The influence of bacteriocin-producing probiotic starter cultures on fermentation time and post-acidification in yoghurt

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By

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inoculaated for making yoghurt. The control yoghurt took 3 hours and 20 minutes to ferment while the Sonicated yoghurt took 4 hours and 25 minutes. The pH of both yoghurts decreased rapidly over the 4 weeks of storage with the control yoghurt having the lowest pH. The control had a higher viable count of *L. delbrueckii* subsp. *bulgaricus* than the sonicated yoghurt. There was a high number of *L. acidophilus* and bifidobacteria in the inoculation and due to the short fermentation time the increase in their population was only slight. The storage trial showed that the probiotic bacteria survived the harsh conditions present in yoghurt. The organic acid concentration was measured. The control batch of yoghurt produced more lactic, butyric and propionic acid than the experimental batches.

Production of antimicrobial substances by *L. acidophilus* was determined against *L. delbrueckii* subsp. *bulgaricus*. Four strains of *L. acidophilus* (La-5, La2404, La2405 and La2406) were used as the producer organism for inhibitory activity against *L. delbrueckii* subsp. *bulgaricus* using modified spot on lawn and agar well-diffusion techniques. Two strains of *L. delbrueckii* subsp. *bulgaricus* (Lb2515, Lb2501) were used as the indicator organism. The four strains of *L. acidophilus* produced inhibitory zones against one strain of *L. delbrueckii* subsp. *bulgaricus* (Lb2515). These inhibitory zones were confirmed to be bacteriocin as no zones appeared when treated with proteolytic enzymes.

In order to determine lysis of *L. delbrueckii* subsp. *bulgaricus* with bacteriocin produced by *L. acidophilus,* one strain of *L. acidophilus* (La-5) was incubated with one strain of *L. delbrueckii* subsp. *bulgaricus* (Lb2515) at inoculation levels of 1%, 5% or 10% for 8 hours. Plate counts and β-galactosidase activity were measured. During incubation, the *L. acidophilus* counts increased and the *L. delbrueckii* subsp. *bulgaricus* counts decreased suggesting that *L. acidophilus* was producing bacteriocin against *L. delbrueckii* subsp. *bulgaricus*. However *L. acidophilus* did not inhibit *L. delbrueckii* subsp. *bulgaricus* enough to stop its growth. Therefore, it was thought, that if the bacteriocin produced by *L. acidophilus* could be concentrated and purified, this could be added as a supplement to yoghurt, to inhibit growth of *L. delbrueckii* subsp. *bulgaricus*.

L. acidophilus was inoculated in MRS broth and incubated for 18 hours. This was then centrifuged and neutralised to pH 6.0. The broth was filtered using a 30kDa ultrafiltration unit and was concentrated approximately 50 times, and the bacteriocin was extracted and purified. This was then added to *L. delbrueckii* subsp. *bulgaricus* at different rates (1%, 5% and 10%) to examine if any inhibition occurred. The results showed that concentrated bacteriocin inhibited *L. delbrueckii* subsp. *bulgaricus.* The 10% sample had the lowest viable counts after 10 hours with a 5 log cycle difference. The next lowest was the 5% sample which also had a 5 log cycle difference. The 1% sample had a 3 log cycle difference.

The bacteriocin was incorporated in milk during inoculation the youghurt and probiotic bacteria at 1% and 2% levels. The fermentation time of all three yoghurts was 3 hours. There seemed to be little difference in the growth of *L. delbrueckii* subsp. *bulgaricus* during fermentation between the three yoghurts. The 1% batch had the highest viable count followed by the control and then the 2% batch. *L. acidophilus* and *B. longum* increased in number slightly during fermentation. The control yoghurt had the highest number of *L. acidophilus* followed by the 1% batch and the 2% batch. The 2% batch had the highest number of *B. longum* followed by the control and the 1% batch. During storage the pH dropped considerably in all yoghurts. The numbers of *L. delbrueckii* subsp. *bulgaricus* decline in all three yoghurts over the 6 weeks of storage. The probiotic bacteria decreased during storage in all yoghurts.

The analysis of organic acids was performed using the HPLC. The lactic acid production increased during fermentation and fluctuated during storage in all three yoghurts. The 1% yoghurt batch had the highest concentration at the end of fermentation followed by the control and the 2% yoghurt. From these experiments, it was observed that the bacteriocin did not inhibit *L. delbrueckii* subsp. *bulgaricus* in the yoghurt. Therefore it was presumed that there was some substance blocking the activity of bacteriocin in yoghurt.

L delbrueckii subsp. *bulgaricus* (1%) was grown with 1%, 5% and 10% levels of bacteriocin for 8 hours in 12% RSM. The results showed that the bacteriocin had no effect on the growth of *L. delbrueckii* subsp. *bulgaricus* bacteria.

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1.0 INTRODUCTION

Consumers are becoming more aware of maintaining their "internal health". The consumer trend is moving towards taking a preventative approach rather than a curative approach to modern health problems. Hence, probiotic functional foods are becoming increasingly popular in the diets of people in Australia, and in other parts of the Western world. The number of probiotic foods is currently much greater in Japanese and European markets than in Australia. Therefore, there is potential in marketing opportunities in Australia and production is expected to grow rapidly. However, studies (Hull *et al.*, 1984; Shioppa *et al.*, 1981; Shah *et al.*, 1995; Shah *et al.*, 2000) have shown that probiotic bacteria are unstable in yoghurt. Recent surveys conducted in Australia (Anon 1992; Rybka and Fleet, 1997; Shah *et al.*, 1995; Shah *et al.*, 2000) and in Europe (Iwana *et al.*, 1993) have shown low viability of probiotic organisms in commercial preparations which have created a negative image about these products. The loss of viability is claimed to be due to a variety of factors including H_2O_2 and acid produced by yoghurt bacteria, dissolved O_2 level in the product (Dave and Shah, 1997) and lack of nutrients in milk to sustain their growth. By enhancing the viability and survival of probiotic bacteria, confidence of the consumers in "probiotic health foods" can be restored.

A number of health benefits associated with the consumption of products containing live probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* have been claimed. Yoghurt starter bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) do not survive or colonise in the gastrointestinal tract. Hence probiotic organisms are often incorporated into fermented milk products such as yoghurt. However, propagation of probiotic bacteria, in particular *Bifidobacterium* is difficult, as the organisms are fastidious and their numbers decline during storage due to post-acidification by yoghurt bacteria, in particular by *L. delbrueckii* subsp. *bulgaricus* (Dave and Shah, 1998a). As a result, many yoghurt manufacturers use starter cultures devoid of *L. delbrueckii* subsp. *bulgaricus* but containing a mixture of *L. acidophilus, Bifidobacterium* and *S. thermophilus* (ABT cultures). However, use of ABT starter cultures increases fermentation time significantly, which is undesirable, given the rigid schedule in modern yoghurt

manufacturing. *L. delbrueckii* subsp. *bulgaricus* produces proteolytic enzymes and is found to support the growth of probiotic bacteria during fermentation by releasing growth factors such as amino acids and peptides by hydrolysing milk proteins. However, their presence is undesirable as the organisms are responsible for post-acidification which results in loss of viability of probiotic bacteria (Dave and Shah, 1998a).

Bacteriocins are proteinaceous compounds produced by lactic acid bacteria that kill or inhibit closely related bacteria (Tagg *et al.*, 1976). There is a tremendous interest in bacteriocins and their role as a preservative in minimally processed foods. They have been found to inhibit food-borne pathogens such as *Clostridium botulinum* and *Listeria monocytogenes*, the latter being able to grow at refrigeration temperatures (Montville and Kaiser, 1993). As bacteriocins are protein and "natural", this will satisfy consumer demand for "fresh" and "preservative free" products.

To date, several *L. acidophilus* strains have been studied for the production and isolation of bacteriocins (Barefoot and Klaenhammer, 1984; Reddy *et al.*, 1983; Toba *et al.*, 1991) and their antimicrobial effects against various pathogens, and to understand the role of these organisms. These studies have shown that some strains of *L. acidophilus* produce bacteriocins against *L. delbrueckii* subsp. *bulgaricus*. In a study by Dave and Shah (1998b), *L. acidophilus* produced bacteriocin against seven strains of *L. delbrueckii* subsp. *bulgaricus*.

This project aimed to develop a process for using the beneficial effects of traditional yoghurt starters (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) for quick fermentation and enhancing viability of probiotic organisms through the control of post-acidification using controlled lysis of *L. delbrueckii* subsp. *bulgaricus* by bacteriocin producing strains of *L. acidophilus*. Specifically the aims of the project were:

1. To investigate ways of lysing *Lactobacillus delbrueckii* subsp. *bulgaricus* to release sufficient growth factors to sustain the growth of selected starter strains of *S. thermophilus* in order to reduce fermentation time of the probiotic organisms (*L. acidophilus* and *Bifidobacterium* sp.),

2. To investigate whether bacteriocin producing strains of *L. acidophilus* can be effectively used to lyse cells of *L. delbrueckii* subsp. *bulgaricus in situ* in order to control post-acidification,

3. To trial bacteriocin producing strains of *L. acidophilus*, together with *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus* and *Bifidobacterium* sp. in commercial yoghurt production to evaluate the (a) lysis of *L. delbrueckii* subsp. *bulgaricus*, (b) viability and survival of *L. acidophilus* and *Bifidobacterium* sp., (c) reduction in fermentation time and (d) control of post-acidification.

Chapter 1 and 2 contain a review of literature, Chapter 3 explains the materials and methods used and Chapter 4 presents and discusses the results. Chapter 5 gives a summary of results and Chapter 6 and 7 discuss future research direction and a list of references, respectively.

2.0 LITERATURE REVIEW

2.1 Yoghurt

2.1.1 History

No one knows exactly when or how yoghurt originated, but apparently when the goat was first domesticated in Mesopotamia about 5000 BC, its milk was stored warm due to the hot climate and naturally formed a curd. Someone with sufficient courage tasted this curd and rendered a favourable verdict (Kosikowski, 1997). The practice of souring milk was eventually refined and incessant curiosity about the agents causing fermentation, led to the discovery of bacteria (Kosikowski, 1997).

2.1.2 Yoghurt around the world

Yoghurt is very popular in Asia and in many countries such as Iran, Iraq and Turkey, it possesses an importance unequalled elsewhere. Yoghurt is made mainly from sheep's milk or a combination of sheep and goat's milk and incubated by placing a blanket over the pan (Kosikowski, 1997). In Chang Tang region of Tibet the Phala nomads use yak, sheep and goat milk for making yoghurt, which can be churned into butter, which is added to salty tea and drunk as a nutritious beverage forty times a day (Kosikowski, 1997).

In certain countries fermented milk foods are favoured over fresh milk for reasons of safety, better flavour, texture and possible beneficial therapeutic effects. In countries where inadequate transport, pasteurization and refrigeration facilities exist, particularly tropical countries, many authorities prefer to sour the milk first. In this manner the presence of lactic acid bacteria and their metabolic end products discourage growth of food poisoning and disease producing bacteria.

In Western countries yoghurt appears in different forms. It is eaten as a dessert, a snack between meals, a complete lunch or as a diet and health food. It is often flavoured with fruit, honey or vanilla and is available in its natural form (Kosikowski, 1997). The increase in consumption of yoghurt in the Western world owes much to the development of its health food image (Early, 1998). Marketing strategies have

concentrated on the availability of reduced/lower fat content, calorific content, extended shelf-life, additive free yoghurt, health promotion of probiotic bacteria and children's yoghurt, which are milder and sweeter in taste (Early, 1998).

2.1.3 Characteristics

Yoghurt is a fermented milk food that is produced from milk or skim milk plus a starter culture containing two bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) that produce lactic acid. According to Australian Standard H8, yoghurt should have a pH 4.5 or below and a titratable acidity greater than 0.9% expressed as lactic acid. The delicate flavour in plain yoghurt is achieved through a bacterial relationship influenced by such factors as acid concentration. Other factors include volatile flavour components in small amounts such as acetic acid, diacetyl and acetaldehyde. The latter produced by *L. delbrueckii* subsp. *bulgaricus*, is a major contributor to the unique flavour of yoghurt (Kosikowski, 1997). Yoghurt has a smooth, light gel texture, however, it can also be produced as a liquid beverage and a solid frozen dessert.

Yoghurt is often flavoured with fruit, honey and essences and may be dyed with acceptable food dyes. Stabilisers are often added to give smoothness characteristics, but no salt is added to yoghurt.

Generally the fat content is about 3% in Australian yoghurt, when it is made with whole milk but there are many varieties of reduced fat yoghurt which have 1.7%, and "no fat" yoghurt, which has a fat content below 0.1%. Yoghurt that is higher in fat has a much smooth texture and richer flavour than the low fat varieties. The texture of yoghurt does vary between varieties and with the yoghurt is manufactured.

2.1.4 Manufacture of yoghurt

The manufacturing process of yoghurt can vary from one manufacturer to another. The composition can vary, as do yoghurt types and types of starter cultures used, which therefore, affect the principles of manufacture. In this section the two main

types of yoghurt making; set and stirred, will be discussed. However, firstly it is important to consider the types of cultures that are used in yoghurt making.

2.1.4.1 Yoghurt cultures

The general function of any starter culture should be to produce sufficient lactic acid in as short a time as possible to ferment milk to pH 4.5 and to give acceptable texture, viscosity and flavour in the final product. Most commercial manufacture involves the use of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. These two bacteria have a relationship that is termed symbiosis (Early, 1998). *S. thermophilus* initiates the fermentation process by stimulation of peptides and free amino acids released from the milk proteins by *L. delbrueckii* subsp. *bulgaricus*. The lactobacilli in turn are stimulated by formic acid produced by *S. thermophilus* (Early, 1998).

Streptococci dominate the early stage of yoghurt fermentation. As the redox potential of the milk medium is reduced and the pH lowered from 6.5 to 5.5, growth of *L. delbrueckii* subsp. *bulgaricus* is enhanced. Below pH 5.0, lactobacilli dominate yoghurt fermentation and produce acetaldehyde and lactic acid, yielding the characteristic yoghurt flavour. Continued acid production lowers yoghurt pH to near 4.6, which induces clotting.

