ≤ 5 hydrolysed skim milk. However, all treatments showed less survival than the

combination of the three treatments together as in sample (C), which was 0.98 log cfu/ml (Table 5.2).

The results demonstrate that the viability of using one of the prebiotics with the DH≤5 hydrolysed skim milk was similar to using the prebiotic alone, where it was % 4.68, 2.67 and 1.31 in samples (A), (B) and (D), respectively, while it was 5.02, 2.49 and 1.86 in hydrolysed skim milk, 5% FSM and 3% ISM, respectively (Table 5.2). However, this viability was increased significantly ($P<0.05$) in sample (C) which was 10.44% and that validated the theory of the positive effect of the synergism of prebiotics and hydrolysed skim milk on the growth and survival of bifidobacteria.

The results shown in Table (5.2) indicate the importance of synergy of prebiotic and hydrolysed skim milk and this is because of the possibility of the release of peptides and amino acids as result of the partially hydrolysed skim milk aids in the survival of *Bifidobacterium lactis* Bb12. This appears especially in the comparison between samples (A) and (B) with sample (D), which indicates of the importance of peptides and amino acids in the survival of *Bifidobacterium lactis* Bb12 and *Bifidobacteria spp.* in general.

When milk is hydrolysed, the amount of peptides and amino acids will increase in this milk (Adachi *et al.*, 1991; Shah, 2000a; Carreira *et al.*, 2004) and this will provide an important source of nitrogen to enhance the growth and survival of bacteria in general and probiotics in milk (Cheng *et al.*, 1984; Zajka and Zbikowski, 1986; Roy *et al.*, 1990).

5.5.3 The effect of the optimum combination of prebiotics and hydrolysed skim milk on the growth and survival of different probiotics in skim milk.

Since bifidobacteria have been highly recognized for their important role in health promotion, a lot of studies have been done to observe their characteristics and over 90 probiotic products on the market have been studied (Shah, 2000a). Different strains and species of bifidobacteria were used in these probiotic products and in order to assess viability of probiotics it was very important to chose the right species and strain of these bacteria in dairy product production. However, the studies have shown different behavior and characteristic for each species during the fermentation period (Abu-Tarboush, 1994; Shah, 2000a) and therefore it is very important to choose the right species of bifidobacteria to incorporate into dairy products (Ventling and Mistry, 1993; Tamime *et al.*, 1995; Gomes and Malcata, 1998; Shah, 2000a). The literature has shown a very positive image of the use of *Bifidobacterium lactis* Bb12 in dairy products over decades (Alander *et al.*, 2001; Trindade *et al.*, 2003; Semjonoves, *et al.*, 2004; Janer *et al.*, 2005), and therefore it has been studied in detail in this research. However, it was very important in this study to find out the different effect of the optimum medium on the growth and survival of different species of probiotic bacteria in fermented milk and to seek the effect of hydrolysed skim milk and

prebiotics on their growth and survival in fermented milk. However, bifidobacteria species are very similar as they are all Gram-positive rods and produce lactic and acetic acid in fermented milk (Abu-Taraboush *et al.*, 1998).

Three species of bifidobacteria; *Bifidobacterium bifidum* NCIMB 702203, *Bifidobacterium infantis* NCIMB 702205 and *Bifidobacterium breve* NCIMB 702257 and one strain of *Lactobacillus spp.* *L. casei* strain shirota were obtained to study the effect of the synergism of prebiotics and hydrolysed skim milk on their growth and survival.

Skim milk was hydrolysed for DH≤5 by trypsin, then 5% FOS and 3% inulin were added to each sample and then pasteurized (milk pasteurized at 70 °C for 15 minutes (chapter 4)) to prepare new media for use in this study, hereafter named the Mix Design Skim Milk (MDSM) product, for assessing the growth and survival of Bifidobacteria and lactobacillus species separately. Each species of probiotic was separately incubated anaerobically in skim milk without any treatments overnight (14-15h) at 37°C to use as inoculums cultures in the MDSM product. A 5% v/v of the overnight culture of each species was inoculated in each sample and the control to start the fermentation process separately. At the end of the fermentation period and when a pH of 4.2±0.03 was reached, all samples were immersed in an ice bath until a temp of 4°C±1 was reached then stored at that temperature for 28 days to assess survival.

The growth rate was studied as the pH changed in MDSM product over the fermentation period of 16 hours. Samples were taken hourly until a pH of 4.2±0.03 was reached and the fermentation was stopped. The survival study has

done by assessing the total count of *Bifidobacterium spp.* and *Lactobacillus casei* strain shirota at the end of fermentation and every 7 days for 28 days. All samples were diluted in MRD and plated on MRS-agar with 5% L- cysteine hydrochloride. The viability percentage was calculated as mentioned in chapter 3 for all samples and controls.

5.5.3.1 Growth of different probiotic bacteria in MDSM product

In Table (5.4) the comparison of the fermentation time in hours and total count of probiotic bacteria as \log_{10} cfu/ml at the end of the fermentation time between the control (skim milk with no treatments) and in the MDSM product showed a better reduction in the fermentation time in all probiotic samples in MDSM product.

However, from Fig 5.4 and Table 5.4 it has shown that *B. breve* NCIMB 702257 has the best growth rate in the MDSM product as it reduced the fermentation time by 11 hours as compared with its control, which was a significant ($P<0.05$) reduction of the fermentation time. *Bifidobacterium infantis* NCIMB 702205 showed better growth in the MDSM product but it did not significantly ($P<0.05$) reduce the fermentation time as it only reduced it by two hours (Table 5.4) and Fig (5.5). *B. bifidum* NCIMB 702203 in the MDSM product did show better growth and the total time needed for the end of the fermentation process was 29 hours, which was reduced by 7 hours as compared with its control. *L. casei* strain shirota in the MDSM product took 25 hours to reach end of the

fermentation, which was a 9-hour reduction in the fermentation time as compared to the control.

The best fermentation times was 25 hours and was achieved with the *B. breve* NCIMB 702257 and *L. casei* strain shirota in the MDSM product with reductions of 11 and 9 hours in the fermentation time as compared to the control, respectively. *B. bifidum* NCIMB 702203 in the MDSM product did show good reduction in the fermentation time by 7 hours and the *Bifidobacterium infantis* NCIMB 702205 showed the least effect in the MDSM product with just 2 hours reduction in the fermentation time as compared with its control (Table 5.4).

Table (5.4) the fermentation time (h) and total count (\log_{10} cfu/ml) at the end of fermentation by *Bifidobacteria spp.* and *Lactobacillus casei* strain shirota in MDSM product.

	Control + <i>B. breve</i> NCIMB 702257	<i>B. breve</i> NCIMB 702257 in MDSM	Control + <i>B. infantis</i> NCIMB 702205	<i>B. infantis</i> NCIMB 702205 in MDSM	Control + <i>B. bifidum</i> NCIMB 702203	<i>B. bifidum</i> NCIMB 702203 in MDSM	Control + <i>L. casei</i> strain shirota	<i>L. casei</i> strain shirota in MDSM
Fermentation time (h)	36	25	36	34	36	29	34	25
Total count \log_{10} cfu/ml at the end of Fermentation	9.26	9.83	9.66	9.76	9.63	9.87	9.72	9.87

Figure (5.4) shows the difference of the pH change over the fermentation period to reach the pH 4.2 ± 0.03 by using *Bifidobacterium bifidum* NCIMB 702203 in the MDSM product and in the control.

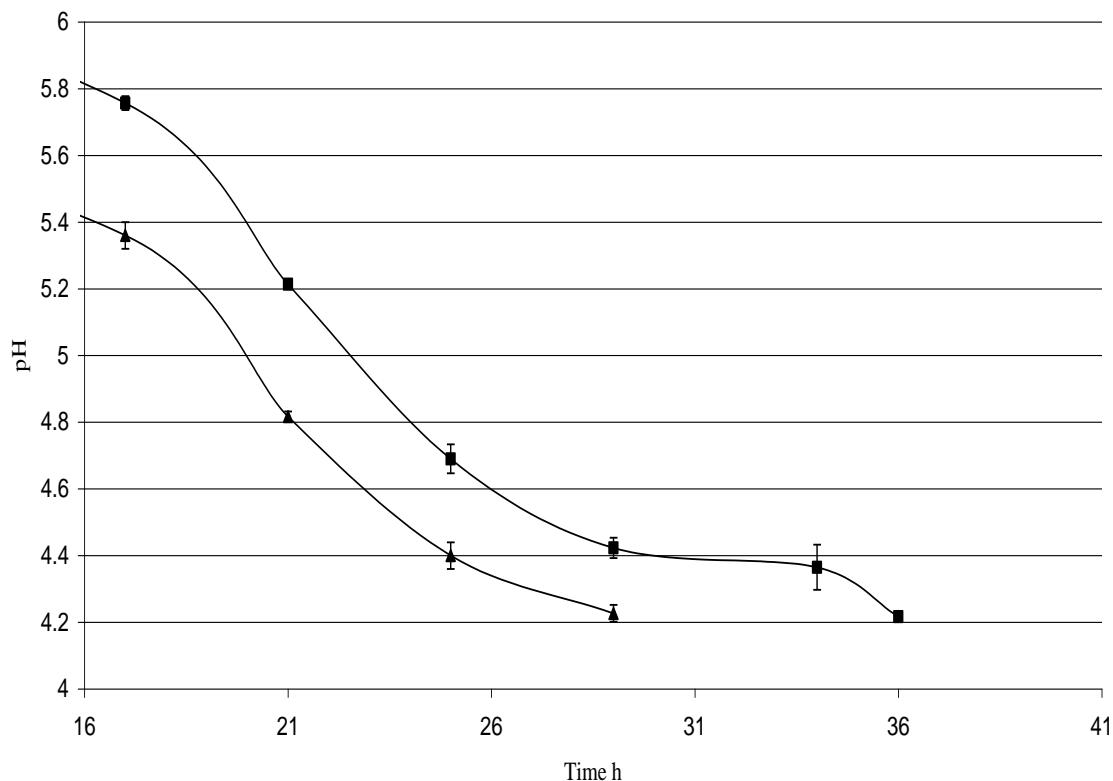


Fig 5.4 the pH change and development of *Bifidobacterium bifidum* NCIMB 702203 growth to reach the fermentation point at $\text{pH } 4.2 \pm 0.03$ in skim milk without any addition (Control) (■) and in MDSM product (▲). Error bars were taken as standard deviation for three samples.

Figure (5.5) showed the difference of the pH change over the fermentation period to reach the $\text{pH } 4.2 \pm 0.03$ by using *Bifidobacterium infantis* NCIMB 702205 in the MDSM product and in the control.

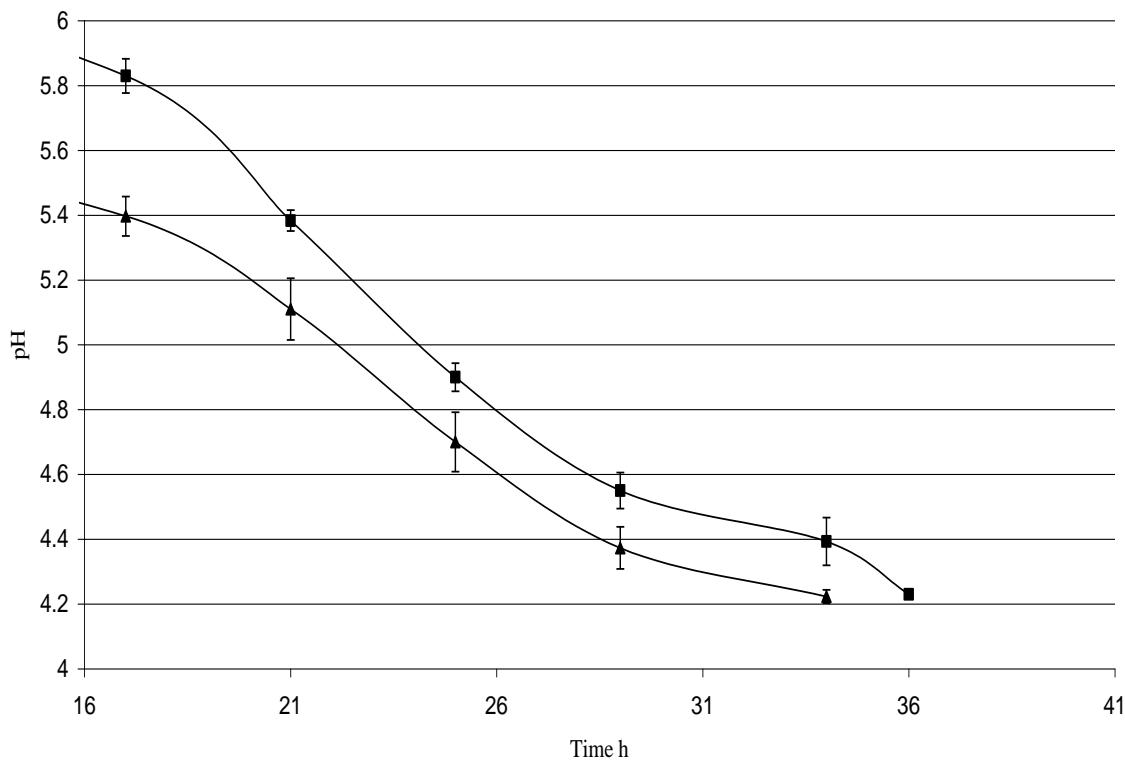


Fig 5.5 the pH change and development of *Bifidobacterium infantis* NCIMB 702205 growth to reach the fermentation point at $\text{pH } 4.2 \pm 0.03$ in skim milk without any addition (Control) (■) and in MDSM product (▲). Error bars were taken as standard deviation for three samples.

Figure (5.6) showed the difference of the pH change over the fermentation period to reach the $\text{pH } 4.2 \pm 0.03$ by using *Bifidobacterium breve* NCIMB 702257 in the MDSM product and in the control.