Other bacteria such as *Lactobacillus helveticus*, and probiotic bacteria such as *Lactobacillus acidophilus*, bifidobacteria and *Lactobacillus casei* are also used in yoghurt as a replacement for *L. delbrueckii* subsp. *bulgaricus* or as a therapeutic starter culture.

Lactobacillus delbrueckii subsp. *bulgaricus* is a robust culture and continues to ferment during cooling leading to excessive lactic acid production. This results in a very sour taste. The trend has been to move away from *Lactobacillus delbrueckii* subsp. *bulgaricus* because of the unfavourable flavour and replace it with what is called an 'ABT' culture which consists of *L. acidophilus*, bifidobacteria and *S. thermophilus*. Another popular starter culture is 'ABC', which consists of *L. acidophilus*, bifidobacteria and *L. casei*. These cultures (ABT and ABC) are used as adjunct starter cultures, while yoghurt bacteria are used as a primary culture.

Yoghurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) are not natural inhabitants of the intestine and cannot survive under acidic conditions and bile concentration encountered in the gastrointestinal tract. Therefore, probiotic bacteria (*L. acidophilus* and bifidobacteria) are added to yoghurt to give therapeutic benefits. Probiotic bacteria will be discussed later in this chapter.

2.1.4.2 Processing

As discussed previously there are many variations in the manufacture of yoghurt. In this section the general process of yoghurt making will be described paying particular attention to set and stirred type yoghurts. Set yoghurt is fermented in its retail container and is undisturbed forming a semi-solid mass. Stirred yoghurt is fermented in a vessel and the coagulum is broken during the cooling and packaging stage, giving a very smooth texture (Early, 1998).

For making yoghurt, milk is heated to approximately 85°C for 30 minutes. This step has several aims: it eliminates or reduces levels of food spoilage microorganisms and reduces the total microbiological population to a level, which will not compromise the growth of the starter culture microorganisms. The most important factor in this heat treatment is that it denatures the whey proteins, β-lactoglobulin and α-lactalbumin, in order to improve the texture and viscosity of the final product and to assist in the prevention of whey separation during shelf life (Kosikowski, 1997; Early, 1998).

After heat treatment, the milk is cooled to a suitable temperature of 45°C for inoculation. At this point, fruit can be added to containers for set type yoghurt. The warm inoculated milk is then poured on top of the fruit mix and allowed to incubate. For stirred type yoghurt, the fermentation is carried out in a large vessel, and fruit is added after fermentation (Kosikowski, 1997; Early, 1998). Fermentation is carried out for both types of yoghurt at 42°C until the pH reaches 4.5, during which the curd should not be disturbed. The yoghurt curd or 'coagulum' begins to form as more lactic acid is produced and the iso-electric point of casein (pH 4.6-4.7) is approached (Early, 1998). Fermentation time does vary between 6-12 hours depending on the bacteria, batch size and milk season.

For stirred yoghurt, once fermentation time is completed, the curd is broken using slow speed paddle agitation for no more than 5-10 minutes (Varnam, 1994). This produces the required body and texture and the yoghurt is then cooled in a two-stage process. The first stage is to cool the yoghurt to 15-20°C, at which point, fruit or other flavours can be added (Varnam, 1994). Second stage cooling is below 5°C, which is achieved in a cold store (Varnam, 1994). Cooling should be carefully controlled, since too rapid cooling leads to syneresis or 'wheying off'. The cooled, stirred yoghurt is then filled into containers and must remain chilled until it reaches the consumer. Yoghurt has a shelf life of approximately 42 days. In the case of set yoghurt, cooling takes place inside the retail container and is started before the final pH is reached. Care must be taken when transferring the yoghurt, as excessive agitation reduces the viscosity and can also cause syneresis (Varnam, 1994).

There are other types of yoghurt, such as thermised yoghurt, which once fermented the yoghurt is pasteurised to kill all yoghurt bacteria. This increases the shelf life considerably, often up to 12-15 weeks. However, this practice is not permissible in Australia as the product is defined as containing live micro-organisms at commercially viable levels on consumption (Early, 1998).

Drinking yoghurt is manufactured much the same way, but has lower total solids and undergoes homogenisation to further reduce viscosity (Varnam, 1994). Frozen yoghurts may be prepared from conventional set or stirred yoghurts, although there will be a higher level of sugar and stabilisers, required to maintain the coagulum during freezing and storage and a small quantity of cream may be added to improve 'mouth-feel'. The yoghurt is frozen in a blast freezer (-20°C) or frozen with aeration in an ice-cream freezer (Varnam, 1994).

2.2 Yoghurt Bacteria

Yoghurt bacteria include *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. These bacteria are commonly used in the production of yoghurt and are part of the Lactic acid bacteria group. The characteristics of lactic acid bacteria and the genus *Lactobacillus* and *Streptococcus* are discussed next.

2.2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of gram-positive bacteria united by a constellation of morphological, metabolic and physiological characteristics. The general description of the bacteria included in the group is gram positive, nonsporing, nonrespiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates (Salminen and von Wright, 1993). The genera included in this group are *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus* and *Vagococcus* (Salminen and von Wright, 1993).

2.2.2 Genus *Lactobacillus*

The members of the genus Lactobacillus are gram-positive, non-sporing bacilli, which can vary from slender long rods to short ones. Lactobacilli have complex nutritional requirements including energy, which they derive via homo-or heterofermentative catabolism of carbohydrates, a carbon source, and a variety of nucleotides, amino acids and vitamins for growth (Hoover and Steenson, 1993; Wood, 1992). This genus has been subdivided into three subgenera determined by the fermentation end products. Homofermentative lactobacilli exclusively ferment hexose sugars to lactic acid by the Embden-Meyerhof pathway. They do not ferment pentose sugars or gluconate. This group includes *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus helveticus*, which are starter cultures. They grow at higher temperatures (>45°C) than lactobacilli in the other groups and are thermoduric. *L. acidophilus* is also a member of this group but is not a starter culture organism (Marth and Steele, 1998).

Facultatively heterofermentative lactobacilli ferment hexose sugars, either only to lactic acid or to lactic acid, acetic acid, ethanol and formic acid when glucose is limited. Pentose sugars are fermented to lactic and acetic acid via the phosphoketolase pathway. This group includes *Lactobacillus casei*, which is not usually used as a starter culture but has beneficial secondary fermentation during cheese ripening (Marth and Steele, 1998).

Obligately heterofermentative lactobacilli ferment hexose sugars to lactic acid, acetic acid or ethanol and carbon dioxide using the phosphoketolase pathway. Pentose sugars are also fermented using this pathway. These lactobacilli can cause undesirable flavour and gas formation during ripening of cheese. Species include *Lactobacillus kefir* (Marth and Steele, 1998).

The organic acids that are produced (lactic and acetic) serve directly as antagonists to other competing microflora by lowering the pH of the surrounding environment. This decrease in pH of their environment allows the lactobacilli to effectively compete and ultimately dominate fermenting ecosystems, since they are more acid tolerant than other organisms, including many pathogenic and spoilage species, as well as other lactic acid bacteria (Hoover and Steenson, 1993).

Lactobacilli are widespread in nature and are found in the oral cavity, gastrointestinal tract, and vagina of humans and animals (Salminen and von Wright, 1993). Many species of lactobacilli have found applications in the food industry. They are generally the most acid-tolerant of the LAB and therefore, will terminate many spontaneous lactic fermentation such as silage and vegetable fermentation.

The species of Lactobacilli, that are important in this project, are *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus*, which belong to the *Thermobacterium* group. *Lactobacillus delbrueckii* subsp. *bulgaricus* is gram-positive with very slender and long rods. It has an optimum temperature of 42°C and grows best under anaerobic conditions; however, it is considered a facultative anaerobe. It is the bacterium responsible for the production of acetaldehyde, the main contributor of the characteristic flavour in yoghurt. It is commonly grown in deMan Rogosa and Sharpe (MRS) broth and in reconstituted skim milk (RSM) with glucose and yeast extract and selective media is MRS broth, pH 5.2. *Lactobacillus acidophilus* will be discussed later in this chapter.

2.2.3 Genus *Streptococcus*

The members of this genus are gram-positive cocci, forming pairs and chains of cells when cultured in liquid media (Wood, 1992). The streptococci have complex nutritional requirements that vary between species but do involve amino acids, peptides, purines, pyrimidines and vitamins as growth factors. Carbohydrates are fermented with major production of lactic acid and minor amounts of acetic and formic acids, ethanol and carbon dioxide (Wood, 1992).

Streptococcus thermophilus is the strain used for yoghurt. Its optimum growth temperature is 37°C and grows aerobically. It can be grown in MRS broth but is better suited to M17 broth in which it can be grown selectively. It also grows well in RSM with glucose and yeast extract.

2.3 Yoghurt bacteria in yoghurt

S. thermophilus initiates the fermentation process by stimulation of peptides and free amino acids released from the milk proteins by *L. delbrueckii* subsp. *bulgaricus* the main amino acid being valine. Milk contains too little of these amino acids and the cocci which are very weakly proteolytic, form the acids too slowly. *S. thermophilus* enhances the growth of the rods by forming formic acid out of pyruvic acids under anaerobic conditions and by a rapid production of carbon dioxide (Walstra *et al.*, 1999). Figure 1 shows an outline of the stimulation of the growth of yoghurt bacteria in milk.

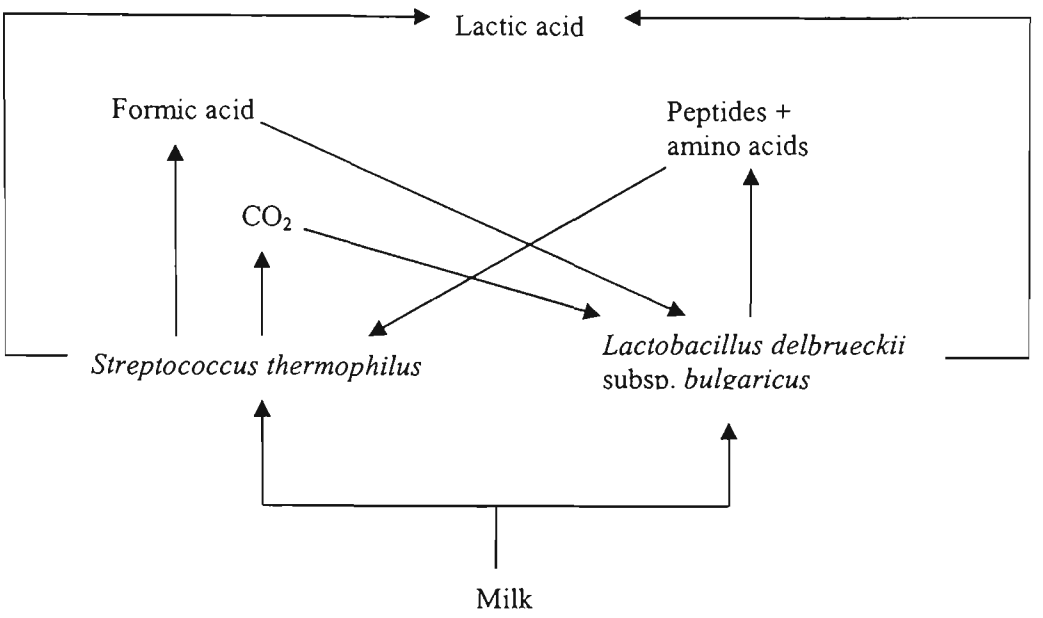

Figure 1. Outline of the stimulation of growth of yoghurt bacteria (Source: Walstra *et al.*, 1999)

As the redox potential of the milk medium is reduced and the pH lowered from 6.5 to 5.5, growth of *L. delbrueckii* subsp. *bulgaricus* is enhanced. Below pH 5.0, lactobacilli dominate yoghurt fermentation and produce acetaldehyde and lactic acid, yielding the characteristic yoghurt flavour. Continued acid production lowers yoghurt pH to near 4.6, which induces clotting. High quality yoghurts have a pH of 4.2 to 4.3 at the time of consumption and possess proper taste and aroma.

2.4 Post-acidification in yoghurt

During refrigerated storage, the lactobacilli in yoghurt continue to produce acid and pH is lowered to <4.0, which results in an excessively sour product. This process is known in the industry as "post-acidification". The extra lactic acid produced causes the pH to decrease further, which makes the environment unsuitable for the survival of probiotic bacteria and gives a very sour flavour, which is not desirable.

The pH also decreases during storage, due to ongoing metabolic activity of yoghurt bacteria, in particular by *L. delbrueckii* subsp. *bulgaricus*. It is for this reason that many yoghurt manufacturers use starter cultures devoid of *L. delbrueckii* subsp. *bulgaricus* but containing *L. acidophilus*, *Bifidobacterium* sp. and *S. thermophilus*

(ABT). However, the use of ABT starter cultures alone, increase fermentation time significantly, which is undesirable, given the rigid schedule in modern yoghurt manufacturing.

Pasteurising the finished yoghurt, to destroy viable starter culture bacteria, can also prevent post-acidification however, pasteurisation of yoghurt is not permitted in Australia.

Lactobacillus delbrueckii subsp. *bulgaricus* does have benefits to the yoghurt making process. If it is excluded the fermentation process becomes very slow and increases the cost of manufacture. The bacteria also produces proteolytic enzymes that is found to support the growth of probiotic bacteria (*L. acidophilus* and *Bifidobacterium*) during fermentation by releasing growth factors such as amino acids and peptides, and also stimulates production of acetaldehyde, acetic acid, diacetyl and lactic acid. Supplementation with exogenous nutrients such as yeast extract, tryptone or deMan, Rogosa and Sharpe (MRS) broth has been found to promote *L. acidophilus* and bifidobacteria growth (Dave and Shah, 1998b). However, the nutrients required for this (i.e. MRS broth) are either not permitted in yoghurt, contribute an off-flavour or are too expensive on a commercial basis. If the growth factors from *L. delbrueckii* subsp. *bulgaricus* could be released into the yoghurt this would be a natural way of supplementing the probiotic bacteria with nutrients.

2.5 Probiotic bacteria

2.5.3 Definition

According to Parker (1974) probiotic bacteria are 'organisms and substances produced by these organisms which contribute to intestinal microbial balance'. Probiotic bacteria was defined by Fuller (1989) as "a live microbial food supplement which beneficially affects the host in improving its intestinal microbial balance". Wood (1992) broadened Fuller's definition as 'a probiotic is a mono- or mixed culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora'.

Probiotic bacteria are lactic acid bacteria that are of human origin, are acid and bile resistant to survive in the intestine, they are able to adhere and colonise in the intestinal tract, are antagonistic against carcinogenic and pathogenic bacteria and are stable during processing and storage (Salminen, 1998).

2.5.4 *Lactobacillus acidophilus*

Lactobacillus acidophilus is a probiotic bacteria commonly used in yoghurt. It is a gram-positive rod but is much shorter than *Lactobacillus delbrueckii* subsp. *bulgaricus*. The cells are non-motile and non-sporulating and proteins in the cell wall may be important in attaching the bacterium to the intestinal wall (Tamime, 1999). *L. acidophilus* requires riboflavin, pantothenic acid, folic acid, and niacin for growth but not other B vitamins (Tamime, 1999). It has an optimum temperature of 37°C and no growth occurs below 15°C and the optimum pH is 5.5-6.0. *L. acidophilus* is presented in Group I as obligately homofermentative the same as *Lactobacillus delbrueckii subsp. bulgaricus*. It can also be grown in MRS broth and in RSM with glucose and yeast extract. It can also be grown selectively in MRS with sorbitol or salicin.

L. acidophilus has been found to have health-promoting properties, which will be discussed later in this chapter. It also produces bacteriocins, which are antibiotic-like substances that may be important in the prevention of pathogenic growth. Bacteriocins are also discussed later in this chapter.

2.5.5 Genus *Bifidobacterium*

The members of the genus *Bifidobacterium* are gram-positive, non-sporing bacilli. The rods of bifidobacteria often have an irregular shape, with a concave central region and swollen ends. It is however not unusual to encounter cells that are coccoid or appear as short bacilli with varying widths. The shape depends very much on the constituents of the media (Tamime, 1999). Bifidobacteria can utilise ammonium salts as sole source of nitrogen. These bacteria also produce an enzyme, fructose-6-phosphate phosphoketolase (F6PPK), known as "bifidus shunt" and this can be used

to identify the genus, however, not all strains produce enough F6PPK for it to be detectable. The fermentation of two molecules of glucose, leads to two molecules of lactate, and three molecules of acetate (Tamime, 1999).

There are currently thirty different strains of bifidobacteria which have been isolated from different sources such as the faeces of humans, animals, birds and sewage, the human vagina, bees and dental caries (Tamime, 1999). Only six species are of interest to the dairy industry for the manufacture of fermented dairy products. These include *Bifidobacterium adolescentis, B. breve, B. bifidum, B. infantis, B. lactis and B. longum* that have been isolated from human subjects. This restriction is based on the assumption that, if an isolate is of human origin, then it should become implanted on the walls of intestines and metabolise in the colon of another human (Tamime, 1999).

The two species used in this study were *B. infantis* and *B. longum*. They grow best at 37°C under anaerobic conditions. The best media is MRS broth with 1% L-cysteine and they can also be grown selectively on MRS-Broth with NNLP and L-cysteine added to it.

2.5.6 Health benefits of probiotic bacteria

A number of health benefits for products containing live probiotic bacteria (*L. acidophilus* and *Bifidobacterium*) have been claimed. As a result, these organisms are increasingly incorporated into dairy products, such as yoghurt. Metchnikoff (1908) in his book "The Prolongation of Life" proposed the theory that the longevity of the Bulgarians was in part due to ingesting large quantities of fermented milks containing lactobacilli. This observation has led to much interest in the role of lactic acid products in alleviation of human and animal disorders. The benefits offered by *L. acidophilus* and bifidobacteria include improvement in intestinal disorders and lactose tolerance, antimicrobial properties, reduction in serum cholesterol, antimutagenic and anti-carcinogenic activities and adherence to intestinal cells (Shah, 2000b; Harding, 1995). These fermented dairy products have also been reported to be effective in the treatment of diarrhea, constipation, colitis, reducing blood cholesterol, pathogenic recolonisation of the intestinal tract, flatulence, gastric acidity and gastroenteritis

(Harding, 1995). Table 1 shows a summary of health benefits associated with probiotic bacteria.

Table 1: Health benefits of probiotic bacteria

Health Benefit	Proposed mechanism
Protection against undesirable organisms	Production of inhibitory compounds i.e. acids, H_2O_2 and bacteriocins.
Improved digestion	Partial breakdown of protein, fat, carbohydrates and improved bioavailability of nutrients.
Control of serum cholesterol	Gut microflora can metabolise cholesterol
Protection against cancer	Probiotic bacteria can inhibit carcinogens and enzymes involved in converting procarcinogens to carcinogens.
Improved immune system	Enhancement of macrophage formation, stimulation of suppressor cells and production of interferon.
Improved lactose digestion	Bacteria can produce enzyme to breakdown lactose.
Prevention of constipation	Improvement in bowel movement and stabilisation of ecological balance in the intestinal tract.
Increased vitamin contents	Synthesis of group B vitamins
Control of vaginal infection	Inhibition of fungi and bacteria responsible for the infection.

(Sources: Marth and Steele, 1998; Dave, 1998; Shah, 2000b; Harding, 1995; Salminen and von Wright, 1993; Fuller, 1992; Goldin and Gorbach, 1987)

2.5.4.1 *Antimicrobial and antimutagenic properties of probiotic bacteria*

Microorganisms that are considered probiotic must have several important characteristics including their ability to produce antimicrobial substances such as organic acids (e.g. lactic and acetic acids), hydrogen peroxide and bacteriocins to suppress the growth of pathogenic and putrefying bacteria.

These probiotic bacteria produce several organic acids such as acetic, lactic and pyruvic acids. Other acids produced in small quantities included citric, hippuric acid, orotic acid and uric acid (Lankaputhra and Shah, 1998). Lactic and acetic acids are the major acids produced and these acids account for more than 90% of the acids produced (Shah, 2000b). It is well documented that organic acids are inhibitory against coliforms, *Salmonella*, and *Clostridia* in vitro, but convincing in vivo evidence is still lacking (Salminen and von Wright, 1993). Several researchers believe that lactic acid is the only antimicrobial agent of any importance and that lowering of pH due to lactic acid or acetic acid produced by these bacteria in the gut has bacteriocidal or bacteriostatic effect (Shah, 2000b). Lankaputhra and Shah (1998) studied the levels of acetic, butyric, lactic and pyruvic acids produced by the probiotic bacteria as determined by HPLC technique. All strains produced these acids with butyric acid being produced by most strains of *L. acidophilus* and bifidobacteria.

Some strains of lactic acid bacteria including lactococci, lactobacilli, leuconostoc and pediococci have the ability to generate hydrogen peroxide during growth and lack of catalase by these bacteria causes its accumulation in growth media (Shah and Dave, 2002). Accumulation of hydrogen peroxide occurs by the action of superoxide dismutase in most lactic bacteria or by manganese ions present in high concentrations in the cytoplasm of bacteria. Hydrogen peroxide has been reported to inhibit the growth of *Staphylococcus aureus, E. coli, Salmonella typhimurium, Clostridium perfringens, Pseudomonas* sp. and other psychrotrophs (Shah and Dave, 2002). Hydrogen peroxide in the presence of organic acids such as lactic acid is more inhibitory to bacteria (Lankaputhra and Shah, 1998).

Some strains of *L. acidophilus* and bifidobacteria have been reported to show antimutagenic and anticarcinogenic properties. The mechanism of antimutagenicity of probiotic bacteria have not been understood or identified so far and the mechanism of

antimutagenicity remains speculative. It has been suggested that microbial binding of mutagens could be the possible mechanism of antimutagenicity (Orrhage *et al.*, 1994).

Lankaputhra and Shah (1998) studied the antimutagenic activity of organic acids against eight mutagens and promutagens. The study found that butyric acid showed the highest anitmutagenic activity against all the 8 mutagens or promutagens. Lactic and pyruvic acids showed lower antimutagenic activities and thus it appears that lactic acid produced by lactic acid bacteria plays a minor role in antimutagenic activity. Therefore probiotic bacteria which produces butyric acid are more likely to provide antimutagenic properties.

2.5.4.2 Inhibition of spoilage organisms

Bacteria that produce these inhibitory characteristics described in section 2.5.4.1 show inhibition against spoilage organisms. Probiotic bacteria show strong antimicrobial properties against Gram positive bacteria such as *Staphylococcus aureus*, *Clostridium perfringens* than against Gram negative bacteria such as *Salmonella typhimurium* and *Escherichia coli* (Shah, 2000b; Hoover and Steenson, 1993). Much interest has been focused on evaluating the sensitivity of *Listeria monocytogenes* to lactic acid bacteria bacteriocins and it has become a model target bacterium in many studies of food preservatives (Hoover and Steenson, 1993). *Listeria monocytogenes* can grow at refrigeration temperatures and is found in meats and dairy foods which is why there is such interest in probiotics as preservatives.

Listeria is sensitive to many bacteriocins including nisin. Daba *et al.* (1991) found that the bacteriocin 'mesenterocin 5' appeared to be specific for *Listeria* species and not active against other lactic acid bacteria. Daeschel and Klaenhammer (1985) found *Clostridium botulinum* to be inhibited by the bacteriocin of *Pediococcus pentosaceus.* Okereke and Montville (1991) investigated it further and observed that some strains of *Lactobacillus planarum, L. lactis* and *P. pentosaceus* inhibited Listeria.

Bruno and Shah (2002) investigated the role of bifidobacteria in the inhibition of pathogenic and putrefactive microorganisms. In this study four strains of bifidobacteria (*B. infantis, B. pseudolongum* and 2 strains of *B. longum*) were grown

with different pathogenic bacteria including *Escherichia coli, Clostridium perfringens, C. chauvoei, C. sporogenes, Candida albicans, Enterobacter aerogenes, Streptococcus agalactiae, S. mitis* and *S. pyogenes.* All bifidobacteria strains inhibited all strains of pathogenic bacteria when grown together. The inhibition was found to be due to the lowering of the pH by the bifidobacteria strains from production of lactic and acetic acids as a result of fermentation of glucose. When the pH of the supernatant was adjusted to neutral the inhibition of growth were absent.

2.5.4.3 Anticarcinogenic activity

Some strains of *L. acidophilus* and bifidobacteria have been reported to show anticarcinogenic properties. The evidence of anticancer effect can be due to decrease in faecal enzymes involved in conversion of procarcinogens to carcinogens. These probiotic bacteria also lower levels of harmful enzymes such as β-glucosidase and β-glucuronidase responsible for catalysing the conversion of harmful amines (Lidback *et al.*, 1992).

Fermented dairy products have also been shown to either inhibit chemically induced colon tumors or transplantable tumor lines in rodents (Fuller, 1992). Goldin and Gorbach (1984 and 1987) have found evidence for the anti-tumor effect of *L. acidophilus.* Oral supplementation of a diet containing viable *L. acidophilus* of human origin and bile resistant caused a significant decline in 3 different fecal bacterial enzymes associated with carcinogenesis. The reduction in fecal enzyme activity was noted in both humans and rats.

Butyric acid is claimed to prevent carcinogenic effects at molecular (DNA) level (Smith, 1995; Tanaka *et al.*, 1990 and Yanagi *et al.*, 1993). Yanagi *et al.* (1993) reported that addition of butyric acid to a diet containing 20% margarine prevented mammary tumour formation by 7,12-dimethylbenz(a) anthracene in rats. Thus, it appears that antimutagenic effects of probiotic bacteria may be due to both inhibition by bacterial cells and production of organic acids, especially butyric acid (Shah, 2000b).

2.5.4.4 Lactose intolerance

Lactose intolerance is a condition in which lactose is not completely digested into its component monosaccharides, glucose and galactose (Shah, 1993). Lactose is cleaved into its monosaccharides by the enzyme β-D-galactosidase, lactose intolerance results from a deficiency of this enzyme. Lactose is the principal carbohydrate in milk and therefore lactose intolerant sufferers do not consume milk or other dairy products. The traditional cultures used for making yoghurt (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) have substantial quantities of β-D-galactosidase and it has been suggested that the consumption of yoghurt may assist in alleviating the symptoms of lactose intolerance. Kilara and Shahani (1976) have reported that during fermentation lactobacilli produce lactase, which hydrolyses lactose in milk to glucose and galactose. Bifidobacteria are resistant to bile, which gives them an increased chance of colonising the gut, and delivering the enzyme to its site of action (Hughes and Hoover, 1991). This would benefit consumers who are lactose intolerant and have to limit their dairy intake.

2.5.4.5. Reduction in serum cholesterol

Cholesterol lowering effects of fermented milk and their culture organisms has been the subject of a number of studies. Studies have shown that consuming certain cultured dairy products can help reduce serum cholesterol level. In the 1974 (Mann and Spoerry, 1974) study revealed that organisms such as *L. acidophilus* could have potential in reducing serum cholesterol in humans. Mann and Spoerry (1974) were investigating the influence of a surfactant (Tween 20) on serum cholesterol levels. They found that when they fed a group of men on a high cholesterol diet fermented milk both groups, that is men who were receiving the surfactant and men who were not, the serum cholesterol level decreased.

Several animal feeding studies have shown that consumption of milk containing cells of *Lactobacillus acidophilus* by animals resulted in lower serum cholesterol levels than in animals which did not receive milk containing the lactobacilli (Danielson *et al.*, 1989; Gilliland *et al.*, 1985; Grunewald, 1982).

Homma (1988) found feeding of fermented milks containing very large numbers of bifidobacteria (10^9 CFU/g) to hypercholesterolemic human subjects resulted in lowering cholesterol from 3.0 to 1.5g/L of blood serum.

Klaver and Meer (1993) reported that removal of cholesterol from the culture medium by *L. acidophilus* RP32 and other species was not due to bacteria uptake of cholesterol but resulted from bacterial bile salt deconjugating activity. The deconjugation of bile acid by the intestinal flora may influence the serum cholesterol level. The deconjuagted bile acid does not absorb lipid as readily as conjugated counterpart leading to reduction in cholesterol level.