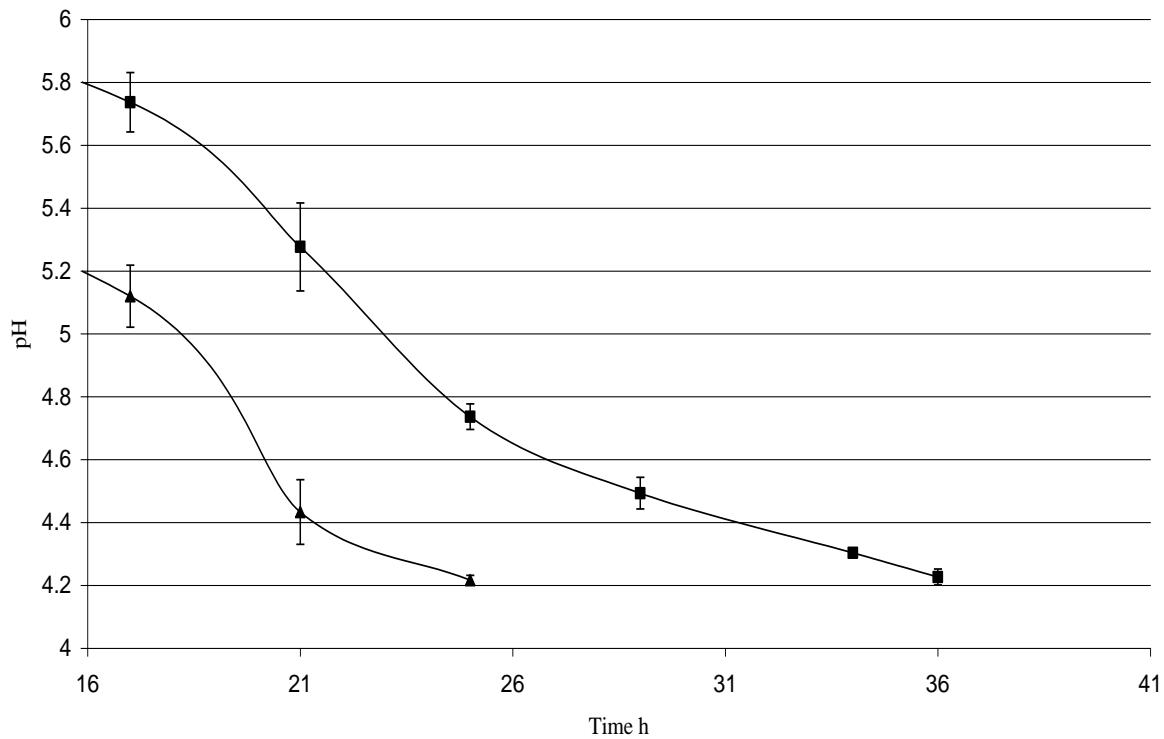


Fig 5.6 the pH change and development of *Bifidobacterium breve* NCIMB 702257 growth to reach the fermentation point at $\text{pH } 4.2 \pm 0.03$ in skim milk without any addition (Control) (■) and in MDSM product (▲). Error bars were taken as standard deviation for three samples.

Figure (5.7) showed the difference of the pH change over the fermentation period to reach the $\text{pH } 4.2 \pm 0.03$ by using *L. casei* strain shirota in the MDSM product and in the control.

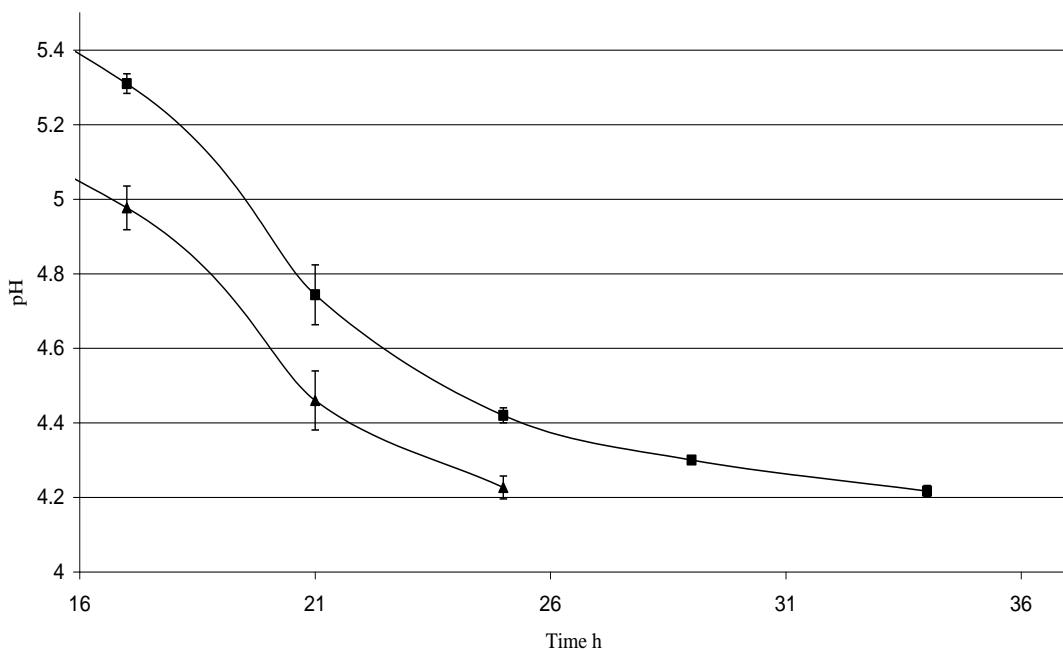


Fig 5.7 the pH change and development of *L. casei* strain shirota growth to reach the fermentation point at pH 4.2 ± 0.03 in skim milk without any addition (Control) (■) and in MDSM product (▲). Error bars were taken as standard deviation for three samples.

All the Bifidobacteria samples with the MDSM product showed better growth than their controls and that was reflected in their total count at the end of fermentation process (Table 5.4), where the total counts as \log_{10} cfu/ml of the *B. breve* NCIMB 702257 and *L. casei* strain shirota were higher than their controls. The same results were found for *B. bifidum* NCIMB 702203 and *B. infantis* NCIMB 702205, with better \log_{10} CFU/ml than their controls (Table 5.4), and that referred to the enhancement of the MDSM product on probiotic bacteria species and validated the theory of the benefit of using a combination of hydrolysed skim milk and prebiotics to enhance the growth of probiotics in milk products.

The difference in the fermentation time for each species, in spite of using the same medium and conditions of the fermentation process, could be due to the difference in the enzymatic ability of each species and their different metabolisms (Desjardins and Roy, 1990).

These results showed a very important and interesting observation to be taken into account into the manufacturing of dairy products, as the choice of the right species of bifidobacteria is very important to get the best growth and the highest total count at the end of the fermentation time. This could enhance the current products to have them achieve a higher number of probiotics and better provide the health benefits of these organisms.

5.5.3.2 Survival and viability of different probiotic bacteria in the MDSM product;

Table (5.5) shows the results of the total count of different probiotic bacteria in the MDSM product and their control samples at the end of the fermentation time at pH 4.2 ± 0.03 and at the end of the refrigerated storage for 28 days at $4^{\circ}\text{C} \pm 1$.

All samples in the MDSM product have shown a significant enhancement ($P < 0.05$) of the survival of the probiotic bacteria over the 28 day storage period at $4^{\circ}\text{C} \pm 1$, but this enhancement varied for the different species of probiotic bacteria. The viability percentage of all *Bifidobacteria spp.* and *L. casei* strain shirota in the MDSM product were very good and significant ($P < 0.05$) as compared with their controls.

However, the viability percentage varied depending on the bacteria species. The best viability percentage was observed with the *B. breve* NCIMB 702257 at 5.62% in the MDSM product, which is 3.8% better than its control sample (Table 5.5). The other *Bifidobacteria spp.* showed less viability percentage than *B. breve* NCIMB 702257 and the results are shown in Table (5.5) with 3.13%, 4.33% and 4.97% in *B. bifidum* NCIMB 702203, *B. infantis* NCIMB 702205 and *L. casei* strain shirota, respectively, where the viability percentages for their control samples were less than 1% (Table 5.5).

Table 5.5 Total counts of different probiotic bacteria and their control in the MDSM product at the pH 4.2 ± 0.03 , at the end of the 28 day refrigerated storage at $4^{\circ}\text{C} \pm 1$ and the viability percentage.

	Total count of probiotic bacteria as \log_{10} cfu/ml							
	Control+ <i>B. breve</i> NCIMB 702257	<i>B. breve</i> NCIMB 702257 in MDSM	Control+ <i>B. infantis</i> NCIMB 702205 in MDSM	<i>B. infantis</i> NCIMB 702205 in MDSM	Control+ <i>B. bifidum</i> NCIMB 702203	<i>B. bifidum</i> NCIMB 702203 in MDSM	Control+ <i>L. casei</i> strain shirota	<i>L. casei</i> strain shirota in MDSM
End of Fermentation at pH 4.2 ± 0.03	9.26	9.83	9.66	9.76	9.63	9.87	9.72	9.87
After 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$	7.52	8.59	7.32	8.40	7.46	8.37	7.63	8.57
Viability %	1.82	5.62	0.45	4.33	0.67	3.13	0.82	4.97

Figure (5.8) shows the total count as \log_{10} cfu/ml change over the refrigerated storage period at 4°C of *Bifidobacterium bifidum* NCIMB 702203 in the MDSM

medium and its control sample which was measured every 7 days over 28 days of storage.

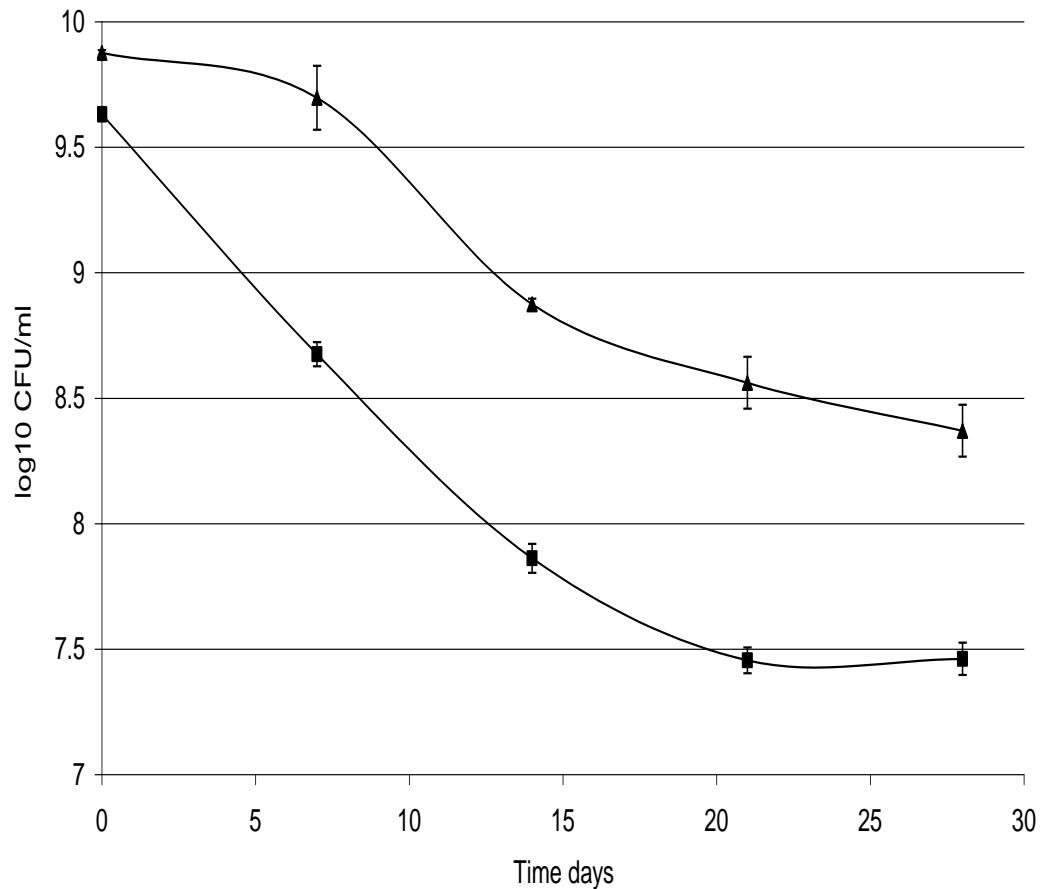


Fig 5.8 the \log_{10} of the viable count of *Bifidobacterium bifidum* NCIMB 702203 in skim milk without hydrolyse (Control) (■) and in MDSM product (▲). Over 28 days refrigerated storage at 4°C. Error bars were taken as standard deviation for three samples.

Figure (5.9) shows the decrease of the total count of *Bifidobacterium infantis* NCIMB 702205 as \log_{10} cfu/ml in the MDSM product and in its control sample over the refrigerated storage period at 4°C.

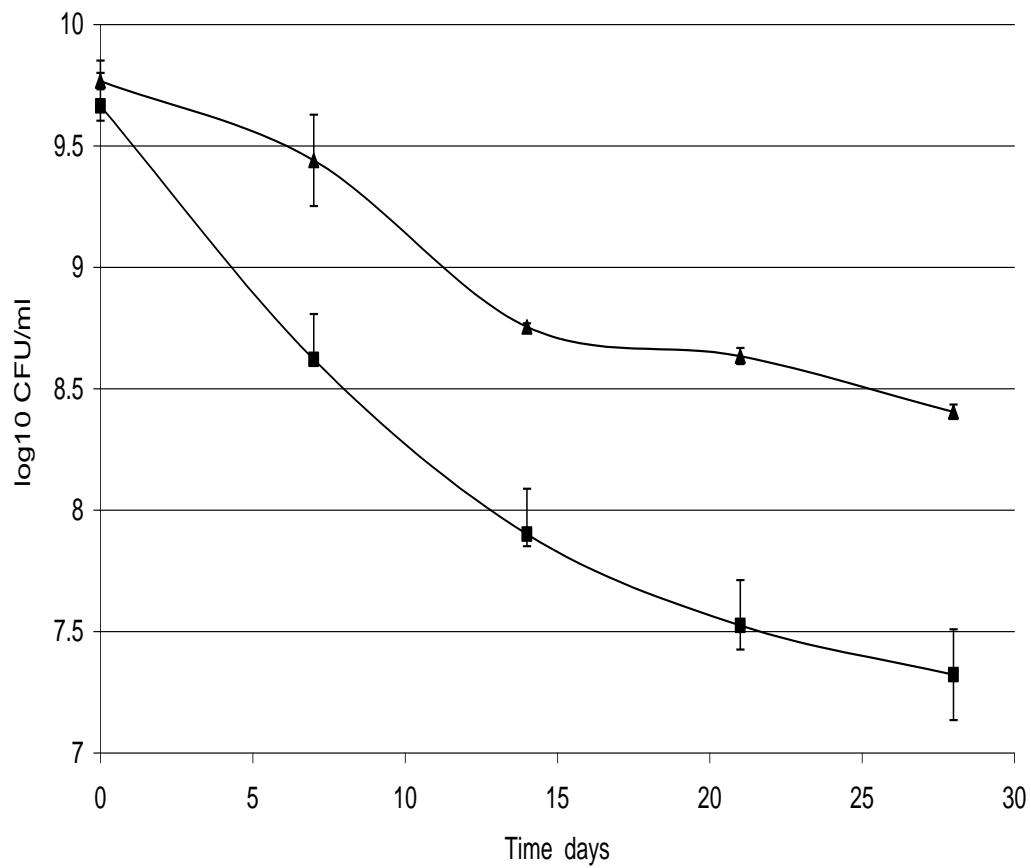


Fig 5.9 the \log_{10} of the viable count of *Bifidobacterium infantis* NCIMB 702205 in skim milk without hydrolysis (Control) (■) and in MDSM product (▲) over 28 days refrigerated storage at 4°C. Error bars were taken as standard deviation for three samples.

The total count change of *Bifidobacterium breve* NCIMB 702257 as \log_{10} cfu/ml are shown in Figure (5.10) in the MDSM product and its control sample over the refrigerated storage period at 4°C.

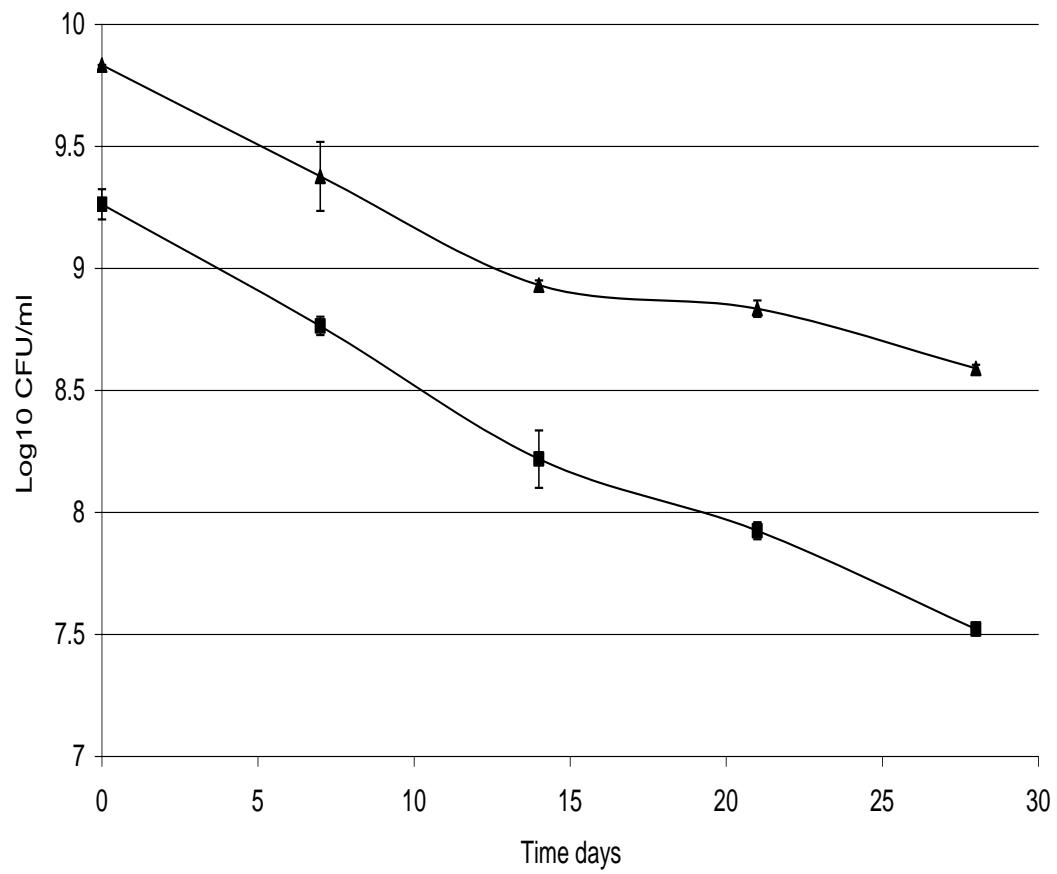


Fig 5.10 the \log_{10} of the viable count of *Bifidobacterium breve* NCIMB 702257 in skim milk without hydrolysis (Control) (■) and in MDSM product (▲) over 28 days refrigerated storage at 4°C. Error bars were taken as standard deviation for three samples.

The *L. casei* strain shirota total counts were taken over the refrigerated storage for 28 days at 4°C in the MDSM product and in its control sample as shown in Figure (5.11).

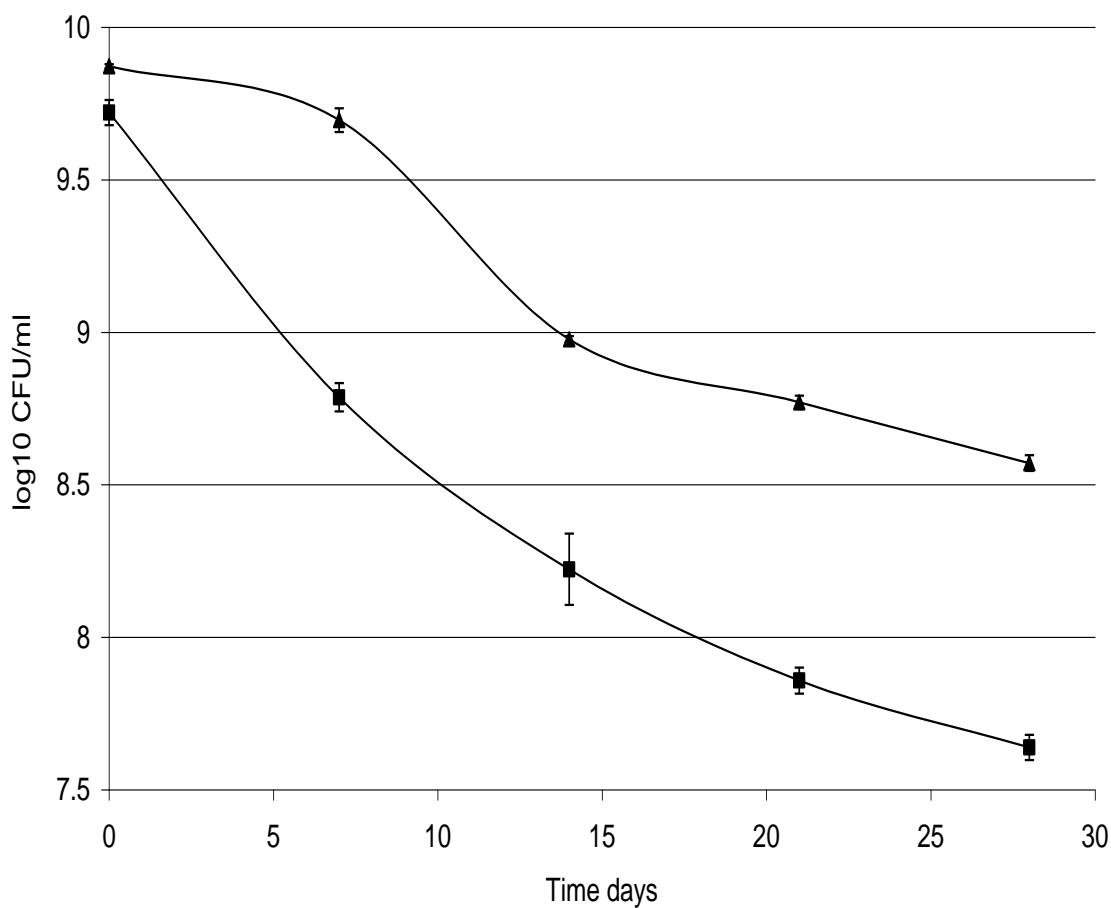


Fig 5.11 the \log_{10} of the viable count of *L. casei* strain shirota in skim milk without hydrolysis (Control) (■) and in MDSM product (▲) over 28 days refrigerated storage at 4°C. Error bars were taken as standard deviation for three samples.

All samples for the different species of probiotic bacteria showed a lower reduction in the total count in MDSM product when compared with the control samples and that maybe due to the presence of peptides and amino acids from the partially hydrolysed skim milk using trypsin in the MDSM product with the presence of FOS and inulin, which provide a good nutrient source for the survival of probiotic bacteria in fermented products.

However, the logarithmic reductions for different probiotic bacteria species in the MDSM product were less than their control samples (Figure 5.12), but the best results were seen with *B. breve* NCIMB 702257 species, where it showed better viability and survival in the MDSM when compared to its control and the other species of probiotic bacteria used in this study (Shahidi *et al.*, 2008). Moreover, there was a decrease in the \log_{10} cfu/ml as shown in Figures (5.8-5.10) that was especially seen at the third week of refrigerated storage at $4^{\circ}\text{C}\pm 1$ with a total count less than $8 \log_{10}$ cfu/ml. Therefore, the MDSM product did show better survival as viability, total count and logarithmic reduction (figure 5.12) over the 28 days refrigerated storage at $4^{\circ}\text{C}\pm 1$, which suggests the importance of the synergism of prebiotic and hydrolysed skim milk in enhancing the survivability of probiotics in fermented products.

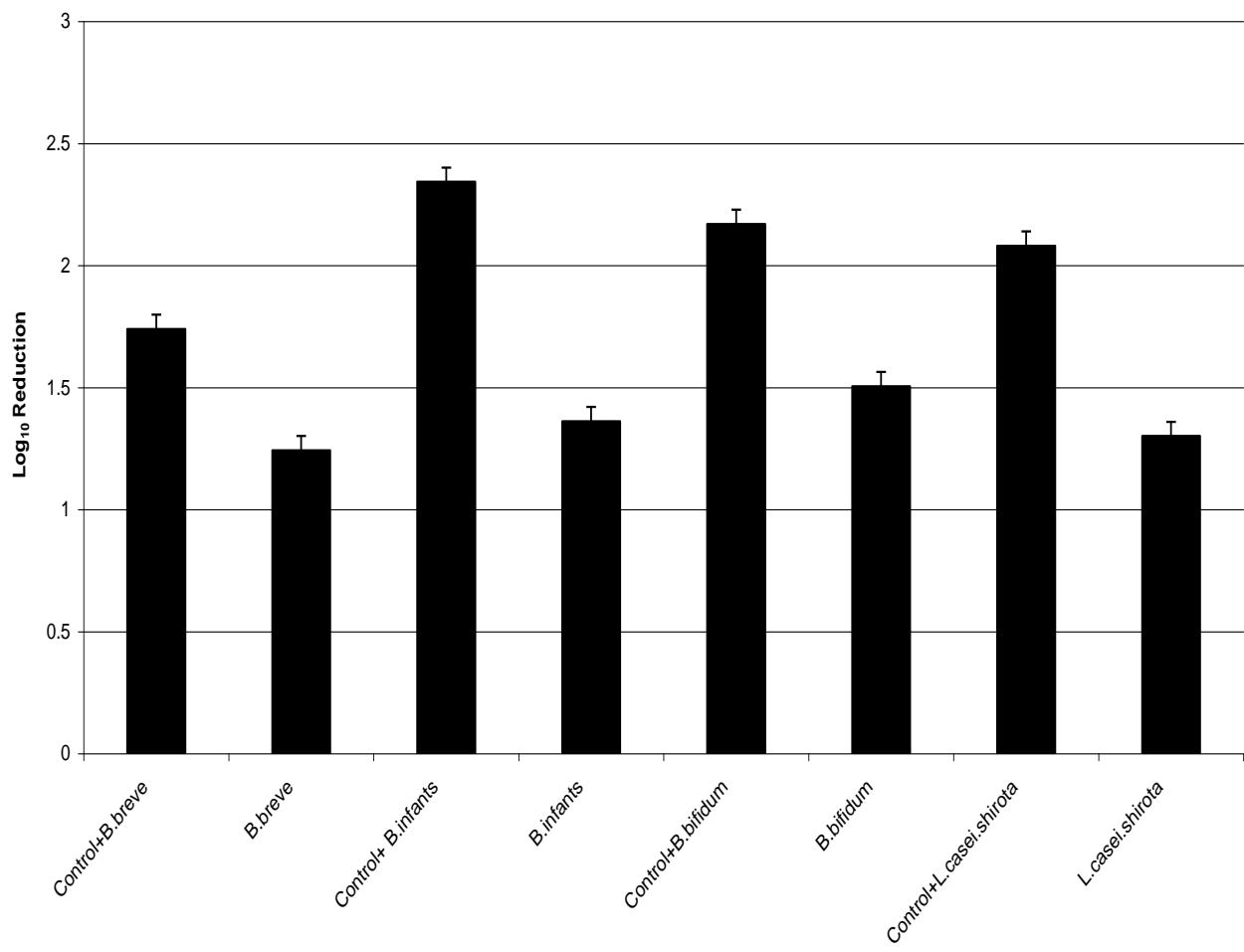


Fig 5.12 the mean logarithmic reduction for three samples of different probiotics as \log_{10} cfu/ml in the control and in the MDSM product over refrigerated storage at $4^{\circ}\text{C}\pm 1$ for 28 days.

Although all samples of different species of probiotic bacteria showed better growth and survival in the MDSM product as compared to their control samples, the growth and survival did vary between these species. The results have shown that *Bifidobacterium breve* NCIMB 702257 has the best growth and survival in

the MDSM product among all the probiotic samples in the MDSM product, which may be due to their special enzymatic ability to utilize the peptides and oligosaccharides available in fermented skim milk. *L. casei* strain shirota was the second best sample of the chosen probiotic bacteria in its growth and survival in the MDSM product, while *Bifidobacterium bifidum* NCIMB 702203 has the minimum promoting effect in the MDSM product for growth and survival.

However, a comparison between the species may help provide a better understanding of the metabolic ability of these different species in the same environment, which will help us seek a greater effect of the synergy of prebiotics and hydrolysed skim milk to enhance the growth and improve the survivability of different species of probiotic bacteria in fermented milk products.

The literature emphasizes the use of *Bifidobacterium lactis* in many dairy products in Europe and America (Meile *et al.*, 1997; Ventura and Zink, 2002; Biedrzycka and Bielecka, 2004; Simpson *et al.*, 2005), and that is mainly due to specific characteristics that they possess in regard to acid production and oxygen tolerance (Perrin *et al.*, 2000; Crittenden *et al.*, 2001a; Maus and Ingham, 2003; Saarela *et al.*, 2004; Talwalkar and Kailasapathy, 2004; Simpson *et al.*, 2005) that make this species suitable for use in dairy products.

This study demonstrated the response of *Bifidobactrium lactis* with commercial label Bb12 to enhance its growth and survival significantly ($P<0.05$) in comparison with its control samples and in comparison to other species of *Bifidobacteria spp.* and *Lactobacillus spp.* in the presence of prebiotic and hydrolysed skim milk (Biedrzycka and Bielecka, 2004). This synergy showed a

significant reduction ($P<0.05$) in the fermentation time in the presence of prebiotic (5%FOS and 3%inulin) in hydrolysed skim milk (for DH<5) by trypsin.

Furthermore, the survival studies showed significant improvement ($P<0.05$) of the *Bifidobacterium lactis* Bb12 survivability in comparison with the other species of probiotic bacteria in the same fermented skim milk product (MDSM product) after refrigerated storage for 28 days at $4^{\circ}\text{C}\pm1$ as the shelf life of most dairy products is around 28 days. This study has provided very important information regarding the use of bifidobacteria in fermented milk (Saito *et al.*, 2002; Bolduc *et al.*, 2006; Martinez-Villaluenga and Gomez, 2007), which is not that common due to their slow growth and survival in dairy products (Dave and Shah, 1998). The idea of using various substances individually as stimulators of growth and survival of these organisms had high interest in the last century and until now more data was still not available (Dave and Shah, 1997b; Christensen *et al.*, 1999; Donkor *et al.*, 2007). Other investigators were always interested in more studies for the selection of more suitable strain and species of bifidobacteria in dairy products, which shows the difficulties of using these organisms in dairy products because of their sensitivity to many factors such as pH, hydrogen dioxide and oxygen (literature review). However, *Bifidobacterium lactis* with the commercial name Bb12 was chosen based on its frequent use in dairy products and many studies worldwide showing its ability to survive and grow better under stressful conditions when compared to other species and strains (Alander *et al.*, 2001; Semjonoves, *et al.*, 2004; Shahidi *et al.*, 2008) in *in vitro* and *in vivo* studies. (Nopchinda *et al.*,

2002; Jain *et al.*, 2004; Chouraqui *et al.*, 2004; Larsen *et al.*, 2006; Mohan *et al.*, 2006).