Research into the potential of *L. acidophilus* to exert hypocholesterolemic effects in humans has indicated tremendous variation among strains of *L. acidophilus* isolated from the human intestinal tract in their ability to assimilate cholesterol (Buck and Gilliland, 1994). Evaluation of strains of *L. acidophilus* currently used commercially in cultured dairy product in the United States had revealed that none of them are particularly active with regard to actively assimilating cholesterol in laboratory media (Gilliland and Walker, 1990). On the other hand, new strains that are very active in this regard have been isolated from the human intestinal tract and thus they may provide greater potential for use as a dietary adjunct to assist in controlling serum cholesterol levels (Buck and Gilliland, 1994).

L. acidophilus and bifidobacteria actively assimilated cholesterol and other organic acids. Reports by Gilliland *et al.* (1985) show that *L. acidophilus* itself may take up cholesterol during the growth in the small intestine and make it unavailable for absorption into the blood stream. The effects of lactic acid bacteria on cholesterol levels are therefore inconsistent and range from a significant reduction to no reduction. The exact mechanism is unknown (Shah, 2000b)

2.5.5 Viability of Probiotic bacteria in yoghurt

For therapeutic benefits, the minimum level of probiotics bacteria in yoghurt has been suggested to be 10^6 viable cells per gram of yoghurt to enable them to survive in the gut and colonise (Tamime and Robinson, 1999). Despite the importance of viability

of probiotic bacteria studies have shown that probiotic bacteria are unstable in yoghurt (Hull *et al.*, 1984; Shioppa *et al.*, 1981). Recent surveys conducted in Australia (Anon, 1992; Shah *et al.*, 1995) and in Europe (Iwana *et al.*, 1993) have shown poor viability. Several factors have been claimed to affect the viability of yoghurt and probiotic bacteria in fermented milk products. The viability of probiotic bacteria in yoghurt depends on the strains used, interaction between species present, culture conditions, production of hydrogen peroxide due to bacterial metabolism, final acidity of the product and the concentrations of lactic and acetic acids. The viability also depends on the availability of nutrients, growth promoters and inhibitors, concentration of sugars, dissolved oxygen and oxygen permeation through package, inoculation level, incubation temperature, fermentation time and storage temperature (Young and Nelson, 1978; Gilliland *et al.*, 1988; Shah, 1997; Conway *et al.*, 1987; Shah and Jelen, 1990; Costello, 1993; Bertoni *et al.*, 1994; Shah *et al.*, 1995; Lankaputhra and Shah, 1995; Lankaputhra *et al.*, 1996b). However the main factors for loss of viability have been attributed to the decrease in the pH of the medium and accumulation of organic acids (Shah, 2000a; Hood and Zottola, 1988).

During production of yoghurt, yoghurt bacteria and probiotic bacteria produce organic acids and the pH is lowered to 4.5 or lower due to legal requirements and in order to produce good quality yoghurt. The amount of lactic acid could vary at the same pH in yoghurt due to the buffering effects of ingredients added to yoghurt mixes (Dave, 1998). Also depending on the extent of growth of bifidobacteria, concentration of acetic acid (which is more toxic compared to lactic acid) would vary in the product (Dave, 1998).

L. acidophilus tolerates acidity, however a rapid decrease in their number has been observed under acidic conditions (Shah and Jelen, 1990, Lankaputhra and Shah, 1995). Bifidobacteria are not as acid tolerant, as the growth ceases below pH 5.0, while the growth of *L. acidophilus* ceases at pH 4.0 (Shah, 1997).

In a study by Dave and Shah (1997a) the viability of yoghurt and probiotic bacteria was assessed during manufacture and 35 days of storage of yoghurt made from four commercial starter cultures. The viability of *L. acidophilus* was affected by the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* whereas bifidobacteria

exhibited better stability in the yoghurt prepared from cultures that contained *Lactobacillus delbrueckii* subsp. *bulgaricus*. The viability of both probiotic organisms improved when the dissolved oxygen concentration was low in the product and the storage temperature affected the viability of bifidobacteria but not *L. acidophilus*.

Joseph *et al.* (1998) studied antagonism between yoghurt and probiotic bacteria isolated from commercial starter cultures and commercial yoghurts using modified spot on lawn and agar well diffuison assays. Zones of inhibition for two *Bifidobacterium* isolates were observed with all *L. acidophilus* strains. The isolates of *L. acidophilus* were resistant and did not show inhibition by any of the four groups of microorganisms. Dave and Shah (1997a) and Shah and Ly (1999) also observed antagonistic relationships between yoghurt and probiotic bacteria

There are certain growth factors that probiotic bacteria require to grow. Milk is considered to be a less than optimal medium for the growth of bifidobacteria. The essential factor which is lacking in cow's milk but present in human milk is *N*-acetyl-D-glucosamine-containing saccharides which are known as the bifidus factors (O'Brien *et al.*, 1960; Glick *et al.*, 1960; Kurmann, 1988; Rasic and Kurmann, 1983; Poch and Bezkorovainy, 1988). Lactulose (4-O-β-D-galactopyransyl-D-fructose) also has a growth promoting effect on bifidobacteria (Mizota *et al.*, 1987; Park *et al.*, 1988).

Kosikowski (1982) suggested the use of sterile milk supplemented with 0.5% Bacto-liver, 0.05% $MgSO_4$ and 0.001% cysteine for growth of bifidobacteria in milk. Marshall *et al.* (1982) fortified milk with whey protein and threonine to provide the bifidobacteria with nutritious medium and lower redox potential. Anand *et al.* (1985) reported good growth of *B. bifidum* in sterile skim milk supplemented with 1% dextrose and 0.1% yeast extract.

Oxygen content is also a critical factor for bifidobacteria as it is anaerobic organism. During yoghurt production oxygen can easily invade and dissolve in the milk (Shah, 2000a). To exclude oxygen during the production of bifidus milk products, special

equipment is required to provide an anaerobic environment. Oxygen can also enter the product through packaging materials during storage (Shah, 2000a).

2.5.6 Improving viability of probiotic bacteria

It is important that the cells remain viable throughout the projected shelf life of a product so that when consumed the product contains sufficient viable cells. One of the important characteristics of probiotic bacteria is their ability to survive the acid in the human stomach and bile in the intestine. Several investigators have studied the survival of *L. acidophilus* and bifidobacteria in the presence of acid and bile salts. Clark *et al.* (1993) studied the survival of *B. infantis, B. adolescentis, B. longum* and *B. bifidum* in acidic conditions and reported that *B. longum* survived the best. Clark and Martin (1994) reported that *B. longum* tolerated bile concentrations of as high as 4.0% whereas Ibrahim and Bezkorovainy (1993) found *B. longum* to be the least resistant to bile.

Many strains of *L. acidophilus* and *Bifidobacterium* sp. lack the ability to survive harsh conditions and may not be suitable for use as dietary adjuncts in fermented foods. Lankaputhra and Shah (1995) have shown that of six strains of lactobacilli, three *L. acidophilus* strains survived best under acidic conditions. Two strains of *L. acidophilus* showed the best tolerance to bile. Among the nine strains of *Bifidobacterium* sp., *B. longum* and *B. pseudolongum* survived best under acidic conditions. *B. longum, B. pseudolongum* and *B. infantis* showed best tolerance to bile. However *B. infantis* survived poorly in acidic conditions and therefore may not be suitable for inclusion as dietary adjuncts. Therefore the selection of appropriate strains on the basis of acid and bile tolerance would help improve viability of these probiotic bacteria strains.

Probiotic bacteria may require the incorporation of micronutrients in yoghurt. Dave and Shah (1998b) investigated the effects of cysteine, whey powder, whey protein concentrate, acid casein hydrolysates and tryptone on the viability of *Streptococcus thermophilus, Lactobacillus acidophilus* and bifidobacteria. It was observed that the addition of cysteine, whey protein concentrate, acid casein and tryptone improved the viability of bifidobacteria but whey powder failed to improve viability. Sodium

dodecyl sulfate-PAGE and amino acid analysis suggested that a nitrogen source in the form of peptides and amino acids improved the viability of bifidobacteria.

Shah and Lankaputhra (1997) sonicated *L. delbrueckii* subsp. *bulgaricus* to release growth factors to support probiotic bacteria. They found that the probiotic bacteria numbers were 2 log cycles higher in yoghurt made with ruptured yoghurt bacteria and was still above the recommended level during 6 weeks of storage.

L. acidophilus produces a bacteriocin against *L. delbrueckii* subsp. *bulgaricus*, which could be used to lyse the bacteria and release the intracellular contents. This would also be a natural way of preserving the product.

2.6 Bacteriocin

Lactic acid bacteria produce a wide variety of antimicrobial proteins including peptide antibiotic, antibiotic-like substances, bacteriocins and bacteriocin-like substances for the inhibition of food-borne pathogens and spoilage organisms (Shah and Dave, 2002). Among the antibiotic like substances, nisin is well characterised. Tagg *et al.* (1976) defined bacteriocins as 'proteinaceous compounds that show antimicrobial activity against closely related species'. This definition holds true for the majority of bacteriocins, however it is now evident that bacteriocins may act beyond closely related species or those confined to the same ecological niche (Shah and Dave, 2002).

Bacteriocins share a number of characteristics and many studies on antimicrobial proteins produced by lactic acid bacteria frequently cite these criteria. There are six criteria suggested by Tagg *et al.* (1976) however these should not be used as inflexible criteria as it has become increasingly clear that few antimicrobial proteins fit all six criteria (Hoover and Steenson, 1993). The six criteria include

- Bacteriocins must be proteins
- Bacteriocins must be bactericidal
- Bacteriocins must have specific binding sites
- Bacteriocins must be plasmid mediated
- Bacteriocins must be produced by lethal biosynthesis

- Bacteriocins must be active against a narrow spectrum of closely related bacteria

Many of these criteria are not essential in the definition of bacteriocins. There are many bacteriocins that are not produced by lethal biosynthesis as they are produced as proteins in the growth phase without the lysis of the producing organism. Klaenhammer (1993) concluded that there are only two true requisites for bacteriocins: their proteinaceous nature and their lack of lethality to cells, which produce them.

Biochemical and genetic studies of bacteriocins produced by lactic acid bacteria have now defined four major classes (Klaenhammer, 1993). Class I bacteriocins are membrane-active and heat stable peptides. They contain lantibiotics that are small ribosomally synthesised polypeptides containing modified amino acids such as lanthionine and 3-methyl-lanthionine (Shah and Dave, 2002; Marth and Steele, 1998). Nisin produced by *Lactococcus lactis* subsp. *lactis* is the most prominent lantibiotic (Hoover and Steenson, 1993). Class I bacteriocin peptides undergo post-translational modifications.

Class II bacteriocins are small hydrophobic peptides which are moderately heat stable (Hoover and Steenson, 1993). This class does not contain unusual amino acids such as lanthionine (Marth and Steele, 1998). To date, many bacteriocins belonging to class II have been identified and characterised such as Lactacin F and B, Lactocin 27, Carnobacteriocins, Brevicin 37 Pediocin PA-1, Sakacin P, Curvacin A and Enterocin A (Shah and Dave, 2002; Hoover and Steenson, 1993; Marth and Steele, 1998). Class II bacteriocin peptides do not undergo post-translational modifications. Class II bacteriocins have two sub-groupings: class IIa bacteriocins are effective against Listeria and thus have potential as antimicrobial agent in food and feed. The majority of bacteriocins produced by *L. acidophilus* are heat stable, low molecular mass, non-lanthiobiotic peptides, which belong to class II.

Class III bacteriocin contain large heat labile peptides. There appear to be numerous members representing this class among the lactobacilli, including helveticin J, acidophilucin A, lactacin A and B and caseicin 80 (Hoover and Steenson, 1993).

Class IV bacteriocins are complex bacteriocins formed by the association of bactericidal proteins with one or more other essential chemical moieties (Shah and Dave, 2002).

Most bacteriocins produced by LAB have narrow antibacterial spectrum confined to species related to producer organisms, whereas some bacteriocins are active against *Listeria* sp. and other food-borne pathogenic and spoilage organisms. Most bacteriocins are hydrophobic and hence can be bound by lipids and phospholipids (Shah, 2002).

The antimicrobial activity of bacteriocins is due to increases in the permeability of the cytoplasmic membrane of target cells causing the dissipation of the proton motive force or disturbing membrane transport and thus inhibiting energy production and biosynthesis of proteins. The mechanism of action of nisin involves binding to the peptidoglycan layer, causing destabilisaton of the membrane by the formation of pores which allow leakage of ions such as a potassium and magnesium and dissipation of the proton motive force (Shah and Dave, 2002).

2.6.1 Bacteriocins produced by Lactic acid bacteria

2.6.1.1 Bacteriocins produced by L. acidophilus

Among lactobacilli *L. acidophilus* has been regarded as a good candidate for use as a dietary adjunct. The chemical nature and structure of antibacterial substances produced by *L. acidophilus* have been studied.

Dave and Shah (1997b) and Joseph *et al.* (1998) have shown that some strains of *L. acidophilus* produce bacteriocins against *L. delbrueckii* subsp. *bulgaricus*. In a study by Dave and Shah (1997b), *L. acidophilus* (LA-1) produced bacteriocin against seven strains of *L. delbrueckii* subsp. *bulgaricus* (2501, 2505, 1515, 2517, 2519, LB-3 and LB-4), one strain each of *L. casei*, (2603), *L. helveticus* (2700) and *L. jugurti* (2819). To date several *L. acidophilus* strains have been studied for the production and isolation of bacteriocins (Barefoot and Klaenhammer, 1984; Reddy *et al.*, 1983; Toba *et al.*, 1991) and to study their antimicrobial effects against various pathogens.

Lactocidin has been identified and purified by chromatography on a silicic acid column from an active fraction in the acid soluble fraction of cultures of *L. acidophilus*. Lactocidin has a broad antibiotic spectrum against Gram negative and Gram positive bacteria (Shah and Dave, 2002).