5.6 CONCLUSION

This study proved the ability to enhance the growth and survival of *Bifidobacterium lactis* Bb12 as compared to other *Bifidobacteria* spp. and *Lactobacillus casei* strain shirota, which provides great new data for the probiotic manufacturing industry to improve the growth and survival of probiotic bacteria in their products in sufficient number at the end of their shelf-life in general and for *Bifidobacterium lactis* Bb12 specifically.

However, the use of bifidobacteria in dairy products raises several issues, such as their ability to produce acetic acid, which could affect the sensory attributes of the products. Ways to improve the taste and other sensory attributes of bifidobacteria dairy products in this study still need to be addressed. Therefore, the next chapter will explore the organoleptic qualities of bifidobacteria in hydrolysed skim milk with added prebiotics.

CHAPTER SIX

SENSORY EVALUATION OF FERMENTED PREBIOTICS SKIM MILK PRODUCT

6.1 INTRODUCTION:

Dairy foods have great consumption worldwide and therefore they have to appeal to consumers. However, the dairy industry has come a long way since its early development last century in developing techniques for judging consumer acceptance of dairy products. The large consumption of dairy products around the world led to an increased interest by dairy industry members to enhance the study, methodology and education in dairy science (Hunter and McEwan, 1998; Lawlor and Delahunty, 2000). The increased interest in formalizing the study of dairy products resulted in the development of standardized methods to judge dairy products in terms of the appearance, flavour, and texture of products on the market and products being tested to bring to market. The dairy market is very competitive now and more attention has to be paid to the ingredients, flavour, texture and having a consistent product on the market to attract and retain consumers. Moreover, huge interest has been generated in the health benefits of probiotics in dairy products, which has exploded the dairy product market, thus making the market very lucrative and competitive now. Bringing a high quality dairy product to market has to remain the main factor to maintain the microbial quality, shelf life, shelf stability or a combination of these elements in current products and new products being tested for marketing (Chapman *et al.*, 2001). Thus, it has been highly important to understand the product's characteristics and its effect on the sensory attributes, which is associated with the manufacturing process, region of production and the season, etc. Other attributes such as the dairy product's flavour, texture and colour (appearance)

are attractive targets for customers, which need to be analyzed using global methods under the term sensory analysis. Sensory analysis has been defined as "a compilation of different tools or tests that can be used for subjective or objective evaluation of food sensory properties." (Drake, 2004)

However, it was very important to recognize factors that affect the sensory properties of milk and dairy products such as heat treatments, oxidation processes, and the transfer of substances from the feed, the transfer of substances from the environment, packaging, storage time and enzymatic and microbial activities (Parliament and McGorrin, 2000). Therefore, several sensory analysis methods are now available to meet the requirements to judge the sensory properties of dairy products. The most common laboratory methods for the sensory analysis of foods are: (1) discriminative tests, (2) descriptive tests and (3) affective tests (Poste *et al.*, 1991).

6.1.1 The Discriminative Test (Difference test)

This test is used to determine whether a difference exists among samples and testing for quality control to find the similarity. This test includes:

A- Triangle Test – The panel attempts to detect which one of the three samples is different from the other two. This method is useful in quality control work to determine if samples from different lots are different, and it is used to find the odd sample, which is different only in the variable studied in these samples, and mask all the other differences.

B- Due-trio test – The panel selects one of the two samples that are different from standard. It has the same application as the triangle test, and although easier to conduct, it is less efficient.

More discriminative tests are used in dairy products such as; two-out-of-five, paired comparison and ranking test methods (Pangborn and Dunkley, 1964; Drake, 2004).

6.1.2 The Descriptive Test

The descriptive test is used to determine the nature and intensity of the differences between samples and to give useful information for dairy research, product development and marketing. It includes flavour profile, texture profile, quantitative descriptors, and attribute ranking methods (Deliza and Macfie, 1996; Russell *et al.*, 2006).

6.1.3 The Affective Test

Affective tests are based on either a measure of preference (acceptance) or a measure from which we can determine relative preference. Test results give an indication of preference (select one over another), liking (degree of like/dislike), or acceptance (accept or reject) of a product (Martens, 1999). It includes paired comparison preference test, hedonic scaling test and ranking test.

- Ranking test requires the panelist to evaluate three or more samples and arrange them in order of preference or liking. Coded samples are presented in different randomized order for each panelist and panelist are asked to rank for overall preference, or the most liked for a specific attribute, such as colour or flavour preference. The total of the rank of

each treatment, and test significance is completed using Friedman test for rank data.

In most cases, a combination of different tests and descriptive sensory analysis is used for problem solving.

6.2 AIM:

This study was conducted to assess the feasibility of obtaining a new bifidobacteria product with high survival and acceptance to the consumer.

6.3 OBJECTIVES:

- Conduct a sensory evaluation on *Bifidobacterium lactis* Bb12 drink in MDSM and chose the best product.
- Mix *Bifidobacterium lactis* Bb12 drink in MDSM product in different percentages with low fat yogurt and chose the best mixture with the best survival.
- Compare the new product with known products on the market and develop it to achieve high level of acceptance by the consumer.

6.4 SENSORY EVALUATION OF FERMENTED PROBIOTIC SKIM MILK

The experiment was conducted in two stages:

1- Phase I – Three samples which contained *Bifidobacterium lactis* strain Bb12 that were obtained from the fermentation process described earlier (Chapter Five) and with the addition of different concentrations of sugar and strawberry flavour were prepared. The various concentrations were used to enhance

their sensory attribute and to choose the most acceptable sample for consumers and make this product as close as possible to the probiotic products on the market.

2- Phase II – The preferred product selected from Phase I was used and mixed with low fat yogurt, which was obtained from the local market. The skim milk yogurt cultures containing *S. thermophilus* and *L. dellbrueckii* ssp. *bulgaricus* were used and special investigation was done to assess the effect of this mixture on the survival of *Bifidobacterium lactis* Bb12 in the product after 28 days of refrigerated storage at $4^{\circ}\text{C}\pm 1$. Once the best mixture media was assessed that provided the best growth and survival media for *Bifidobacterium lactis* Bb12, the sensory attributes of this mixture were tested and compared to the preferred mixture of Phase I with a well known product sample chosen from the local market and made with bifidobacteria and strawberry flavour.

For all samples tested above in the two phases, the Ranking Test was used for the sensory attributes comparison including; appearance, taste, mouth feel and overall preference.

The concentrations of sugar and strawberry flavour that were used in phase I was chosen after studying the ingredients in products currently on the market and the minimum and maximum concentrations of each were studied.

Fermented skim milk using *Bifidobacterium Lactis* Bb12 as culture in the MDSM product was modified by adding sugar and strawberry flavour in different concentrations to obtain the best product for the growth and survival of

Bifidobacterium lactis Bb12 while maintaining consumer acceptance attributes. After conducting these experiments to chose the most suitable concentrations of sugar and strawberry, three products having the most optimal design were as follows: sample (A) with (5% sugar, 3% strawberry flavour), (B) with (2.5 % sugar, 2% strawberry flavour) and (C) with (1% sugar, 1% strawberry flavour), prepared in the laboratory under aseptic conditions and using food grade level ingredients. Thirty volunteers where chosen to taste the three different samples listed above. Ranking sensory analysis was used and attributes such as appearance, taste, mouth feel, and overall acceptability (preference) were tested in this experiment (Poste *et al.*, 1991). Volunteers were given a questionnaire and were asked to rank the three products (A, B, and C) according to their preference with regard to each sensory attribute listed above.

After evaluation of the product from the first phase, further study was done and more sensory tests were conducted for the second stage, but using a standard product purchased from the market with Bifidobacteria and strawberry flavour.

6.5 MATERIAL AND METHODS

For this study all materials used were food grade compared to the previous experiments.

6.5.1 ENZYME PREPARATIONS:

Trypsin from porcine source (Trypsin T069) was obtained from Biocatalyst Limited, Wales, UK and the strength prepared from this enzyme was similar to

the one used in previous experiments with the exception of this being food grade. The use of trypsin in the pretreatment of milk was shown not to have any negative effects on the sensory attributes of dairy products, “and the set yoghurt prepared from trypsin-treated milk with no more than 5% DH showed either a small or no improvement in texture and sensory properties” (Kumar *et al.*, 2000).

6.5.2. SKIM MILK TREATMENT AND FERMENTATION:

Skim milk was purchased locally and hydrolyzed by trypsin for 10 minutes then immersed in hot water at 90°C for 10 minutes to inactivate the enzyme. Next, 5% FOS and 3% inulin were added to the sample (Food Grade FOS and inulin were obtained from ORAFTI active food ingredient, Belgium) and pasteurized at 70°C for 15 minutes (Shin *et al.*, 2000a), then cooled to 37°C to start the fermentation process. *Bifidobacterium lactis* Bb12 5% v/v as the culture was used for all MDSM samples in the fermentation process (Food Grade *Bifidobacterium lactis* Bb12 Culture was obtained from Chr. Hansen, UK). The fermentation was carried out in an anaerobic incubator at 37°C until a pH 4.2 ± 0.03 was reached in 24 h and the fermentation was stopped by immersing the samples in ice bath to $4^{\circ}\text{C} \pm 1$, which was the temperature used for serving the samples in the sensory test room.

6.5.3. PREPARATION OF TEST SAMPLES:

After the fermentation process was finished and the fermented product by *Bifidobacterium lactis* Bb12 was obtained and stored at $4^{\circ}\text{C} \pm 1$ over night, sugar and flavour were added to improve the acceptability of this product to potential

consumers. Custar sugar, which is fine and dissolves easily, was purchased locally and different concentrations were used until optimal sweetness was achieved. Furthermore, strawberry flavour was used to improve the flavour of the product (Strawberry flavour 12238, obtained from H.E. Stringer Limited. Tring, Hertfordshire, UK) and it contained the following ingredients: water, propylene glycol E1520, Natural Identical Flavouring substances, Thickening agent E413, colour E122, preservative E211.

For the first stage of the sensory test, *Bifidobacterium lactis* Bb12 fermented skim milk was used as the primary product and three samples were prepared by changing the concentrations of sugar and strawberry flavour added to each sample. Each sample was then coded with a number and presented in a different order to each test subject for the sensory test.

The three samples used contained the following concentrations of sugar and strawberry flavour: sample A was coded (538) and consisted of *Bifidobacterium lactis* Bb12 fermented skim milk with (5% sugar and 3% strawberry flavour); sample B was coded (465) with (2.5% sugar and 2% strawberry flavour); and sample C was coded (880) with (1% sugar and 1% strawberry flavour).

Thirty volunteers were recruited for this study and the ranking taste test was used to compare the acceptance of the probiotic drink with different additives used. The ranking used to classify the attributes of the product such as appearance, taste, mouth feel, and overall acceptability (preference) ranged from the most liked sample, average and least liked in this experiment (Poste *et al.*, 1991). Volunteers were given a questionnaire and were asked to rank the three

products (A, B, and C) according to their preference with regard to each sensory attribute listed above.

Sample of the questionnaire used for phase I and II for the ranking test is presented in Figure (6.1). All results of the ranking test were analyzed using the Friedman test for rank data (Poste *et al.*, 1991).

For the second stage of the experiment, the *Bifidobacterium lactis* Bb12 drink chosen from the first stage of the sensory evaluation was improved by adding sugar and strawberry flavour and coded number (540), the second product of this stage was a mixture of *Bifidobacterium lactis* Bb12 drink in MSDM product, which was used in different mixtures with ready low fat yogurt made using yogurt culture obtained from the local market, and this mixture is presented (Table 6.1).

PROBIOTIC DRINKS

Rank the three samples given for **preference**. You must prefer sample for each attribute and rank it as 1 (most liked), 2 (second most liked) and 3 (least liked). Place the code number in the appropriate line.

Taste in this order: **880** **465** **538**

Appearance: Most liked 1.....

2.....

Least liked 3.....

Taste: Most liked 1.....

2.....

Least liked 3.....

Mouth feel: Most liked 1.....

2.....

Least liked 3.....

Overall preference: Most liked 1.....

2.....

Least liked 3.....

Feel free to make your comments on any of the samples or attributes

.....
.....

Figure 6.1 Questionnaire used for the ranking test in Phase I, and II of the sensory evaluation study.

Table 6.1 the different mixture % w/w of *Bifidobacterium lactis* Bb12 drink in MDSM product with low fat yogurt made using normal yogurt cultures.

Sample number	Mixture % w/w of <i>Bifidobacterium lactis</i> Bb12 drink in MDSM product and low fat yogurt	
	Proportion or ratio of <i>B. lactis</i> Bb12 drink % w/w	Proportion or ratio of low fat yogurt % w/w
1	20	80
2	40	60
3	50	50
4	60	40
5	80	20

The purpose of using different mixtures was to investigate the effect of the various mixtures on the survival of *Bifidobacterium lactis* Bb12 in all mixtures over the refrigerated storage period at 4°C ±1. This study has done to choose the most optimal mixture for improving the survival of *Bifidobacterium lactis* Bb12 at the end of the shelf life of the product, and then apply it to the sensory evaluation test and coded as number (388).

The third sample was a probiotic drink product obtained from the local market, which is made with *Bifidobacterium spp.* and strawberry flavour (Vitality, Muller) and coded as number (684).

6.5.4 STUDYING THE SURVIVAL OF *Bifidobacterium Lactis* Bb12 IN THE MIXTURE WITH LOW FAT YOGURT:

Low fat yogurt purchased locally and made with yogurt cultures *S. thermophilus* and *L. delbrueckii* subsp. *Bulgaricus* was used and applied to different mixtures of this yogurt with the *Bifidobacterium lactis* Bb12 drink in MDSM product over 28 days refrigerated storage at 4°C ±1 to assess survival. From each sample and every 7 days, samples were plated on MRS-NNLP agar with 0.05% L-cysteine hydrochloride as described in Dave and Shah (1996) for the enumeration of *Bifidobacteria* spp. In the yogurt mixture and with anaerobic incubation at 37°C for 72h, the media were supplemented with nalidixic acid (15 mg/L), neomycin sulphate (100 mg/L), lithium chloride (3g/L), and paromomycin sulphate (200 mg/L) (Dave and Shah, 1996; Tharamaraj and Shah, 2003). *S. thermophilus* agar (ST agar) media with aerobic incubation at 37°C for 24h was used for enumeration of *S. thermophilus* in the yogurt (Tharamaraj and Shah, 2003). This way was a more economical use of M17 as reported in Dave and Shah (1996). MRS agar modified to pH (5.2) was used for enumeration of *L. delbrueckii* subsp. *bulgaricus* from the yogurt mixture and with an anaerobic incubation at 45 °C for 72h (Tharamaraj and Shah, 2003, Casteele *et al.*, 2006). The total count of each species was taken in the different mixture percentages over the 28 days.

6.5.5 STATISTICAL ANALYSIS:

Statistical analysis of data for ranking sum of each attribute (appearance, taste, mouth feel and overall preference) was performed by the Friedman test and the

value of Chi-square χ^2 were found with 3 degrees of freedom for $\alpha = 0.05$. (Postel *et al.*, 1991).

6.6 RESULTS AND DISCUSSION:

6.6.1 Sensory evaluation of Phase I:

Figures 6.2-6.5 show the ranking sum of each sample of the products made of *Bifidobacterium lactis* Bb12 drink in MDSM product with different concentrations of sugar and strawberry flavour. The ranking sum for the appearance of the three samples shows no significant difference, and all the samples were very close but still the best appearance was ranked with sample A (538) Figure (6.2), then sample C (880), and last was sample B (465).

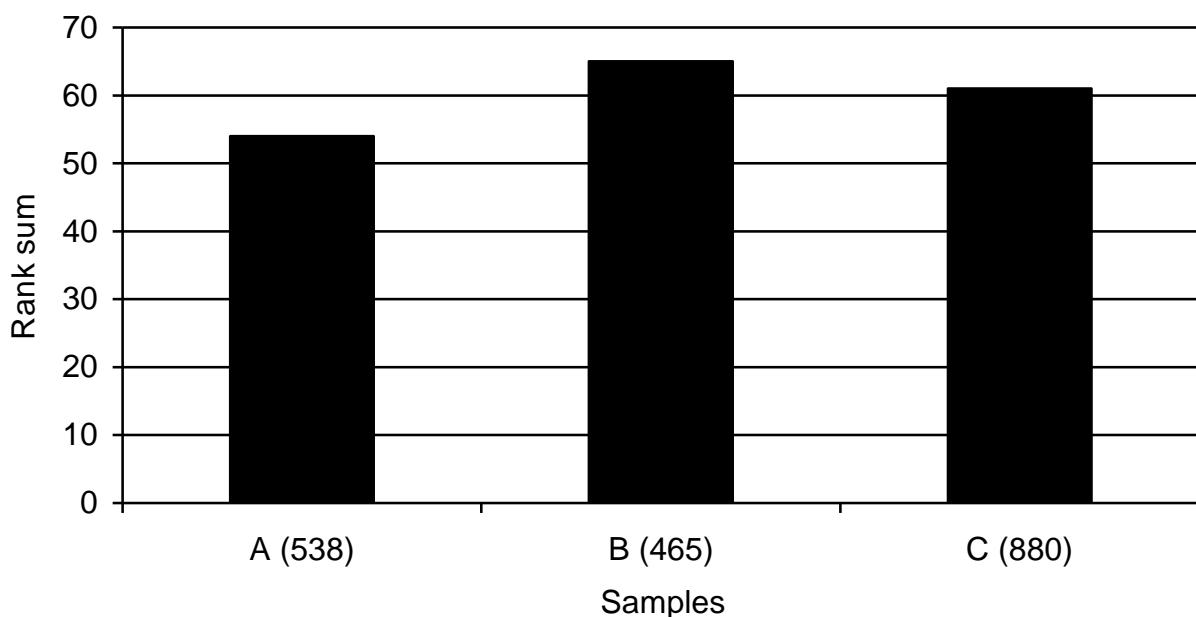


Figure 6.2 Ranking sum of the appearance for three samples made of *Bifidobacterium lactis* Bb12 drink in MDSM product with; A (538), B (465) and C (880) after evaluation by 30 panelists.

The three samples show a more significant difference in taste. It was significant in sample A (538) ($P < 0.05$) as compared with the other two samples and the difference was not significant ($P > 0.05$) between sample B (465) and C (880) according to the 30 panelists, which makes sample A (538) have the best taste, and this sample had the highest concentrations of sugar and strawberry flavour Figure (6.3).

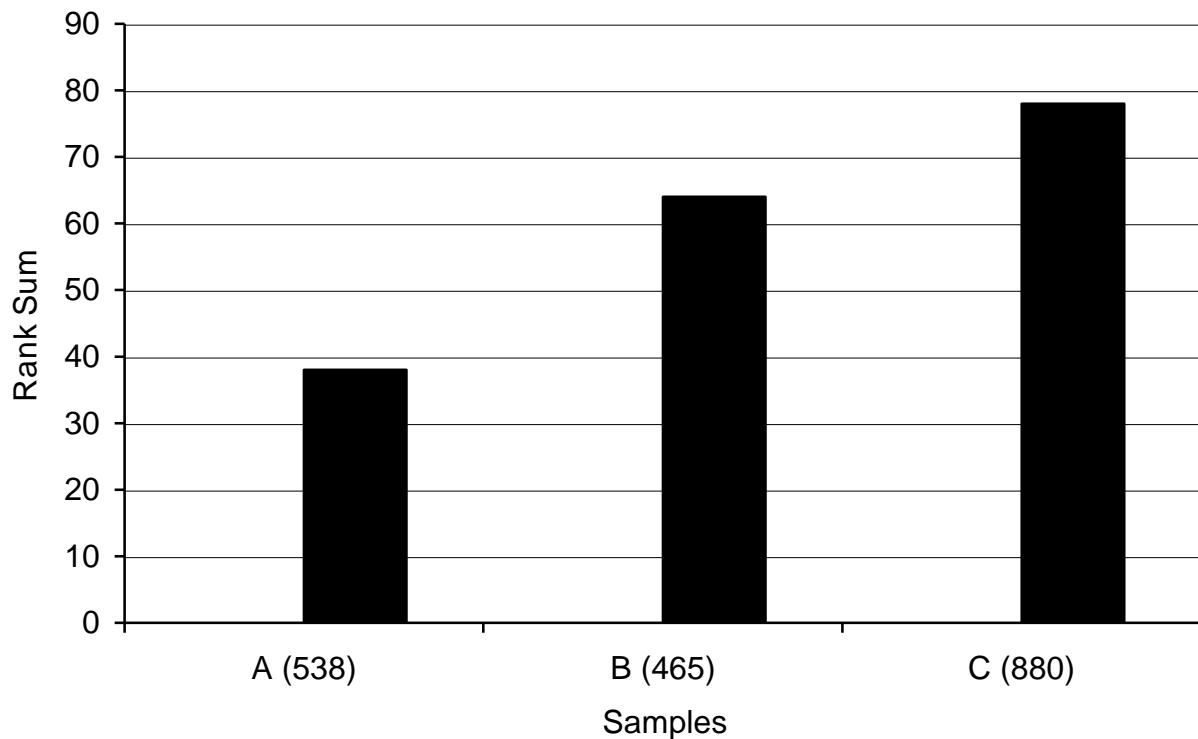


Figure 6.3 ranking sum of taste for three samples made of *Bifidobacterium lactis* Bb12 drink in MDSM product with; A (538), B (465) and C (880) after evaluation by using 30 panelists.

Sample A (538) was ranked significantly ($P<0.05$) the best mouth feel as compared to samples B and C as illustrated in Figure (6.4).

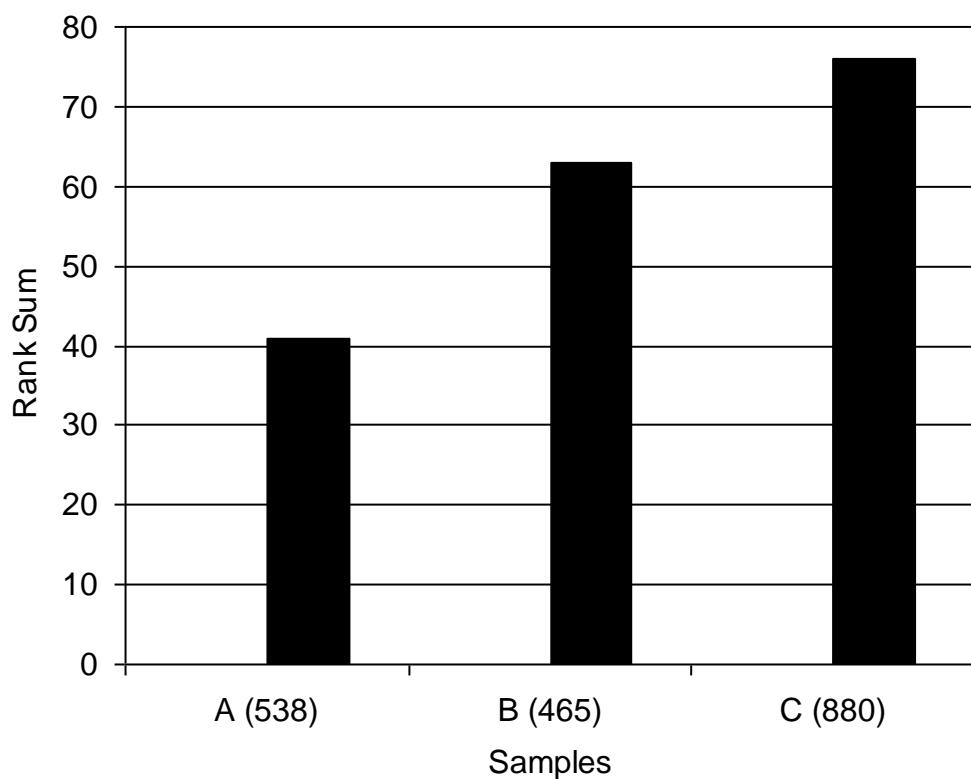


Figure 6.4 ranking sum of mouth feel for three samples made of *Bifidobacterium lactis* Bb12 drink in MDSM product with; A (538), B (465) and C (880) after evaluation by using 30 panelists.

Moreover, the use of overall preference for the sensory evaluation gives an idea about the sample that was most preferred by the panelists as illustrated in Figure (6.5). Sample A was preferred and the difference was significant ($P<0.05$).

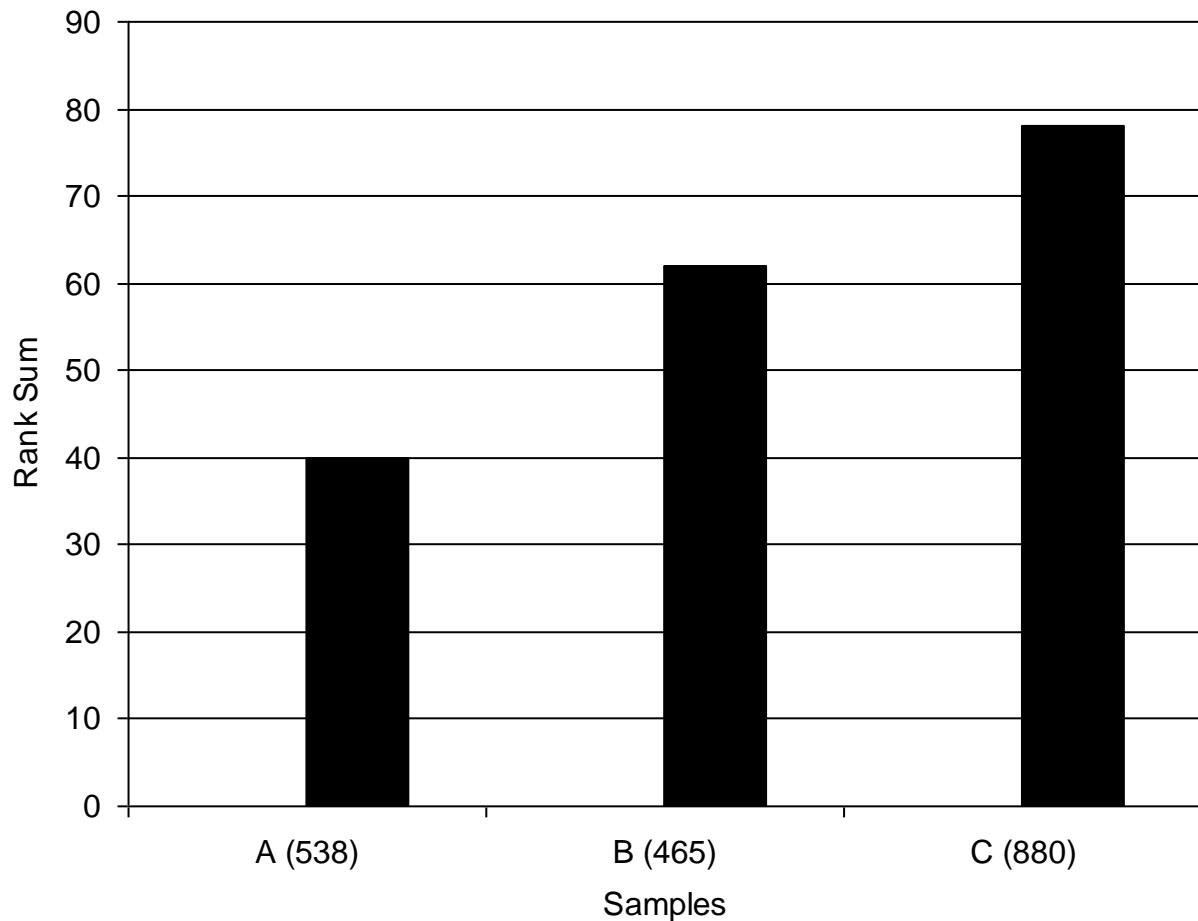


Figure 6.5 ranking sum of the overall preference for three samples made of *Bifidobacterium lactis* Bb12 drink in MDSM product with; A (538), B (465) and C (880) after evaluation by using 30 panelists.

The result indicate that the *Bifidobacterium lactis* Bb12 drink in sample A with 5% sugar and 3% strawberry flavour was the most acceptable, whereas sample B with 2.5 % sugar and 2% strawberry flavour and C 1% sugar and 1% strawberry flavour were least acceptable and did not differ between themselves significantly ($P<0.05$).

These results gave an indication of the best sample of each of the prepared samples by using the same Bifidobacteria (*Bifidobacterium lactis* Bb12) and just changing the concentrations of sugar and strawberry flavour in each sample. Therefore, more investigation applied to compare this sample with a sample from the local market and adding another sample made by adding yogurt to improve the acceptance by the consumers.

6.6.2 Sensory evaluation in Phase II:

Figures 6.6-6.10 show the effect of different mixtures of the *Bifidobacterium lactis* Bb12 drink from MDSM product (Chapter 5) and low fat yogurt on the survival of the different bacteria available in the mixture, such as *Bifidobacterium lactis* Bb12, from the probiotic drink which was made in MDSM product and the *S. thermophilus* and *L. delbrueckii* Subsp. *bulgaricus* as cultures in the low fat yogurt in the total mixture.

All the results obtained show an increase in the survival of *Bifidobacterium lactis* Bb12 by increasing the *Bifidobacterium lactis* Bb12 drink of the MDSM product percentage in the mixture until a 50:50 ratio was reached and where the survival

of *Bifidobacterium lactis* Bb12 in the mixture of *Bifidobacterium lactis* Bb12 drink and low fat yogurt starts to decrease, as shown in figures (6.6- 6.10).

Figure (6.6) shows the total count change of *Bifidobacterium lactis* Bb12, *S. thermophilus* and *L. dellbrueckii* ssp. bulgaricus over 28 days storage at 4°C ±1 in prepared mixture of 20:80 v/v of MDSM product and low fat yogurt.