Barefoot and Klaehammer (1983) and Barefoot *et al.* (1994) observed Lactacin B which is produced by *Lactobacillus acidophilus*. It is heat stable and only detected in cultures maintained at pH 5.0 to 6.0. The bacteriocin was found to inhibit *L. delbrueckii* subsp. *bulgaricus, L. helveticus and L. lactis*. It was also observed that lactacin B only demonstrated antagonism against lactobacilli.
Shah and Dave (1999) investigated the bacteriocin acidophilicin, which is produced by *L. acidophilus* LA-1. It was found to be heat stable and had a molecular mass of 54kDa. This bacteriocin was isolated and purified using a two-stage fractionation with ammonium sulfate. It was also found that bacteriocin production was affected by pH but showed activity over a wide range of temperatures.

2.6.1.2 Bacteriocins produced by bifidobacteria

Bifidobacteria have a higher antibacterial activity compared to lactobacilli. However, most bifidobacteria do not produce any antibacterial substances other than lactic acid and acetic acid. Only few reports are available on the nature of the antimicrobial activity of bifidobacteria and studies on the chemical nature and structure of antibacterial substances produced by bifidobacteria are still in infancy stage (Shah and Dave, 2002).

Yildirim and Johnson (1998) and Yildirim *et al.* (1999) reported on the Bifidocin B a bacteriocin produced by *Bifidobacterium bifidum* NCFB 1454. This bacteriocin was found to be resistant to organic solvents and heat, and showed activity after storage at -20°C and -70°C for 3 months. With a molecular mass of about 3.3 kDa Bifidocin B was active against some food-borne pathogens and food spoilage bacteria such as *Listeria, Enterococcus*, *Bacillus, Lactobacillus Leuconostoc* and *Pediococcus* species. The bacteriocin was active against Gram positive bacteria but not Gram negative bacteria.

Anand *et al.* (1984) and Anand *et al.* (1985) reported a bacteriocin named bifidin, which is produced by *B. bifidum* 1452. This bacteriocin was found to be heat stable at 100°C for 30 min and inhibited the growth of *E. coli, Bacillus cereus, Staphylococcus aureus, Micrococcus flavus* and *Pseudomonas fluorescens*.

2.6.1.3 Bacteriocins produced by Lactococcus and Pediococcus

There are many bacteriocins that are produced by *Lactococcus* and *Pediococcus*. The best characterised and have found ways in food application are nisin and pediocin. Nisin is produced by *Lactococcus lactis* subsp. *lactis* and is one of the most studied bacteriocin. Nisin was discovered in 1928 and has received commercial application in the food industry (Shah and Dave, 2002). It is used in processed cheese, hard cheese, milk, yoghurt, cottage cheese, bacon and smoked fish (Marth and Steele, 1998; Shah and Dave, 2002). Nisin is heat stable and can be added before heat processing or canning of foods and as it is a polypeptide any residues remaining in foods are digested. It can inhibit *Bacillus subtilis, Salmonella typhimurium, E. coli, C. sporogenes, C. tyropbutyricum Listeria Staphylococcus, Lactobacillus, Micrococcus, Pediococcus* and *Leuconostoc* (Hoover and Steenson, 1993). Nisin causes cellular death by affecting cytoplasmic membrane and proton motive force (Marth and Steele, 1998; Shah and Dave, 2002; Hoover and Steenson, 1993). It is part of class I as it contains the amino acids lanthionine and is therefore called a lantibiotic (Marth and Steele, 1998).

Pediocin is produced by *Pediococcus pentosaceus* (previously known as *Pediococcus cerevisiae*) and *Pediococcus acidilactici* has also been reported. These bacteria inhibit growth of some strains of Gram positive bacteria including *Pediococcus, Lactobacillus, Leuconostoc* and *Bacillus* (Marth and Steele, 1998; Shah and Dave, 2002; Hoover and Steenson, 1993). The inhibitory substance produced by *P. pentosaceus* is known as pediocin A and that produced by *P. acidilactici* is called pediocin AcH (Hoover and Steenson, 1993). Both pediocins have been used in food systems such as the production of soft cheeses to control the growth of *Listeria monocytogenes* (Marth and Steele, 1998; Shah and Dave, 2002).

2.6.2 Applications of bacteriocins

There is a tremendous interest in bacteriocins and their role as a preservative in minimally processed foods. The discovery of psychrotrophic pathogens such as *Listeria monocytogenes* that grow at refrigeration temperatures has cast doubt over the safety of minimally processed refrigerated foods (Hoover and Steenson, 1993). Bacteriocins have been found to inhibit food-borne pathogens such as *Listeria* and *Clostridium botulinum* and as bacteriocins are protein and "natural", this will satisfy consumer demand for "fresh" and "preservative free" products (Montville and Kaiser, 1993).

Today only nisin has found practical application and is currently being used worldwide. Pediocin has also received industrial attention for control of *Listeria.* The bacteriocins of *Lactobacillus acidophilus* have received much attention and could possibly be another dietary adjunct. The bacteria is currently added to yoghurt for their health promoting benefits and to date several *L. acidophilus* strains have been studied for the production and isolation of bacteriocin and their antimicrobial effects against various pathogens. Such *L. acidophilus* strains (e.g. LA-1) could be used to lyse *L. delbrueckii* subsp. *bulgaricus* to release intracellular enzymes in order to catalyse the production of essential peptides and amino acids. This would then act as growth factors for *S. thermophilus, L. acidophilus* and *Bifidobacterium* i.e., *in situ* production of growth factors without post-acidification. Morgan *et al.* (1997) studied the effect of increasing starter cell lysis in cheddar cheese using a bacteriocin-producing adjunct. Cheeses manufactured with the bacteriocin-producing adjunct exhibited increased cell lysis, elevated concentrations of free amino acids and higher sensory evaluation scores than cheese manufactured without the adjunct.

3.0 MATERIALS AND METHOD

3.1 Bacterial Strains

Pure cultures of *Streptococcus thermophilus* 2014, *Bifidobacterium infantis* 1912 (VUP 13518) and *Bifidobacterium longum* 1941 (VUP 13514) were obtained from Victoria University Culture Collection. *Lactobacillus acidophilus* (LA-5, 2504, 2505, 2506) and *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515 and 2510 were obtained from Chr. Hansen Pty. Ltd. (Bayswater, Australia). Two freeze dried commercial cultures "Robust" and "Mild" were obtained from National Foods Ltd. The strain numbers have been concealed for confidential reasons. "Robust" contains a *Streptococcus thermophilus* and very robust *Lactobacillus delbrueckii* subsp. *bulgaricus* culture. "Mild" contains a *Streptococcus thermophilus* and a mild *Lactobacillus delbrueckii* subsp. *bulgaricus*.

3.2 Maintenance of Microorganisms

Working cultures were maintained in 12% reconstituted skim milk (RSM) supplemented with 1% glucose and 0.5% yeast extract (RGY) or in DeMann, Rogosa and Sharpe broth (MRS broth). *Bifidobacterium* required 1% L-cysteine as well.

All lactic acid bacteria were stored as liquid stock cultures in RSM supplemented with glucose (1%) and yeast extract (0.5%). Aliquots were taken and mixed with 20% glycerol and stored in cryovials at -80°C and -20°C until required. When required, a vial was thawed and grown for 24-48 hours. Three transfers were carried out before bacteria were used and bacteria were grown for 18 hours after each transfer. After 20 transfers a fresh culture was taken from original frozen stock culture to avoid changes in morphology.

3.3 Media preparation

3.3.1 Peptone water

Peptone water (0.15%) was prepared by dissolving 1.5g of peptone medium (Oxoid, Australia) in 1 litre of distilled water and dispensing 9 mL aliquots into McCartney bottles followed by autoclaving at 121°C for 15 minutes at 15 psi. Sterile peptone water was stored at room temperature.

3.3.2 MRS agar and broth

MRS broth was used for growing *L. acidophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus.* MRS broth and 1% L-cysteine hydrochloride was used to grow bifidobacteria. To prepare MRS agar (Oxoid, Australia) 10g of bacteriological agar (Oxoid, Australia) was added to the broth, which was sterilised at 121°C for 15 minutes at 15 psi.

3.3.3 Selective medium for *L. acidophilus*

For selective enumeration of *L. acidophilus,* MRS-sorbitol agar was used. This was based on the method developed by Lankaputhra and Shah (1996a). To prepare MRS-sorbitol agar, 26g of MRS broth without carbohydrate (Amyl media), 40 mL of MRS supplement (Amyl media) and 10g of agar were mixed and 900mL of water was added. This was then autoclaved at 121°C for 15 minutes at 15 psi. A 10 % sorbitol (Oxoid) solution was made and 100 mL was filter sterilised into 900mL of MRS agar (no carbohydrates) in a laminar flow. Once pouring was complete these plates were incubated anaerobically at 37°C for 72 hours.

3.3.4 Selective medium for *Lactobacillus delbrueckii* subsp. *bulgaricus*

For selective enumeration of *Lactobacillus delbrueckii* subsp. *bulgaricus,* MRS agar at pH 5.2 was used. To prepare this medium, 52g of MRS broth (Oxoid) and 10g of bacteriological agar were mixed with 1 L of water. When dissolved, the pH was

adjusted to 5.2 using 5M NaOH. This was autoclaved at 121°C for 15 minutes at 15 psi. After pouring these plates were incubated anaerobically at 42°C for 72 hours.

3.3.5 Selective medium for *S. thermophilus*

For selective enumeration of *S. thermophilus,* M17 agar was used. To prepare M17 agar, 23g of M17 broth was mixed with 10g agar, 50mL of M17 supplement and 950 mL of distilled water. It was autoclaved at 121°C for 15 minutes and 15 psi and once cooled and poured these plates were incubated aerobically at 37°C for 24 hours.

3.3.6 Selective medium for bifidobacteria

For selective enumeration of bifidobacteria MRS- NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate) (Sigma Chemicals Company) was used. To prepare MRS-NNLP agar, 52g of MRS broth was mixed with 10g of agar and 1L of water. After autoclaving (121°C for 15 minutes and 15 psi) and cooling to 45°C, filter sterilized 1% NNLP and 1% L-cysteine hydrochloride were added. After pouring and setting, plates were incubated anaerobically at 37°C for 72 hours.

3.3.7 Preparation of serial dilution for spread and pour plating

One gram of sample was added to 9mL of 0.15% peptone water and vortexed. Then 1mL of this dilution was transferred to a second bottle of 9 mL of 0.15% peptone water and series of ten-fold dilutions were prepared (10^3 to 10^7). Enumeration was carried out using the pour plate technique or spread plate technique. Duplicate plates were then incubated at appropriate temperatures. Plates containing 25 to 250 colonies were counted and recorded as log of colony forming units per gram of sample.

3.4 Yoghurt Preparation

3.4.1 General yoghurt making

All yoghurt was made as follows with various changes, which will be explained, later. The process for yoghurt making is shown in Figure 2. Milk for yoghurt making was made by dissolving 12% reconstituted skim milk powder in distilled water. This was heated to 85°C for 30 minutes. The milk was allowed to cool to 45°C and then inoculated with starter culture. The inoculated milk was then poured into100mL cups and incubated at 42°C. The pH was measured every 2 hours until a pH of 4.5 was reached. The yoghurt was then removed and stored at 4°C for 4-6 weeks for storage trials.

3.4.2 Yoghurt making using commercial cultures

Two freeze dried commercial cultures "Robust" and "Mild" were obtained from National Foods Ltd. The "Robust" culture was added at the rate of 14g per 500L while the "Mild" culture was added at the rate of 17.2g per 500L. The yoghurts were made the same as described in Section 3.4.1 and were stored at 4°C for 4 weeks. The viability of yoghurt bacteria and the pH changes were assessed during fermentation and storage.

3.4.3 Yoghurt made with commercial culture and *L. acidophilus* LA-5 and *Bifidobacterium infantis* 1912

The culture was diluted first in 100 ml of 12% RSM for better dispersion and were added at the same rate as in 3.4.2. Probiotic bacteria, *L. acidophilus* LA-5 and *Bifidobacterium infantis* 1912 were grown for 18 hours in RSM supplemented with glucose and yeast extract and added at a rate of 1%. The yoghurts were made as described in section 3.4.1 and were stored at 4°C for 4 weeks. The viability of yoghurt bacteria and probiotic bacteria was assessed during fermentation and storage and changes in pH were monitored.

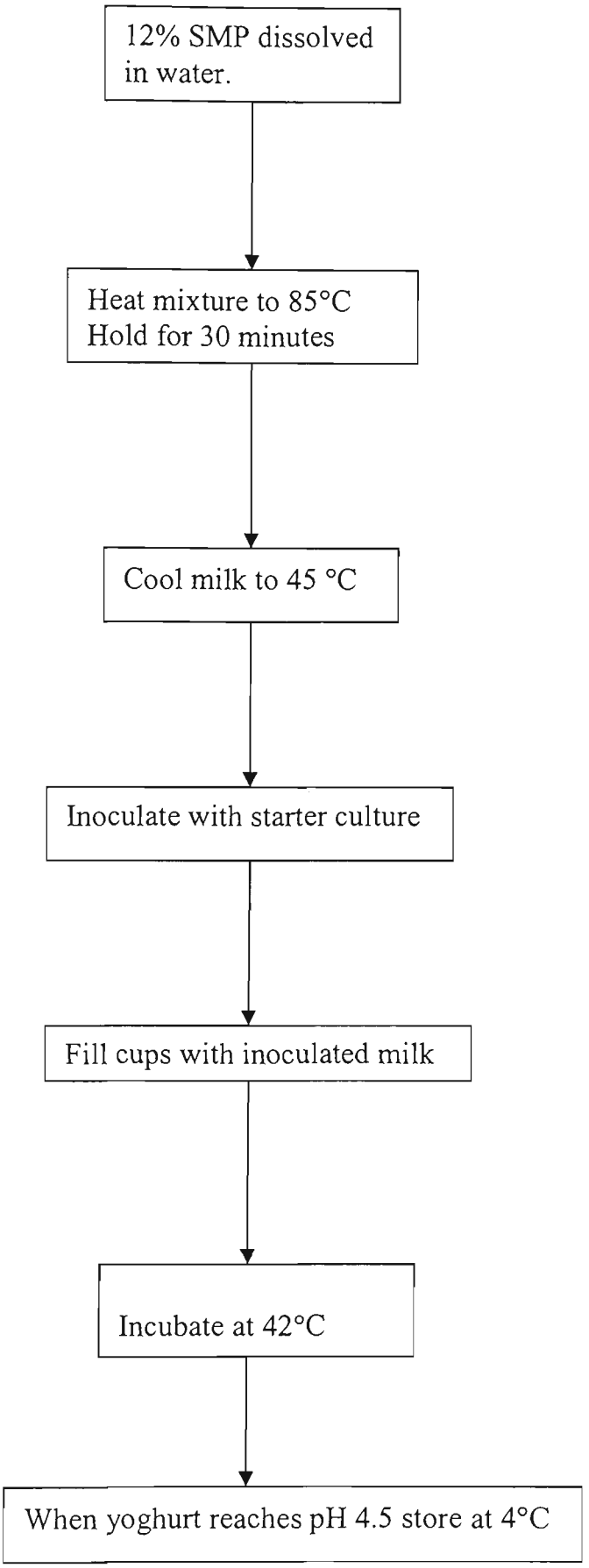

Figure 2. Standard procedure for preparation of yoghurt.