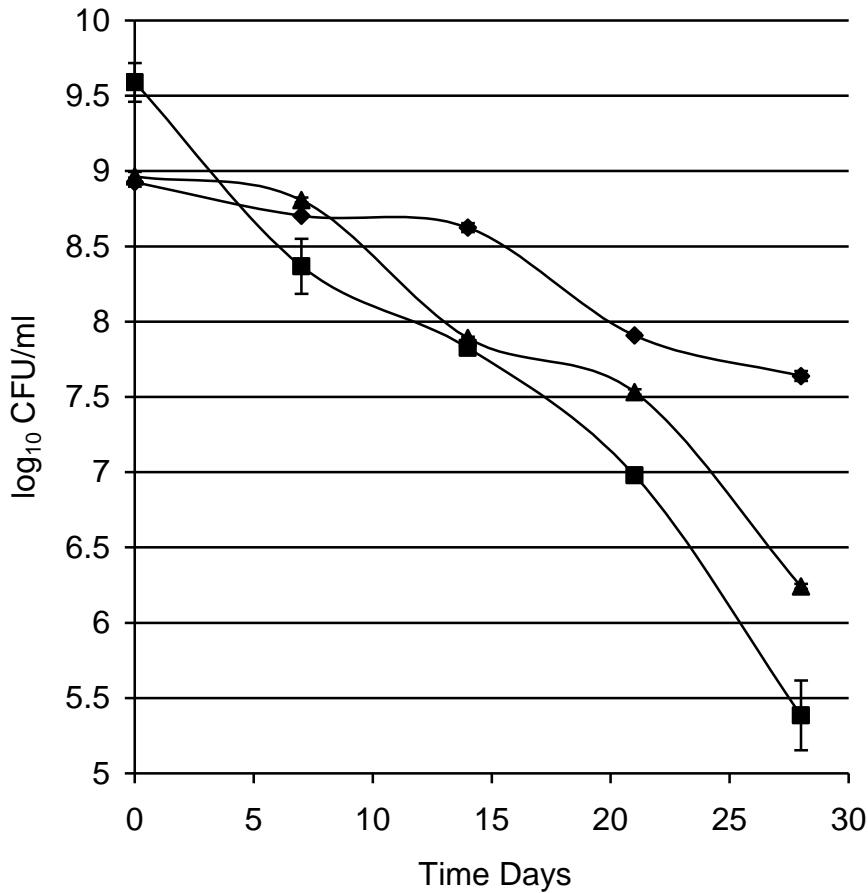


Figure 6.6 the \log_{10} cfu/ml of *Bifidobacterium lactis* Bb12 (■), *S. thermophilus* (♦) and *L. dellbrueckii* ssp. bulgaricus (▲) in sample (1) 20:80 % v/v of *Bifidobacterium lactis* Bb12 drink in MDSM and yogurt made from normal culture over 28 days refrigerated storage at 4°C ±1. Error bars were taken as standard deviation for three samples.

Figure (6.7) shows the total count change of *Bifidobacterium lactis* Bb12, *S. thermophilus* and *L. delbrueckii* ssp. bulgaricus over 28 days storage at 4°C ±1 in prepared mixture of 40:60 v/v of MDSM product and low fat yogurt.

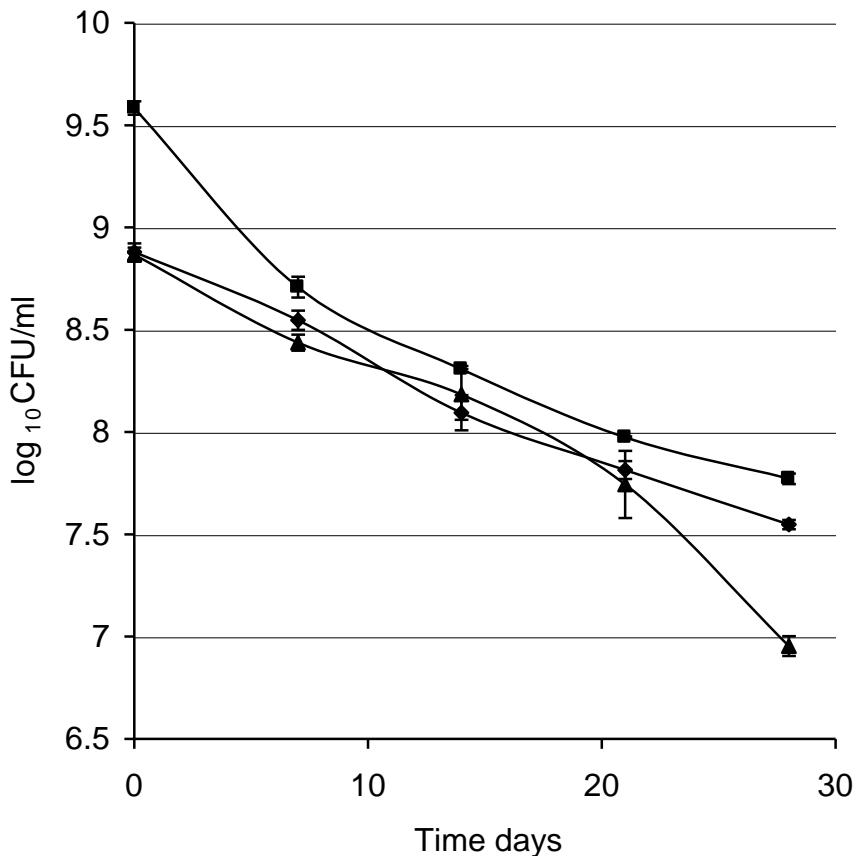


Figure 6.7 the log₁₀ cfu/ml of *Bifidobacterium lactis* Bb12 (■), *S. thermophilus* (♦) and *L. delbrueckii* ssp. bulgaricus (▲) in sample (2) 40:60 % v/v of *Bifidobacterium lactis* Bb12 drink in MDSM and yogurt made from normal culture over 28 days refrigerated storage at 4°C ±1. Error bars were taken as standard deviation for three samples.

Figure (6.8) shows the total count change of *Bifidobacterium lactis* Bb12, *S. thermophilus* and *L. delbrueckii* ssp. bulgaricus over 28 days storage at 4°C ±1 in prepared mixture of 50:50 v/v of MDSM product and low fat yogurt.

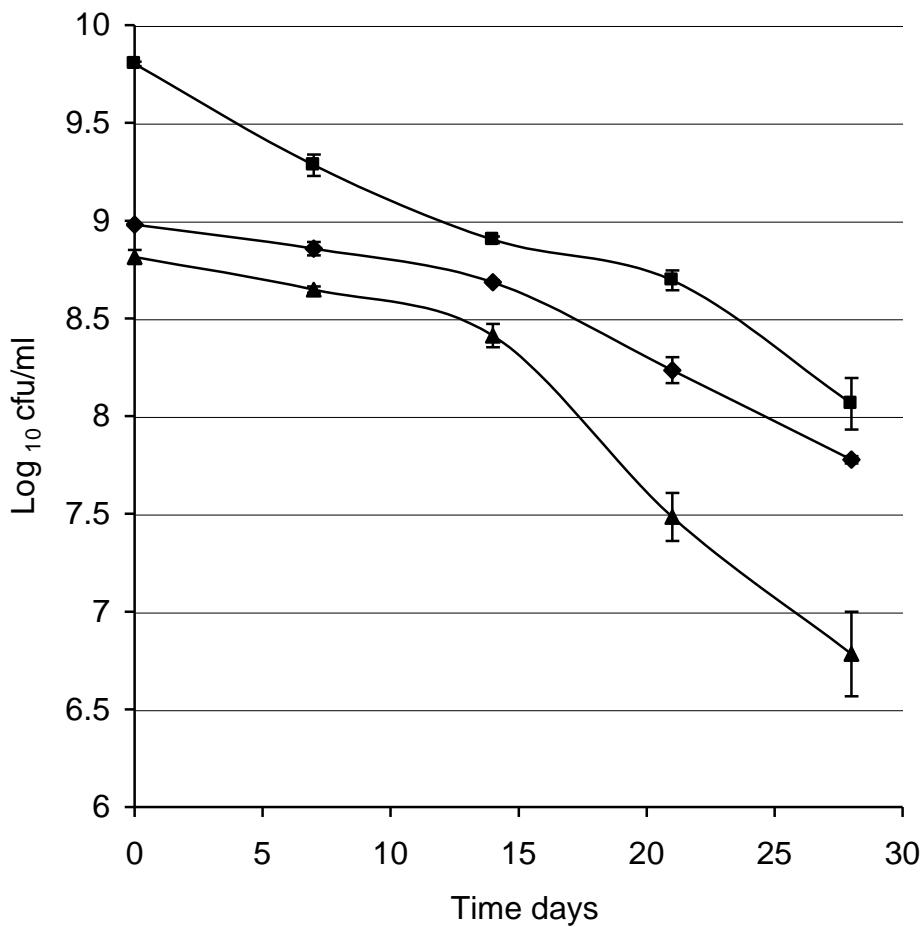


Figure 6.8 the \log_{10} cfu/ml of *Bifidobacterium lactis* Bb12 (■), *S. thermophilus* (♦) and *L. delbrueckii* ssp. *bulgaricus* (▲) in sample (3) 50:50 % v/v of *Bifidobacterium lactis* Bb12 drink in MDSM and yogurt made from normal culture over 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$. Error bars were taken as standard deviation for three samples.

Figure (6.9) shows the total count change of *Bifidobacterium lactis* Bb12, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* over 28 days storage at $4^{\circ}\text{C} \pm 1$ in prepared mixture of 60:40 v/v of MDSM product and low fat yogurt.

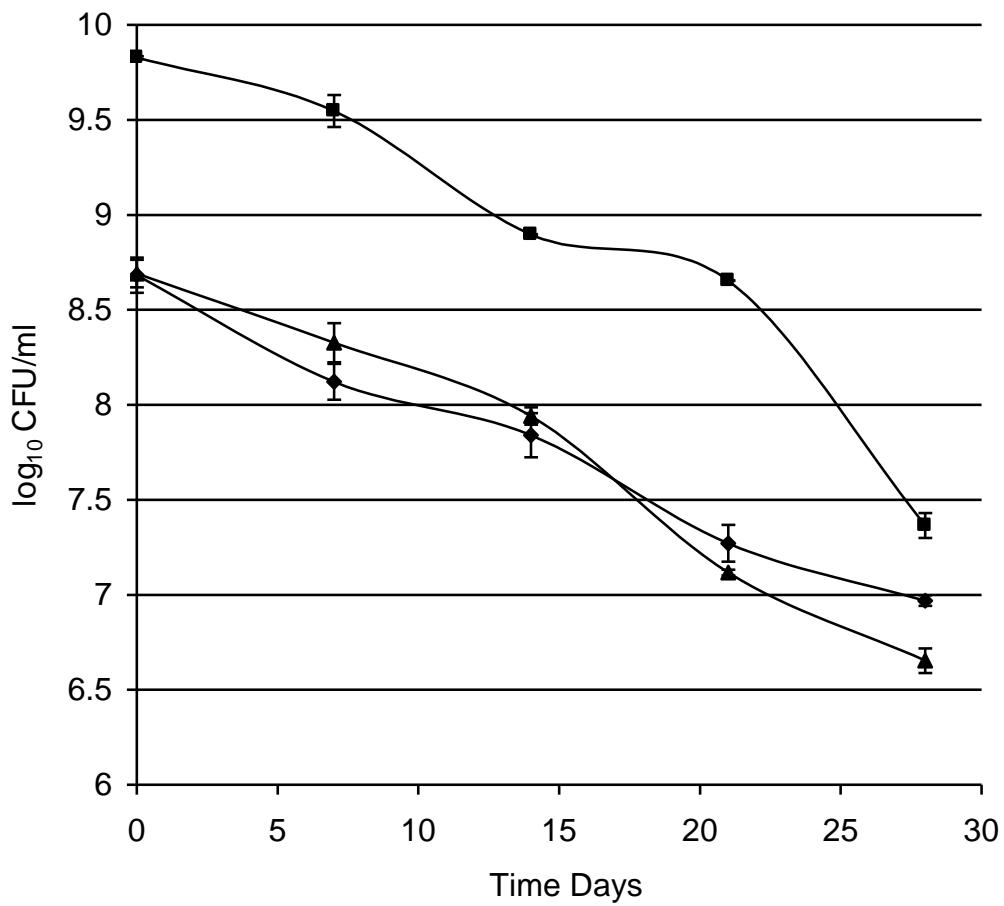


Figure 6.9 the \log_{10} cfu/ml of *Bifidobacterium lactis* Bb12 (■), *S. thermophilus* (♦) and *L. delbrueckii* ssp. *bulgaricus* (▲) in sample (4) 60:40 % v/v of *Bifidobacterium lactis* Bb12 drink in MSDM and yogurt made from normal culture over 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$. Error bars were taken as standard deviation for three samples.

Figure (6.10) shows the total count change of *Bifidobacterium lactis* Bb12, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* over 28 days storage at $4^{\circ}\text{C} \pm 1$ in prepared mixture of 80: 20 v/v of MDSM product and low fat yogurt.

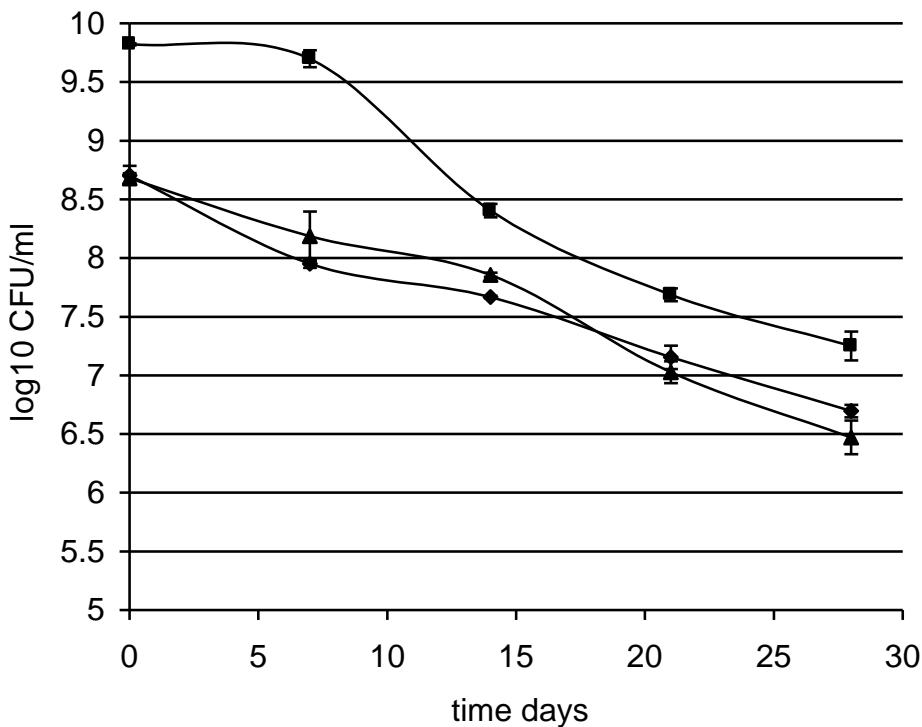


Figure 6.10 the \log_{10} cfu/ml of *Bifidobacterium lactis* Bb12 (■), *S. thermophilus* (♦) and *L. delbrueckii* ssp. *bulgaricus* (▲) in sample (5) 80: 20 % v/v of *Bifidobacterium lactis* Bb12 drink in MSDM and yogurt made from normal culture over 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$. Error bars were taken as standard deviation for three samples.