3.4.4 Yoghurt made with commercial bacteria and *L. acidophilus* and *Bifidobacterium longum* 1941

This yoghurt was prepared as described in section 3.4.3 with *Bifidobacterium longum* 1941 replacing *Bifidobacterium infantis* 1912. Yoghurts were stored at 4°C for 6 weeks. The viability of yoghurt and probiotic bacteria and pH change were assessed during manufacture and storage.

3.4.5 Yoghurt made with sonicated *Lactobacillus delbrueckii* subsp. *bulgaricus*

Lactobacillus delbrueckii subsp. *bulgaricus* was grown in MRS broth at 42°C for 18 hours, centrifuged and cells were collected and suspended in sterile Milli Q water. The cells were washed and centrifuged three times. The final volume was made up to 10mL. A Branson Sonifier 450 (Branson Ultrasonics Corporation, Eagle Road, Danbury, CT, USA) was used to sonicate the cells. The sonicator was set for 50% output and samples were sonicated for 4 minutes, with a period of 30 seconds for cooling after each minute of sonication. The samples were kept in an ice bath and the temperature did not rise above 15°C.

A control yoghurt was made using unsonicated cultures of *S. thermophilus* 2014, *L. acidophilus* LA-5, *B. infantis* 1912 and *Lactobacillus delbrueckii subsp. bulgaricus* 2515 at a rate of 1% each. The experimental yoghurt contained *S. thermophilus* 2014, *L. acidophilus* LA-5, *B. infantis* 1912 and sonicated *Lactobacillus delbrueckii subsp. bulgaricus* 2515 at a rate of 1% each. The yoghurts were made as described in section 3.4.1 and were stored at 4°C for 4 weeks. The viability of yoghurt and probiotic bacteria and pH change were assessed during manufacture and storage.

3.4.6 Yoghurt made with bacteriocin

Yoghurt was prepared as described in section 3.4.1 with the following modifications. The control batch was prepared as above, while two experimental batches were made that included the same bacteria plus 1% and 2% concentrated bacteriocin respectively.

The viability of yoghurt and probiotic bacteria and pH change were assessed during manufacture and storage.

3.5 Time interval specification

The "0 h" time represents the observations taken immediately after the addition of starter culture in the milk. The "end of fermentation" represents the observations taken after yoghurt reached pH 4.5 before transfer into cold storage. The "Week 1", "Week 2", etc. represent the analysis of yoghurt after that many weeks of storage.

3.6 Analyses

3.6.1 pH

The pH values of the yoghurt and media were measured at 20°C using a HI 8417 pH meter (Hanna Instruments, New South Wales, Australia). Calibration was done daily using fresh pH 4.0 and 7.0 standard buffers.

3.6.2 OD readings

Optical density was measured using a Pharmacia spectrophotometer (LKB Biochrom, England) at 620nm.

3.6.3 Organic acid determination using HPLC

The organic acids analysed included lactic acid, acetic acid, orotic acid, propionic acid, butyric acid, formic acid and uric acid. The acids were quantified using High Performance Liquid Chromatography (HPLC, Varian Australia Pty. Ltd., Mulgrave, Australia) according to the modified method by Scalabrini *et al.* (1998) and Shin *et al.* (2000) with some modifications.

For extraction of acids, 5g of yoghurt samples were diluted with 4.93mL of 0.009N H_2SO_4 and 70µL of 15.8M HNO_3 was added. This was mixed and digested for 30

minutes. A 1ml aliquot was centrifuged at 14,000 rpm (5415C centrifuge, Crown Scientific, Germany) for 10 minutes. The supernatant was pipetted off and placed into a HPLC vial and capped. Samples were stored at 4°C until required.

Standards of each acid were made using distilled water except for orotic and uric acids, which were dissolved in 0.1M NaOH and measured using the HPLC. A standard curve was produced from the area obtained. Single standard and a mixed standard were also injected to help determine retention time. The retention time for orotic acid 7.9-8.1 min, lactic acid was 12.8-13.1 min, formic acid 13.2-13.6 min, uric acid 14.2-14.7 min, acetic acid 14.8 min, propionic acid 17.4-18.0 min and butyric acid 21.0-21.7 min. The standard curve regression coefficients were 1 for lactic, acetic, propionic, butyric and formic acids and 0.9998 for orotic acid and 0.9996 for uric acids.

The levels of organic acids were measured using a Aminex HPX-87H ion exchange column with a SSI 505LC-column oven at 65°C. A Varian 9100 autosampler, a Varian 9012 solvent delivery system and a Varian variable wavelength UV-Vis 9050 detector was used. Varian Star software (Varian, Australia) was also used. Detection was carried out at 210nm with a sample injection rate of 50uL and a run time of 30 minutes. The mobile phase was 0.009N H_2SO_4 with a flow rate of 0.6mL per minute.

3.6.4 Assay of β-galactosidase

To determine the enzyme activity, a modified version of the method by Shah and Jelen, (1990) was used. Solutions of 0.005M o-nitrophenyl-β-D-galactopyranoside (ONPG) were prepared with 0.1% phosphate buffer at pH 7, and 250µL of sample culture was incubated with 1.25mL of ONPG solution for 15 min at 37°C. The reaction was stopped by adding 1mL of 1M cold sodium carbonate. The amount of o-nitrophenyl released was measured with a spectrophotometer at 420nm against a blank consisting of 1.25mL ONPG, 250µL MRS broth no culture and 1mL of 1M sodium carbonate. The unit of lactase activity was estimated as the enzyme which liberated one µmole o-nitrophenol from ONPG per min per gram samples at 37°C.

3.6.5 Microbiological analysis

The pour plate method as mentioned in section 3.3.7 was used to determine viability of yoghurt and probiotic bacteria. Selective enumeration was used as described in sections 3.3.2 to 3.3.6.

3.7 Detection and assay of inhibitory activity

The detection of inhibitory activity was done by the spot on lawn technique (Dave, 1997). Twenty-five millilitres of 1% agar was poured into a sterile petri dish. When set, wells were cut using the end of a cut 6mm pipette. The bottom of the wells was sealed with sterile 0.9% agar. Fifty microlitres of an active bacteriocin producing organism were put in the wells. The plates were left for 2 hours for diffusion. The wells were then filled with 1% agar and then overlaid with approximately 10mL of 0.9% agar seeded with 1% indicator organism. Plates were incubated at 37°C for 24 hours. If any inhibitory substances were present, a clearing zone formed around the wells.

The nature of the inhibitory substance produced was determined by the well-diffusion technique (Dave, 1997). Agar (0.9%) was cooled to 45°C after autoclaving. This agar was inoculated with 1% of active indicator bacteria and 25mL of it was poured into a petri dish and wells were cut in the solidified agar. The producer organism was centrifuged (4500 rpm, 12 minutes, 4°C) (5415C Centrifuge, Crown Scientific, Germany) and the supernatant was filter sterilised using a 0.45μm membrane. The supernatant was divided into three portions for different treatments. The first portion was untreated, the second portion was neutralised to pH 6.0 using 5M NaOH and the third portion was neutralised (pH 6.0) and treated with catalase (final concentration 0.05-0.1μg/mL) and incubated for 2 hours in a 37°C water bath. Fifty microlitres of each sample was pippetted into wells and left for 2 hours for diffusion. Plates were incubated at 37°C for 24 hours.

The purpose of neutralising and treating with catalase was to remove the inhibitory effects caused by acid and hydrogen peroxide. If a zone still appeared after these

treatments then the next step was taken to determine if the inhibitory substance was a bacteriocin.

Plates were prepared with 0.9% agar that had been seeded with 1% indicator bacteria. When solidified, 6mm wells were cut as before. The producer organism was centrifuged and filtered as above to obtain a cell free extract. The supernatant was neutralised (pH 6.0) and treated with catalase and one of the following enzymes, trypsin (final concentration 1mg/mL), papain (0.5mg/mL), proteinase K (0.2mg/mL) and crude protease (1mg/mL). The treated samples were incubated for 2 hours at 37°C. Fifty microlitres of sample was added to the wells and left to diffuse for 2 hours and then incubated at 37°C for 24 hours. If no zone appeared, it was determined that the inhibitory substance was sensitive to proteolytic enzymes. This would confirm that a bacteriocin was present, as it is proteinaceous in nature.

3.8 Concentrating and Purification of Bacteriocin

3.8.1 Concentration bacteriocin using ultra-filtration

L. acidophilus was grown for 18 hours in MRS broth at 37°C. This was centrifuged at 5000rpm for 15min at 4°C (Beckman J2-HS centrifuge, Beckman Instruments Inc., Palo Alto, CA, USA) to remove cells. The supernatant was neutralised to pH 6.0 and was concentrated using a ultra-filtration (UF) unit with molecular weight cut off (MWCO) of 30kDa.

A Vivaflow 50 unit (Vivascience, Germany) was used to filter MRS broth for samples less than 5L. Two Vivaflow 200 units were used for samples between 5L and 10L. These units were used with a Millipore pump (Millipore Corporation, MA, USA) and an Easy load head (Masterflex, USA). The pump was kept constant at 100kPa.

The concentration ratio for samples less than 5L was approximately 20 and for samples more than 5L was approximately 50.

3.8.2 Purification of bacteriocin

The bacteriocin was fractionated with ammonium sulphate according to the method of Dave and Shah (1997). A 40% saturation was used with 243g of ammonium sulphate added to 1L of liquid. Ammonium sulphate was added slowly and each addition of salt was made only after the previously added amount had completely dissolved. This was then left to stir for 2 hours at 4°C. The mixture was then centrifuged (Beckman J2-HS centrifuge, (Beckman Instruments Inc., Palo Alto, CA, USA) at 8000 rpm for 10 minutes. The precipitate was removed from the supernatant and weighed for yield determination. The precipitate was then dissolved in 0.1M sodium citrate buffer (pH 6.0) at a rate of 0.1g of protein per millilitre of sodium citrate buffer. This solution was dialysed using 14000-16000 MWCO dialysis tubing against 0.001M sodium citrate buffer (pH 6.0) for 2 days at 4°C. The bacteriocin solution was then autoclaved at 121°C for 15 minutes and was stored at 4°C for immediate use or -20°C for storage.

3.9 Lysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* in different media

3.9.1 Lysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* in MRS broth

Lactobacillus delbrueckii subsp. *bulgaricus* was grown with different levels of bacteriocin in MRS broth. The levels of bacteriocin used were 1%, 5% and 10% and these were grown with 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*. A control of 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* without bacteriocin was also used. Once the MRS broth was inoculated, they were incubated for 10 hours at 42°C. Samples were taken at hours 0, 6 and 10 to measure the viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus*.

The similar experiment was conducted except, this time lower levels of bacteriocin were used. The levels of bacteriocin used were: 0.5%, 1% and 2% and these were grown with 1% *Lactobacillus delbrueckii* subsp. *bulgaricus*. A control of 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* without bacteriocin was again used. Once the MRS broth was inoculated, it was incubated for 8 hours at 42°C and samples

were taken every 2 hours to measure the viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus*.

3.9.2 Lysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* in RSM

This experiment is similar to the method described in section 3.9.1. The levels of bacteriocin used were: 1%, 5% and 10% and these were grown with 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*. A control of 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* was also made. Once the 12% RSM was inoculated and incubated at 42°C for 8 hours, samples were taken every 2 hours to measure viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus*.

3.9.3 Lysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* in RSM without casein

Three percent RSM was prepared and the casein was removed according to the method by Uemura *et al.* (1998). Hydrochloric acid (4M) was added to the 3% RSM until the pH reached 4.5 where casein precipitated. The RSM was then centrifuged at 13000 rpm for 10 min and the supernatant was separated and neutralised to pH 6.5. The casein free medium was then autoclaved at 121°C for 15 minutes.

3.10 Growth curves of different strains of *Lactobacillus acidophilus*

Four different strains of *L. acidophilus* were grown in MRS broth over 18 hours at 37°C. The bacteria were inoculated using the formula

$$\frac{0.1 \times \text{no. of mL}}{\text{OD reading of active culture}}$$

After inoculation, the samples were placed in a 37°C incubator and OD readings and plate counts were taken every 2 hours beginning at 0 hour and ending at 18 hours. OD readings were measured using a spectrophotometer at 620nm.

3.11 Sources of chemicals, reagents and microbiological media

3.11.1 Chemicals and reagents

All chemicals and reagents were obtained from Sigma-Aldrich Chemicals (Australia).

3.11.2 Microbiological media

All microbiological media were obtained from Oxoid Ltd. (Hampshire, England) or from Amyl-Media Pty. Ltd. Dandenong, Australia.

3.12 Equipment and Instruments

3.12.1 Anaerobic jars

Anaerobic jars with forty two plate capacity and catalysts were obtained from Oxoid. H_2 and CO_2 generating sachets (Oxoid) were used to create an anaerobic environment.

3.12.2 pH

The pH of yoghurt and media was measured using a HI 8417 pH meter (Hanna Instruments, New South Wales, Australia).

3.12.3 Centrifuge

Beckman J2-HS centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) was used for centrifuging large samples of about 10-1000mL. For volumes smaller than 2mL, a 5415C centrifuge (Crown Scientific, Germany) was used. For samples between 2-50mL and requiring an rpm of less than 4000 a Sorvell RT7 bench top centrifuge (Dupoint) was used.

4.0 RESULTS AND DISCUSSION

4.1 The effects on probiotic bacteria in yoghurt when grown with Commercial yoghurt strains

4.1.1 Growth characteristics of two different commercial strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus.*

Two commercial strains of yoghurt were obtained from National Foods Ltd. and yoghurt was made according to the method described in section 3.4.2. The results are shown in Table 2. The yoghurt inoculated with the "Mild" culture took 9 hours to ferment and the yoghurt inoculated with the "Robust" culture took 6.5 hours. The Mild yoghurt had a much lower count of *Lactobacillus delbrueckii* subsp. *bulgaricus* than the Robust yoghurt in the initial stage of fermentation but did increase by 2 log during fermentation. The Robust yoghurt increased by 3 log. The counts of *S. thermophilus* were similar in both yoghurts but the Mild yoghurt had a higher count at the end of fermentation due to the longer fermentation time.

During storage the viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the Mild yoghurt decreased slightly in the week 1 but were stable up to week 4. The *S. thermophilus* counts were also stable throughout the storage period. The pH of the Mild yoghurt decreased by 0.1 in the first week of storage and then remained stable for the next three weeks.

The viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the Robust culture increased in the first week of storage, which is normal, as this bacteria can grow at lower temperatures. The counts then decreased slightly during week 2 and week 4 of storage but remained in the same log scale. The counts of *S. thermophilus* increased during storage but also stayed in the same log scale. The pH of the Roust yoghurt decreased considerably in the first week of storage and dropped again during the second week. It, however, did increase in week 4.

The Robust culture had a higher viable count of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the initial inoculation which is why the fermentation time was less than that for Mild bacteria. The pH of the Robust yoghurt was much lower during storage

than that for the Mild yoghurt which is possibly due to the higher population of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the Robust yoghurt. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in both yoghurts remained stable during storage, although the *Lactobacillus delbrueckii* subsp. *bulgaricus* in the Robust yoghurt decreased slightly and this could be due to the lower pH of the yoghurt. Dave and Shah (1997a) also observed a decrease in *Lactobacillus delbrueckii* subsp. *bulgaricus* during storage, which may have been due to a drop in pH.

4.1.2 The effect on probiotic bacteria in yoghurt when fermented with commercial bacteria

Lactobacillus acidophilus LA-5 and *Bifidobacterium infantis* 1912 were inoculated with commercial bacteria in yoghurt as described in section 3.4.3. The results of viable counts and pH are presented in Tables 3 and 4. The Mild yoghurt took 7 hours to ferment and the Robust yoghurt took 5 hours. The Mild yoghurt had a higher population of *S. thermophilus* than the Robust yoghurt at the beginning of fermentation but at the end of fermentation the Robust yoghurt had higher *S. thermophilus* counts. There was a slightly higher viable count of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the Robust yoghurt and this increased by 2 log at the end of fermentation. The variable count in the Mild yoghurt increased 1 log scale during incubation.

The probiotic bacteria viable count was higher in the Mild yoghurt than in the Robust yoghurt at the end of fermentation. This would be due to the longer incubation time for the Mild yoghurt. The inoculation size of the bifidobacteria in the Robust yoghurt was lower than that for the Mild yoghurt which could be why this bacteria did not increase in number as much as the Mild yoghurt did.

The storage trial results over 4 weeks are presented in Table 4. The pH of the Mild yoghurt was stable during the storage trial with a slight drop at week 3. The pH of the Robust yoghurt decreased during week 1 to week 3 but went up again at week 4.

The count of *S. thermophilus* decreased slightly in both yoghurts during storage and had very similar counts at the end of the storage periods. The *Lactobacillus*

delbrueckii subsp. *bulgaricus* viable counts in the Mild yoghurt decreased until week 3 when there was a 1 log increase and in week 4 the counts decreased again. It is not known what caused this increase in week 3. The *Lactobacillus delbrueckii* subsp. *bulgaricus* in the Robust yoghurt decreased throughout the storage trial with a 1 log drop in week 4.

In the Mild yoghurt, the *L. acidophilus* was stable until week 3 when the population decreased slightly and then decreased over 1 log scale in week 4. The bifidobacteria increased nearly 2 log during the 4 weeks of storage. This was unexpected as probiotic bacteria have problems with viability in yoghurt but the Mild bacteria may have improved the environment for the bifidobacteria. In the Robust yoghurt, the *L. acidophilus* decreased 3 log over the 4 weeks with a sharp drop at week 4. The bifidobacteria fluctuated slightly during the storage trial.

In general, the Mild yoghurt had a higher population of probiotic bacteria than the Robust culture. This is possibly due to the milder *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteria and the higher pH in the Mild yoghurt. The pH drop in the Robust yoghurt is higher and the "Robust" culture is possibly creating a difficult environment for the probiotic bacteria to survive. Dave and Shah (1997a) observed in some commercial strains that *L. acidophilus* remained well within the recommended limit throughout a 35 day storage period when *Lactobacillus delbrueckii* subsp. *bulgaricus* was absent from fermentation. When *L. acidophilus* was fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* viability of *L. acidophilus* was lost after 20 days. They also observed that bifidobacteria was not as affected by *Lactobacillus delbrueckii* subsp. *bulgaricus* as *L. acidophilus* was and the bifidobacteria remained above the recommended limit for 35 days of storage.

*4.1.2.1 Organic acid production in yoghurt when fermented with commercial yoghurt strains and probiotic bacteria (*L. acidophilus *LA-5 and* B. infantis *1912).*

The organic acid content of the yoghurts made with commercial yoghurt strains and probiotic bacteria (*L. acidophilus* LA-5 and *B. infantis* 1912) is presented in Tables 5 and 6. There was no acetic acid produced in either yoghurt as the bifidobacteria was

only fermented for no more than 7 hours and it requires over 12 hours to produce acetic acid.

The lactic acid production in both yoghurts increased during fermentation and during storage. The Robust yoghurt produced significantly more lactic acid than the Mild yoghurt, which would be due to the higher *Lactobacillus delbrueckii* subsp. *bulgaricus* count in the Robust yoghurt. Dave and Shah (1997a) also observed an increase in lactic acid concentration during storage in yoghurts grown with *Lactobacillus delbrueckii* subsp. *bulgaricus, S. thermophilus* and probiotic bacteria and without *Lactobacillus delbrueckii* subsp. *bulgaricus*.

In the Robust yoghurt butyric acid was found at the end of fermentation and increased in the first week of storage and then remained stable. There was no butyric acid produced in the Mild yoghurt.

Formic acid was found at the beginning of fermentation in both yoghurts. In the Mild yoghurt, the formic acid concentration increased during fermentation and then decreased during storage. There was no formic acid detected in the Robust yoghurt after the initial sample. Formic acid is used as a growth factor for the starter bacteria and since the "Robust" culture contains quick growing *Lactobacillus delbrueckii* subsp. *bulgaricus* the formic acid would have been used up during fermentation (Walstra *et al.*, 1999). The "Mild" culture, however, is not as fast growing and may not require the same amount of formic acid.

The concentration of orotic acid was not significantly different and decreased slightly during fermentation and then remained constant throughout storage. Orotic acid is used as a growth factor by the yoghurt starter cultures, most probably by *Lactobacillus delbrueckii* subsp. *bulgaricus* (Tamime and Robinson, 1999).

There was no propionic acid produced in the Mild yoghurt but in the Robust yoghurt propionic acid was detected after fermentation that increased in the first week of storage and remained constant for the rest of the storage trial. Propionic acid is produced by the yoghurt starter bacteria as a volatile flavour compound (Tamime and

Robinson, 1999). It is possible that the "Mild" culture was not able to produce any propionic acid, as it was a fairly weak culture.

Uric acid is present in milk as a result of normal bovine biomedical processes and does not change during fermentation or storage (Navder *et al.*, 1990). This result is reflected in both yoghurts shown in Tables 5 and 6. The concentration remained between 32 and 35µg/g of yoghurt during fermentation and storage. The results were however significantly different in week 1 and 4.

4.1.2.2 Conclusion

This experiment has shown that the viability of probiotic bacteria is effected by the 2 different commercial cultures. The Mild culture appears to give a good environment for the bifidobacteria but not the *L. acidophilus*. The Robust culture does not provide a good environment for either bacteria as the viable counts were below the population required to give any health benefit (approx. $1x10^6$).

This experiment was repeated using a different strain of bifidobacteria, which is supposed to be more stable.

4.1.3 Probiotic bacteria in yoghurt using *Bifidobacterium longum* 1941

Lactobacillus acidophilus LA-5 and *Bifidobacterium longum* 1941 were inoculated with commercial bacteria in yoghurt as described in section 3.4.4. The results of viable counts and pH are presented in Tables 7 and 8. The Mild yoghurt took 7 hours to ferment and the Robust yoghurt took 5 hours. The Mild yoghurt had a higher population of *S. thermophilus* than that of the Robust yoghurt at the beginning of fermentation but at the end of fermentation the Robust yoghurt had higher *S. thermophilus* counts. There was a higher viable count of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the Mild yoghurt, however, there was a slight increase in population at the end of fermentation. This is possibly due to the weak nature of the bacteria. The viable count in the Robust yoghurt increased 2 log scale during incubation, as a result there was a shorter fermentation time.

The probiotic bacteria viable count was higher in the Mild yoghurt than in the Robust yoghurt at the end of the fermentation. This was also seen in the previous experiment. This would be due to the longer incubation time for the Mild yoghurt and the "Mild" starter culture. In the Mild yoghurt the *L. acidophilus* viable counts increased by 1 log and the *B. longum* viable counts increased by 2 log. In the Robust yoghurt, the *L. acidophilus counts* increased by 1 log and the *B. longum* only slightly increased in number.

The storage trial results are presented in Table 8. The pH of the Mild yoghurt decreased during storage but not as much as that of the Robust yoghurt. There was a slight increase in pH at week 6 but the reason for this is unclear.

The *S. thermophilus* remained constant in both yoghurts during storage and had very similar counts. The *Lactobacillus delbrueckii* subsp. *bulgaricus* counts in the Mild and Robust yoghurts decreased until week 3 when there was a 4 log decrease at week 4. There could have been a problem with the selective agar or a problem with the refrigerator as in week 5 the counts increased 2- 3log.

In the Mild yoghurt, the *L. acidophilus* viable count fluctuated for the first 4 weeks of storage and then dropped to 1.21×10^3. The *L. acidophilus* did not perform well in the Robust yoghurt and the populations decreased rapidly during storage. *B. longum* also fluctuated during storage in both yoghurts, particularly in the Robust yoghurt dropping to 3.3×10^4 at week 3 but then increased to 1.01×10^5. The Mild yoghurt finished the storage trial with a viable count for bifidobacteria of 1.65×10^5.

The Mild yoghurt had a higher population of probiotic bacteria than the Robust culture which was also seen in the previous experiment and again this is possibly due to the milder *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteria and the higher pH in the Mild yoghurt. The "Robust" culture is possibly creating a difficult environment for the probiotic bacteria to survive.

4.1.3.1 *Organic acid concentration in yoghurt when fermented with commercial yoghurt strains and probiotic bacteria (L. acidophilus LA-5 and B. longum 1941).*

The organic acid concentration of the yoghurts made with commercial yoghurt strains and probiotic bacteria (*L. acidophilus* LA-5 and *B. longum* 1941) are presented in Tables 9 and 10. There was no acetic acid produced in either yoghurt as the bifidobacteria was only fermented for no more than 7 hours and it requires over 12 hours to produce acetic acid. There was no butyric acid produced in the Mild yoghurt but in the Robust yoghurt butyric acid was found at the end of fermentation and increased throughout storage.

Formic acid was found in both yoghurts at the beginning of fermentation. In the Mild yoghurt the formic acid increased during fermentation and then decreased during storage. There was no formic acid detected in the Robust yoghurt after fermentation.

The lactic acid production in both yoghurts increased during fermentation and during storage. The Robust yoghurt had a significantly higher concentration of lactic acid than the Mild yoghurt up to week 3. During weeks 4-6 the difference in concentration was not significantly different.

The concentration of orotic acid was significantly different at the end of fermentation and in weeks 1-3. The concentration decreased during fermentation in both yoghurts and then remained constant throughout storage.
There was no propionic acid produced in the Mild batch but in the Robust yoghurt propionic acid was detected after fermentation and then increased in the first week of storage and then remained constant. Uric acid was detected in both yoghurts and remained constant during fermentation and storage. The concentration was significantly different in at the end of fermentation and in weeks 3, 4 and 6.

The organic acids produced by the experimental yoghurts were compared to organic acids produced in commercial yoghurts. Table 11 presents the organic acid content in three different commercial yoghurts. The commercial yoghurts had more lactic acid than the experimental yoghurts, possibly due to a longer fermentation time at the

factory. There were similar amounts of uric acid in the commercial yoghurts and the experimental batches. This was as predicted as uric acid is normally present in milk. There were higher amounts of orotic acid and butyric acid in the commercial yoghurts. The butyric acid was considerably higher as there was none detected in the Mild yoghurts and the most in the Robust yoghurt was 90.72μg/g of yoghurt. There was no formic or acetic acid detected in any of the commercial yoghurts and only the Ski yoghurt detected any propionic acid.

4.1.3.2 Conclusion

The results of the organic acid concentration in these yoghurts are consistent with the production of organic acid in the yoghurts described in section 4.1.2. The Robust culture produced more lactic acid than the Mild culture in both experiments and formic acid was used up during fermentation of the Robust culture but was only slowly used in the Mild yoghurt. There was no butyric acid produced in the Mild yoghurt but this was produced by the Robust culture. This shows that the Mild yoghurt is a slow grower where as the Robust culture is very fast.

These experiments also show that the viability of probiotic bacteria is affected by the 2 different commercial cultures. The probiotic bacteria in both experiments did not survive the full storage trial. The Mild culture appears to give a better environment for the bifidobacteria, but not the *L. acidophilus*. However, the fermentation time with Mild cultures is much longer. The Robust culture does not provide a good environment for either bacteria as the viable counts were below the population required to give any health benefit (approx. $1x10^6$). However, the fermentation time is shorter.