Figure (6.11) shows the logarithmic reduction of total count over the 28 days refrigerated storage at $4^{\circ}\text{C} \pm 1$.

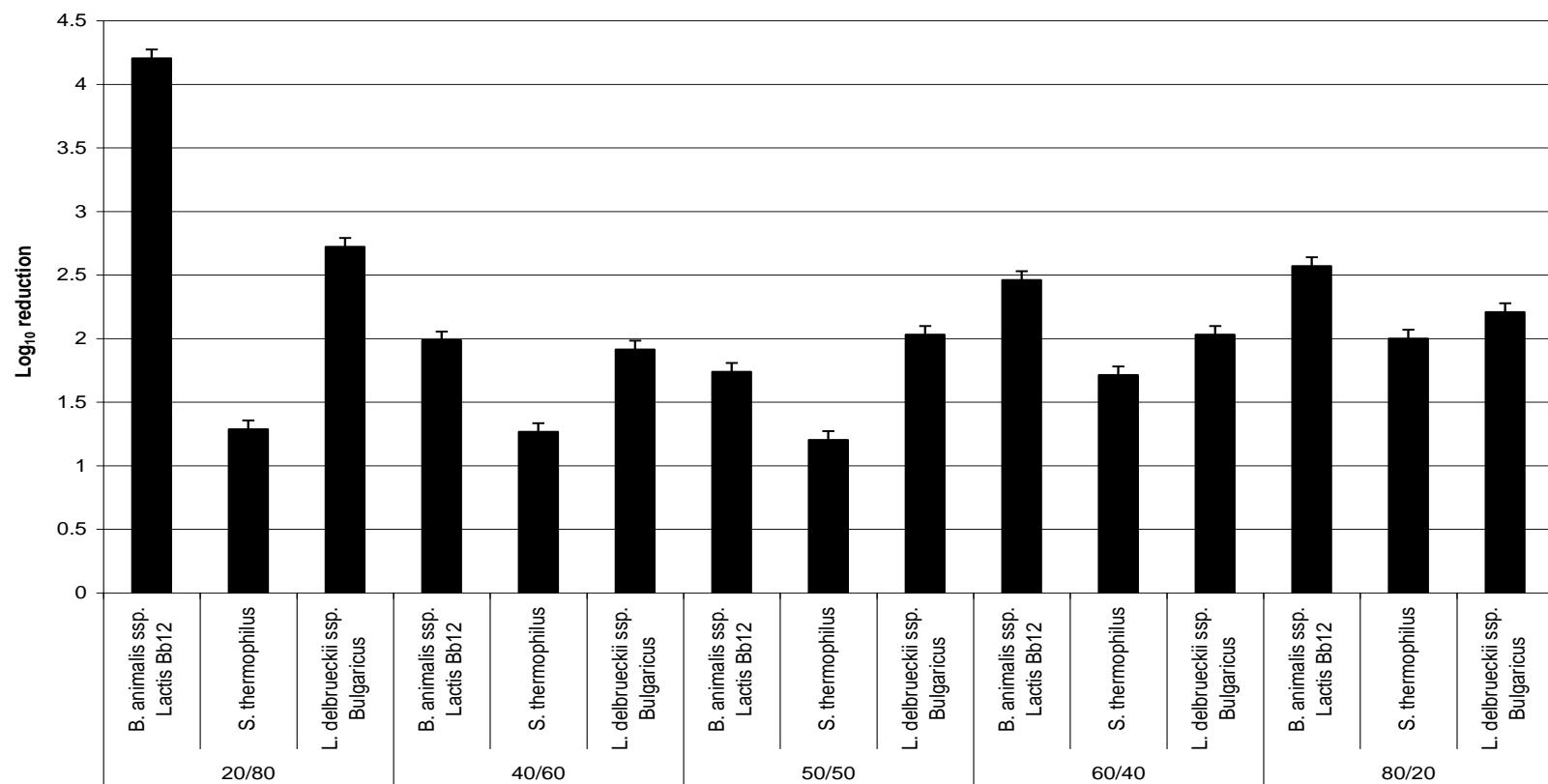


Figure 6.11 the mean logarithmic reduction for three samples of *Bifidobacterium lactis* Bb12, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in the different mixtures made of *Bifidobacterium lactis* Bb12 drink and yogurt made with normal culture over 28 days of refrigerated storage at 4°C±1.

The best is the sample which used 40% of *Bifidobacterium lactis* Bb12 drink in the MDSM product and 60% of the low fat yogurt and the logarithmic reduction as shown in Figure (6.11).

Therefore, the mixture 40:60 *Bifidobacterium lactis* Bb12 drink in MDSM product and low fat yogurt were used as samples in the sensory evaluation after adding the best concentration of sugar and strawberry flavour from the first stage, coded E (388) which consisted of (40:60 *Bifidobacterium lactis* Bb12 drink: low fat yogurt with 5% sugar and 3% strawberry flavour).

The best sample of phase I was sample A (538), but in this phase, it was coded as D (540), which consisted of (*Bifidobacterium lactis* Bb12 drink with 5% sugar and 3% strawberry flavour).

The third sample, a commercial probiotic containing Bifidobacteria and strawberry flavour, was coded as F (684).

All samples were examined using the ranking test and 20 panelists completed the questionnaire. The order of the ranking test was changed for each panelist.

Figure (6.12) shows the difference in the appearance of the three samples and it shows a significant difference ($P<0.05$) between sample (D) and (E) but it is not significant ($P>0.05$) between sample (E) and (F) or (D) and (F) and that refers to the close acceptance of the appearance of the two samples that had been prepared in the laboratory compared to the sample from the market (F).

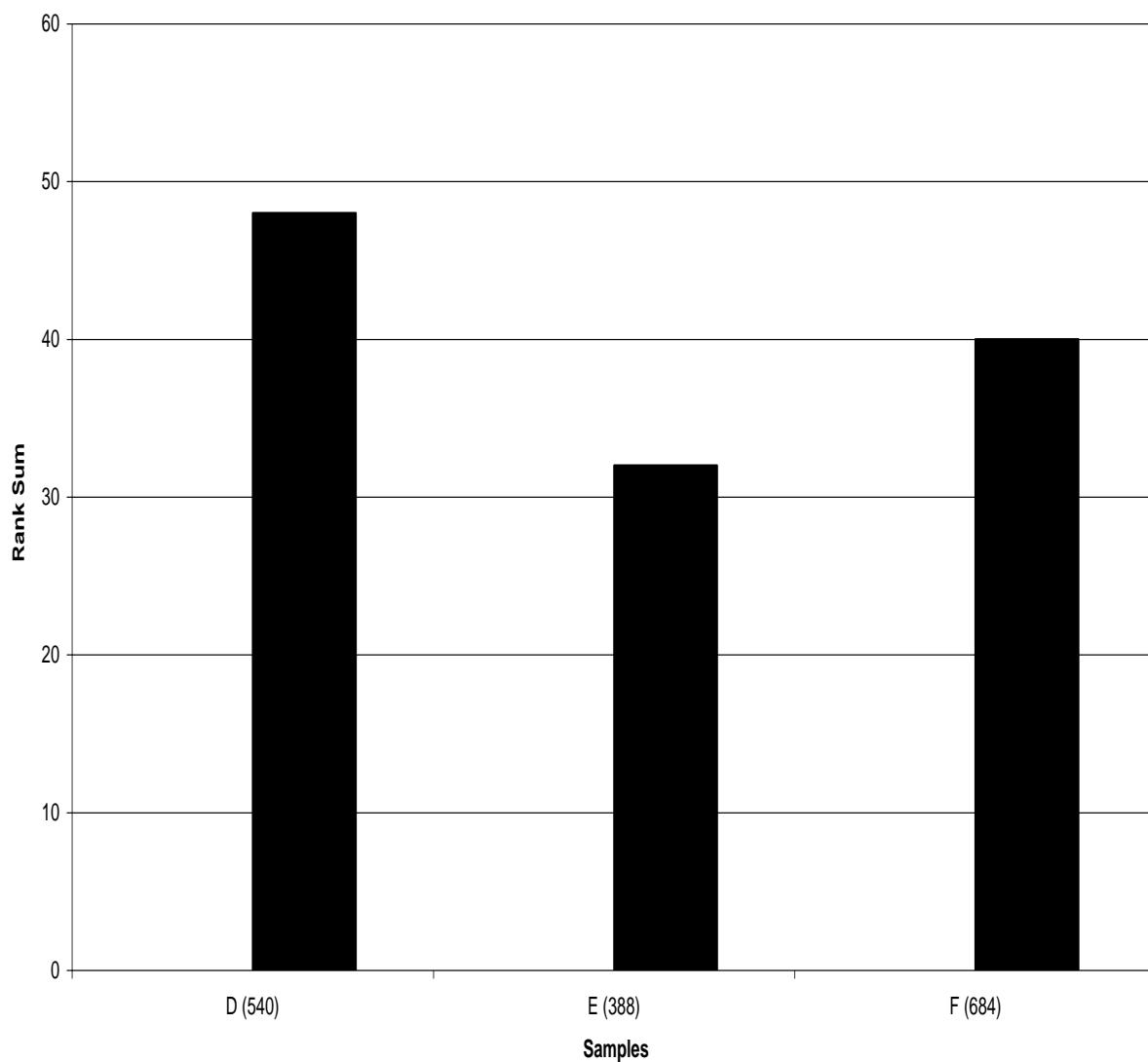


Figure 6.12 ranking sum of the appearance for three different samples D (540) with *Bifidobacterium lactis* Bb12 drink with 5% sugar and 3% strawberry flavour; E (388) with 40:60 *Bifidobacterium lactis* Bb12 drink, low fat yogurt with 5% sugar and 3% strawberry flavour; and F (684) with commercial probiotic containing Bifidobacteria and strawberry flavour, after evaluation by 20 panelists.

The evaluation of the taste sensory is shown in Figure (6.13)

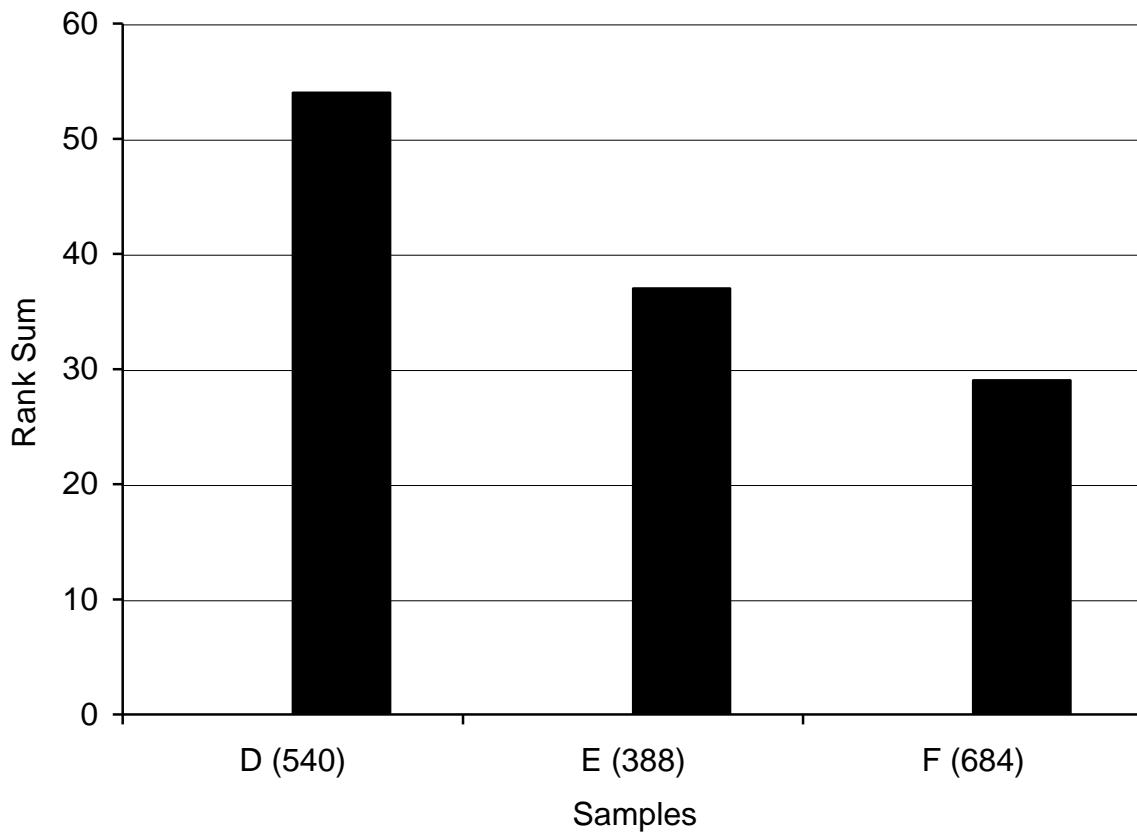


Figure 6.13 ranking sum of the taste for three different samples D (540) with *Bifidobacterium lactis* Bb12 drink with 5% sugar and 3% strawberry flavour; E (388) with 40:60 *Bifidobacterium lactis* Bb12 drink, low fat yogurt with 5% sugar and 3% strawberry flavour; and F (684) with commercial probiotic product containing Bifidobacteria and strawberry flavour, after evaluation by 20 panelists.

This indicates a significant difference ($P<0.05$) between sample (D) and each of (E) and (F) but the difference is not significant ($P>0.05$) between (E) and (F), which indicates that the acceptance of sample (E) is very close to (F) which has good sale in the market. Therefore, the taste of the *Bifidobacterium lactis Bb12* drink in MDSM could be improved by mixing it with yogurt in a 40:60 % ratio and this made it very acceptable for the consumers.

Mouth feel Figure (6.14) was also one of the sensory evaluations that was examined for the three samples and a significant difference ($P<0.05$) was found between sample (D) and samples (E) and (F) but these differences were not significant ($P>0.05$) between samples (E) and (F) which indicates that the mouth feel of sample E is very close and accepted well by consumers.

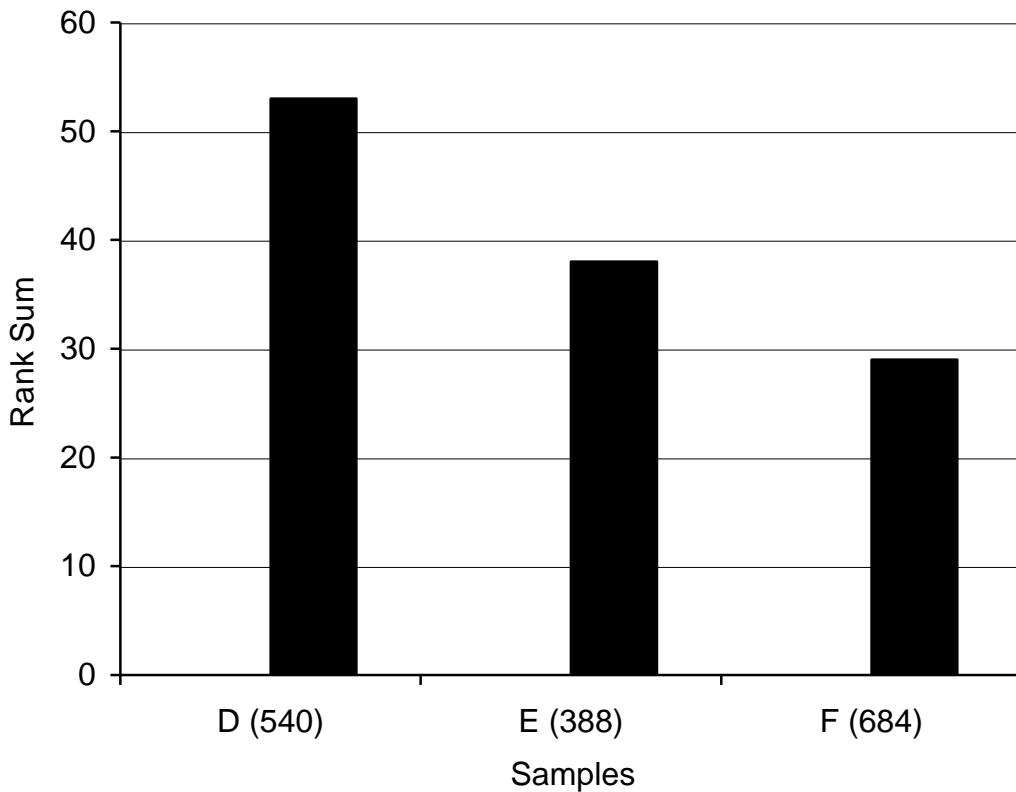


Figure 6.14 ranking sum of the mouth feel for three different samples D (540) with *Bifidobacterium lactis* Bb12 drink with 5% sugar and 3% strawberry flavour; E (388) with 40:60 *Bifidobacterium lactis* Bb12 drink, low fat yogurt with 5% sugar and 3% strawberry flavour; and F (684) with commercial probiotic containing Bifidobacteria and strawberry flavour, after evaluation by 20 panelists.

The overall preference as in Figure (6.15) shows significant differences ($P<0.05$) between samples (D), (E) and (F) but the difference was not significant ($P>0.05$) between samples (E) and (F) which means that the overall preference of sample (E) was very close to sample (F) from the local market. This indicates that the overall preference can be improved with *Bifidobacterium lactis* Bb12 drink by mixing it with low fat yogurt and sugar and strawberry flavour (De souza *et al.*, 2007).

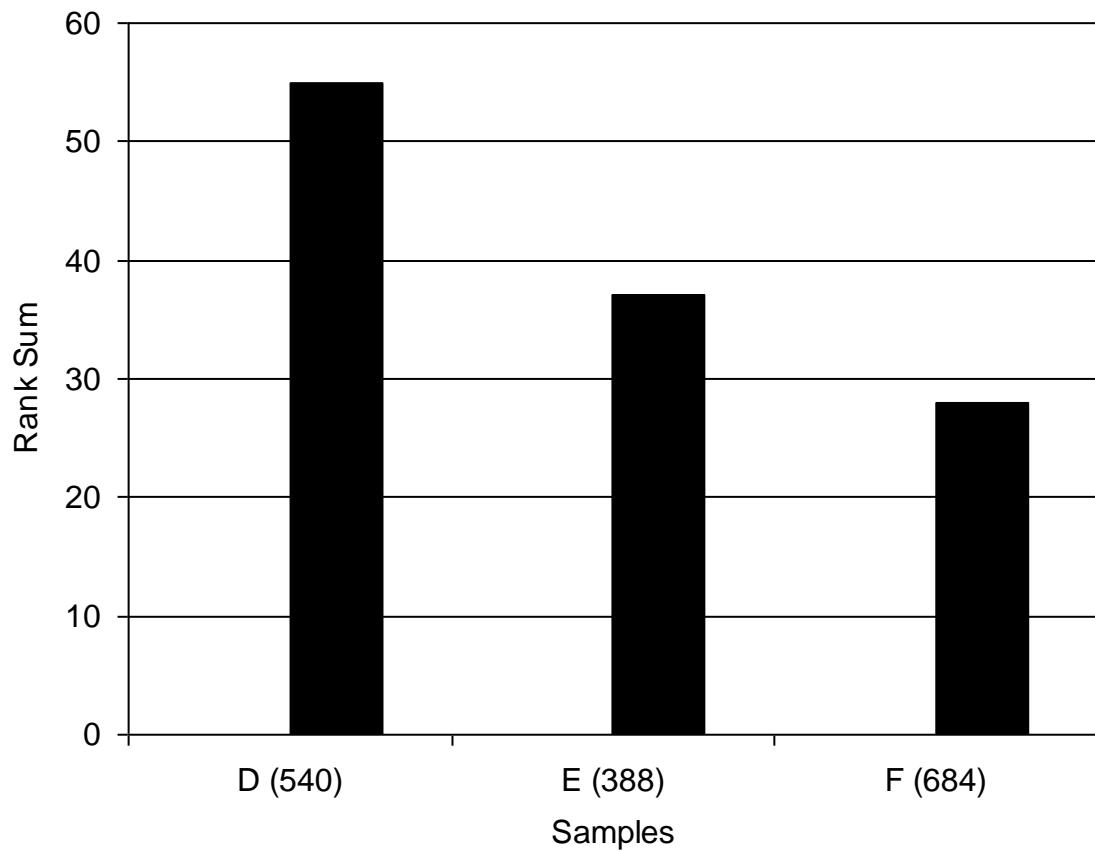


Figure 6.15 ranking sum of the overall preference for three different samples D (540) with *Bifidobacterium lactis* Bb12 drink with 5% sugar and 3% strawberry flavour; E (388) with 40:60 *Bifidobacterium lactis* Bb12 drink, low fat yogurt with 5% sugar and 3% strawberry flavour; and F (684) with commercial probiotic containing Bifidobacteria and strawberry flavour, after evaluation by 20 panelists.

The results indicate that the product made of *Bifidobacterium lactis* Bb12 drink in MDSM product with 5% sugar and 3% strawberry flavour was rated as highly acceptable and the overall preference was improved by mixing it in the percentage of 40:60 % of *Bifidobacterium lactis* Bb12 drink and low fat yogurt. The acceptance was very close from the ranking sum to the product well sold in the market, and the sample (D) which is just *Bifidobacterium lactis* Bb12 drink in MDSM product with 5% sugar and 3% strawberry flavour were significantly recognized as less accepted than samples (E) and (F).

6.7 CONCLUSION:

These studies were applied to find a probiotic drink made using *Bifidobacterium lactis* Bb12 with high survival and viability at the end of its shelf life.

In Phase I of this study it was found that using the *Bifidobacterium lactis* Bb12 drink made in medium, with high stimulation for their growth and survival (Chapter 5), referred to as MDSM product (hydrolyzed skim milk for DH<5 supplemented with 5% FOS and 3% inulin) was further improved by adding 5% sugar and 3% strawberry flavour (sample A). This sample has shown a significant difference from all the ranking sums that have been studied for the sensory evaluation attributes, such as appearance, taste, mouth feel and overall preference. In Phase II, it was apparent that the acceptance of sample A, (referred to as sample D in phase II) was less acceptable but when mixed with yogurt at 40:60 showed to have better acceptability and survival with the least logarithmic loss (reduction) of the total count of *Bifidobacterium lactis* Bb12. This applied in the sensory evaluation with a commercial sample containing Bifidobacteria and strawberry flavour (Vitality, Muller) as sample (F). This comparison has shown very good acceptance of the sample (E) that was proven to have a good medium for the growth and survival of *Bifidobacterium lactis* Bb12 and to be accepted by consumers.

CHAPTER SEVEN

OVERALL CONCLUSIONS AND FUTURE STUDIES

7.1 OVERALL CONCLUSIONS:

The primary aims of the experiments described in this thesis were:

- To test the different effects of using a range of prebiotics in different percentages, especially Fructooligosaccharides (FOS) and inulin, and to figure the best concentration of each of those prebiotics on the growth and survival of *Bifidobacterium lactis* Bb12.
- To find the best hydrolysis time of skim milk by trypsin on the growth and survival of *Bifidobacterium lactis* Bb12.
- To examine the synergistic effect of the prebiotics and the hydrolysed skim milk on the growth and survival of *Bifidobacterium lactis* Bb12 and develop the best environments with fixed degree of hydrolysis (DH) of the skim milk with exact concentrations of FOS and inulin to have the highest growth and survival rate of *Bifidobacterium lactis* Bb12.
- To use this environment (product) which was designed by the mix of hydrolysed skim milk with FOS and inulin, Mix Design Skim Milk (MDSM) product, to examine its effect on the growth and survival of different probiotics from different stains and species to validate their beneficial effect on growth and survival of probiotic bacteria, such as *Bifidobacterium bifidum* NCIMB 702203, *Bifidobacterium infantis* NCIMB 702205, *Bifidobacterium breve* NCIMB 702257, and *Lactobacillus casei* strain shirota.
- Apply the MDSM product to get a new product with a high number of *Bifidobacterium Lactis* Bb12 and acceptance by consumers.

This study clearly highlighted the importance of the growth and survival of probiotics in dairy products. The use of prebiotics and hydrolyzed milk has been the focus of many studies over the last century to enhance the growth and survival of probiotic bacteria in general and *Bifidobacterium* spp., in particular.

Most of the reported selective/differential prebiotics gave mixed results, in which some prebiotics show a different effect on the growth of target bacteria. For example, most of the reported prebiotics that were tested, such as fructooligosaccharides (FOS) and inulin (Chapter 3) have been shown to enhance the growth and survival of *Bifidobacterium Lactis* Bb12. The differences among the various prebiotics are probably due to each reported study using different probiotic strains. Moreover, individual strain sensitivities can further lead to differing counts on different prebiotics. Additionally the hydrolyzed milk had significant results on the growth of the target bacteria and a very good result on bifidobacteria, depending on the degree of hydrolysis and the condition of the hydrolysis process.

This study has shown that the only prebiotics which gave good recovery and reliable results with the strains *Bifidobacterium lactis* Bb12 used in this study were FOS and inulin, as different prebiotics used in different concentrations have different effects on the growth and survival of *Bifidobacterium Lactis* Bb12 on dairy products. FOS a selective prebiotic for *Bifidobacterium Lactis* Bb12, which has shown the greatest effect on the growth and survival of bifidobacteria at 5% w/w (Chapter 3). In addition, inulin has shown its best effect on the growth and survival of bifidobacteria at 3% w/w concentrations.

Also, considering the increasing demand and popularity of probiotic fermented milk among consumers and the recommendations for the minimum dosage of probiotic bacteria to confer health benefits, it is essential that research is directed into producing a standard product to provide uniform counts regardless of individual strain differences or manufacturing processes.

Although *L. acidophilus* and *Bifidobacterium Lactis* Bb12 are the most popular probiotic bacteria, there are very limited studies on probiotic fermented milk containing *Bifidobacterium Lactis* Bb12. Additionally not many probiotic-fermented milk products containing *Bifidobacterium lactis* are available commercially.

The use of hydrolysed milk has been reported in many studies with different strains of probiotic bacteria and different dairy products.

In this study, using hydrolyzed skim milk by trypsin at different ratios and for varying hydrolysis times were the key to find the best degree of hydrolysis to enhance the growth and survival of *Bifidobacterium lactis* Bb12 in fermented milk.

The use of trypsin at rate 1:20000 E:S for DH<5 hydrolysis process of the skim milk was shown to have the greatest effect on the growth and survival of *Bifidobacterium lactis* Bb12 (Chapter 4).

In this regard, this study was able to successfully manufacture probiotic fermented milk with *Bifidobacterium Lactis* Bb12 with a higher than recommended probiotic count on the day of manufacture.

The poor survival of *Bifidobacterium Lactis* Bb12 over the shelf life (Chapter 2) indicated that techniques were needed to protect bacteria from deleterious factors in fermented milk and increase their viability. Among the various options for improving the survival of probiotic bacteria, the effect of prebiotics and/or hydrolyzed skim milk on the growth and survival of probiotic bacteria were studied, as they were a more attractive option in terms of enhancing the growth and survival of probiotic bacteria from all the adverse conditions in fermented milk products.

Accordingly, the synergistic effect of prebiotics and hydrolyzed skim milk has shown a very good effect on growth and survival of other different strains of probiotic bacteria, such as *Bifidobacterium bifidum* NCIMB 702203, *Bifidobacterium infantis* NCIMB 702205, *Bifidobacterium breve* NCIMB 702257, and *Lactobacillus casei* strain shirota (Chapter 5). This study has lead to the development of a combination of prebiotics and hydrolysed protein termed Mix Design Skim Milk (MDSM) product (Chapter 5) that has been shown as beneficial by using of 5% FOS and 3% inulin with hydrolyzed skim milk for DH<5 by trypsin at rate 1:20000 E:S. This media has been shown successful in enhancing the growth and survival of *Bifidobacterium Lactis* Bb12.

The use of the MDSM product in new products with high count of *Bifidobacterium Lactis* Bb12 was studied in comparison with a commercial product (Chapter 6).

In summary, this study was able to successfully find new products and techniques that give reliable and accurate counts of probiotic bacteria. Therefore, this study successfully achieved its aims and objectives.

This study shows the possibility to produce new products with high numbers of *Bifidobacterium lactis* Bb12, which are still accepted for consuming, by using a combination of prebiotics and hydrolyzed skim milk.

7.2 FUTURE DIRECTIONS FOR THIS STUDY

7.2.1 EVALUATE PREBIOTICS:

Currently, fermented milk industries are growing rapidly. As already discussed in this study, due to the regulatory standards set by food authorities worldwide, the development of standard products with new prebiotics will be always an issue for the producer and the consumers. However, it is suggested to use novel combinations of prebiotics in dairy products and study the different effects on the growth and the survival of *Bifidobacterium* spp., which will also include the possible effects on the sensory evaluations of the products.

7.2.2 STUDY THE HYDROLYSIS PROCESS EFFECT

This study successfully demonstrated that hydrolyzed skim milk has a great effect on the growth and survival of *Bifidobacterium lactis* Bb12. However, more research should be done using SDS-page and HPLC to isolate and identify the peptides and the amino acids produced during the hydrolysis process and study their individual effects on the growth and survival of *Bifidobacterium* spp.

7.2.3 SYNERGISTIC STUDY

It is recommended to study any synergistic effect between individual peptides identified using HPLC and prebiotics.

7.2.4 SENSORY EVALUATIONS:

It is recommended that manufacturers use a sensory analysis technique to evaluate and optimize consumer acceptability of new fermented milk formulations

CHAPTER EIGHT

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