4.2 The effect of sonicating *Lactobacillus delbrueckii* subsp. *bulgaricus* on the survival of probiotic bacteria in yoghurt

It is known that *Lactobacillus delbrueckii* subsp. *bulgaricus* releases growth factors such as amino acids and peptides. These have been found to support the growth of probiotic bacteria (*L. acidophilus* and *Bifidobacterium*) during fermentation. If the

growth factors from *L. delbrueckii* subsp. *bulgaricus* could be released into the yoghurt this would be a natural way of supplementing the probiotic bacteria with nutrients.

In order to allow *Lactobacillus delbrueckii* subsp. *bulgaricus* to release its growth factors, the bacteria was sonicated according to the method described in section 3.4.5 prior to yoghurt making. The results of pH and variable counts are presented in Table 12 and the organic acid concentration results are in Table 13 and 14.

The control yoghurt took 3 hours and 20 minutes to ferment while the sonicated yoghurt took 4 hours and 25 minutes. The pH of both yoghurts decreased rapidly over the 4 weeks of storage with the control yoghurt having the lowest pH. If the Mild and Robust yoghurts are compared to these ones the fermentation time is quite different. The Robust yoghurt had a fermentation time of 5 hours and in this experiment the fermentation times are much shorter. This is probably due to the use of fresh cultures. Freeze dried cultures need time to activate since they are coming from such a cold environment to a warm one where as the fresh bacteria has been grown for 18 hours at optimum temperature.

S. thermophilus increased by 1 log in both the control and the experimental yoghurt during fermentation. During storage the bacteria numbers remained relatively constant. The control had a higher viable count of *Lactobacillus delbrueckii* subsp. *bulgaricus* than the sonicated yoghurt which is to be expected as the *Lactobacillus delbrueckii* subsp. *bulgaricus* was sonicated, however, it was thought that the sonication would have decreased the amount of bacteria even further. The bacteria may be fairly strong and have only been damaged and able to repair itself during fermentation. The bacteria numbers during storage decreased every week but were more noticeable in Week 4 in the sonicated yoghurt when it decreased by nearly 1 log.

The probiotic bacteria performed very well in both yoghurts. There was a high number of *L. acidophilus* and bifidobacteria in the inoculation and due to the short fermentation time there was not a large increase in population. The storage trial showed that the probiotic bacteria survived the harsh conditions that were present. The pH of the control and experimental yoghurt were both very low and yet the

probiotic bacteria were stable throughout the 4 weeks of storage. It is difficult to explain as it was thought that the sonicating the *Lactobacillus delbrueckii* subsp. *bulgaricus* had provided the probiotic bacteria with the nutrients to grow but then the control yoghurt would have a much lower population of probiotic bacteria. It could be concluded that the *L. acidophilus* and bifidobacteria had been very active in the inoculating culture since the number of colonies is very high compared to the other inoculations that were done in previous experiments. The inoculations in both yoghurts were in the 10^7 log scale where the Mild and Robust yoghurts discussed in section 4.1.2 and 4.1.3 were in the 10^5 log scale.

Shah and Lankaputhra (1997) sonicated *L. delbrueckii bulgaricus* to release growth factors to support probiotic bacteria. They found that the probiotic bacteria were 2 log cycles higher after fermentation in yoghurt made with ruptured yoghurt bacteria and was still above the recommended level during 6 weeks of storage.

The organic acid concentration is presented in Tables 13 and 14. There was no acetic acid produced by either yoghurt, as there was not enough time for the bifidobacteria to produce it. Butyric acid was present at the initial stage of fermentation and then increased during fermentation and during storage. The control produced significantly more butyric acid than the sonicated yoghurt.

Formic acid was present at the beginning of fermentation but disappeared at the end of fermentation and none was detected during storage. The control had significantly higher content of lactic acid during fermentation and storage. The concentration increased during fermentation and the first week of storage, and remained stable during storage. The same pattern occurred in the experimental yoghurt.

Orotic acid decreased during fermentation in both yoghurts. The sonicated yoghurt had a higher concentration of orotic acid during storage but was only significantly different in weeks 2 to 4.

Propionic acid was detected at the initial stage of fermentation in the control batch and then its level increased. During the first week of storage there was an increase in concentration of propionic acid but then the level fluctuated throughout the rest of the

storage. In the experimental yoghurt there was no propionic acid detected at the beginning of fermentation but some was produced during the fermentation. There was an increase in propionic acid during storage but this was significantly lower than the control. Uric acid was present in both yoghurts and the experimental batch was significantly different at the end of fermentation and in weeks 2, 3 and 4.

The organic acid content shows that there were differences in the fermentation of these yoghurts. The control produced more lactic, butyric and propionic acid than the experimental. This could be due to the bacteria growing faster and higher population of *Lactobacillus delbrueckii* subsp. *bulgaricus*, which would cause the shorter fermentation time. The experimental yoghurt had more orotic acid, which would mean the bacteria did not use as much of it during fermentation. There was only a slightly lower population of *Lactobacillus delbrueckii* subsp. *bulgaricus* in sonicated product but perhaps the bacteria were repairing themselves during fermentation hence the longer fermentation time.

Sonication of the *Lactobacillus delbrueckii* subsp. *bulgaricus* could possibly improve the viability of probiotic bacteria in yoghurt however it would be expensive and impractical. The use of fresh bacteria is also impractical in a manufacturing plant. *L. acidophilus* is known to produce bacteriocin against *Lactobacillus delbrueckii* subsp. *bulgaricus* and if this could be utilised to lyse *Lactobacillus delbrueckii* subsp. *bulgaricus* and release growth factors for probiotic bacteria then this could be a more practical way of improving yoghurt.

4.3 Antimicrobial substances produced by yoghurt and probiotic bacteria

4.3.1 Growth characteristics of *Lactobacillus acidophilus*

To help select a *L. acidophilus* strain for further experiments, four different strains of *L. acidophilus* were grown over 18 hours to determine growth patterns. Optical density was measured at 620nm. The strains used were: *L. acidophilus* LA-5, *L. acidophilus* 2404, *L. acidophilus* 2405 and *L. acidophilus* 2406. The growth pattern is shown in Figure 3.

As the result show, *L. acidophilus* LA-5 and LA2404 had the highest growth at 37°C in MRS broth. *L. acidophilus* LA2406 also showed good growth, however, the growth was slower as compared to strains LA-5 and LA2404. LA2405 did not grow well and had a final OD reading of only half as that of LA-5. This strain may be fairly weak as the initial inoculation required 3.2mL to reach an OD reading of 0.1 as compared to the other strains of 1.8mL. LA-5 and LA2404 seem to be very robust and find the conditions optimal for growth. In general, the lag phase was between 0 and 2 hours and then all strains continued into the log phase between 2 and 6 hours. After 6 - 8 hours, all strains entered the stationary phase.

4.3.2 Screening of *Lactobacillus acidophilus* against *Lactobacillus delbrueckii* subsp. *bulgaricus* for bacteriocin production

Four strains of *L. acidophilus* were screened for antimicrobial activity against two strains of *Lactobacillus delbrueckii* subsp. *bulgaricus*. The four *L. acidophilus* strains (LA-5, 2404, 2405 and 2406) were used against *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515 and *Lactobacillus delbrueckii* subsp. *bulgaricus* 2501. The spot-on-lawn test as described in section 3.7 was used to determine if any antimicrobial substances were present. The inhibitions of *Lactobacillus delbrueckii* subsp. *bulgaricus* by *L. acidophilus* strains are presented in Table 15. The largest average zone was produced by LA-5 against *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515. *L. acidophilus*-2404 also showed a large zone against *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515. LA2405 showed the smallest zones, which also showed weak growth (Figure 3). This would indicate that this strain is very weak and would be of no benefit in this study. *L. acidophilus* produced zones against *Lactobacillus delbrueckii* subsp. *bulgaricus* 2501 with the largest zone being produced by *L. acidophilus*-LA5. From this study, it was determined that *L. acidophilus*-LA-5 produced an antimicrobial substance against *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515 and the next step was taken to determine what kind of antimicrobial substance was present.

4.3.3 Determination of inhibitory substance

To determine the inhibitory substance present the well diffusion test (Section 3.7) was used. The cell free extract was treated with sodium hydroxide, catalase, papain, proteinase K and crude protease in order to understand the nature of the antimicrobial substance. Zone of inhibition could be produced by lactic acid, and treatment with sodium hydroxide, will neutralize the acid effect and if the zone still appears the effect may be due to H_2O_2 or bacteriocin. Table 16 shows the zones of inhibition by *L. acidophilus* against *Lactobacillus delbrueckii* subsp. *bulgaricus*. There were zones present in the neutralized sample, which determines that acid, was not the cause of inhibition. The sample treated with catalase also had a zone, which suggested that hydrogen peroxide was not the cause of inhibition either. The zones disappeared when samples were treated with protein enzymes, which confirmed an active protein compound was involved in the inhibition of *Lactobacillus delbrueckii* subsp. *bulgaricus*. According to Tagg *et al.* (1976) bacteriocins are bactericidal or bacteriostatic compounds containing a biologically active protein moiety. Thus in this study *L. acidophilus*-LA5 produced one or more bacteriocins against *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Dave and Shah (1997b) and Joseph *et al.* (1998) have shown that some strains of *L. acidophilus* produce bacteriocins against *L. delbrueckii* subsp. *bulgaricus*. In a study by Dave and Shah (1997b), *L. acidophilus* (LA-1) produced bacteriocin against seven strains of *L. delbrueckii* subsp. *bulgaricus* (2501, 2505, 1515, 2517, 2519, LB-3 and LB-4).

4.3.4 Antagonism between yoghurt and probiotic bacteria

This step aimed to determine if there was any antagonism between yoghurt and probiotic bacteria. The spot-on-lawn test (section 3.7) was used to determine any inhibition and the well diffusion test (section 3.7) was used to determine the type of inhibition. The results of the spot-on-lawn test are presented in Table 17 and the results of the well diffusion test are in Table 18.

As shown in Table 17, *L. acidophilus*-LA-5 produced an inhibitory substance against *L. delbrueckii* subsp. *bulgaricus* 2515, *Bifidobacterium longum* 1941, and *Bifidobacterium infantis* 1912. *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515 inhibited *L. acidophilus LA-5,* both bifidobacteria strains, but not *S. thermophilus*. *S. thermophilus* produced inhibitory substances against both strains of bifidobacteria but not against *Lactobacillus delbrueckii* subsp. *bulgaricus*. There appeared to be a zone against *L. acidophilus* LA-5, but it was unclear. *Bifidobacterium infantis* 1912 and *Bifidobacterium longum* 1941 showed slight inhibition to *L. acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus*.

Table 18 shows the nature of inhibitory substances produced by yoghurt and probiotic bacteria to various enzymes and pH. *Lactobacillus delbrueckii* subsp. *bulgaricus*, *S. thermophilus, B. infantis* and *B. longum* showed slight inhibition to *L. acidophilus*. Even after acid and catalase effect were removed, there were still zones present, but it is interesting that the zones still appeared even after treatment with enzymes. This means that no proteinaceous compounds were present and therefore no bacteriocin was detected. The substance would have been a bacteriocin like substance (BLIS). The cause of these zones is unknown. *L. acidophilus* produced a zone against *Lactobacillus delbrueckii* subsp. *bulgaricus* and this was proven to again be bacteriocin as there were no zones present when treated with proteolytic enzymes. There were no zones of inhibition observed by other probiotic or yoghurt bacteria listed in Table 17.

The zones in Table 17 are bigger than these presented in Table 18. For example in Table 17 *L. acidophilus* produced a 16.67mm zone against *Lactobacillus delbrueckii* subsp. *bulgaricus* but in well diffusion test (Table 18) there was only a 9.67mm zone before it was treated. These results are similar to those reported by Joseph *et al.* (1998) that a transfer of organisms, in the early stationary phase (18 hours) into a fresh medium with optimum nutrients and favourable pH, could have enhanced the production of the BLIS on the solid agar medium. Eckner (1992) reported that antimicrobial substances sometimes are only produced on solid media.

4.7 Assessment of viability of *Lactobacillus delbrueckii* subsp. *bulgaricus* grown with various inocula sizes of *Lactobacillus acidophilus*

The effect of bacteriocin producing *L. acidophilus* on *Lactobacillus delbrueckii* subsp. *bulgaricus* was observed. Various inocula sizes of *L. acidophilus* were grown with *Lactobacillus delbrueckii* subsp. *bulgaricus,* and cell density (Figure 4), β-galactosidase (Figure 5) and viable counts (Table 19) were measured. The experiment, as described in Section 3.5, used the inoculum sizes of 1, 5 and 10%. Figure 4 shows the changes in the cell density of *L. acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. It shows that the sample inoculated with 10% *L. acidophilus* had a higher cell density reading followed by the 5% sample, 1% sample and then the *L. acidophilus* control and the *Lactobacillus delbrueckii* subsp. *bulgaricus* control, respectively. This is consistent as the 10% sample contains more bacteria than the other samples. The 5% sample had less growth than the 10% and the 1% sample had less than the 5% but more than the controls. *L. acidophilus* had a higher cell density than that of *Lactobacillus delbrueckii* subsp. *bulgaricus,* which is probably due to the difference in growth temperature. The experiment was conducted at 40°C as it is in between the optimum temperatures of *L. acidophilus* (37°C) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (42°C). This temperature was chosen to give *Lactobacillus delbrueckii* subsp. *bulgaricus* the opportunity to grow, as it does not perform as well when grown below optimum temperature.

The changes in β-galactosidase are shown in Figure 5. This experiment was used to measure the lysis of *Lactobacillus delbrueckii* subsp. *bulgaricus*. It was thought, that as *Lactobacillus delbrueckii* subsp. *bulgaricus* produces more β-galactosidase than *L. acidophilus* and β-galactosidase is intracellular, a higher reading would show, that *Lactobacillus delbrueckii* subsp. *bulgaricus* was being lysed by *L. acidophilus*.

However, Figure 5 shows that the *Lactobacillus delbrueckii* subsp. *bulgaricus* control had the lowest amount of β-galactosidase except at the eighth hour when it had the same as the 1% sample and only marginally lower than the *L. acidophilus* control. This is possibly due to the differences in growth temperature, as this experiment was not conducted at the optimum temperature of *Lactobacillus delbrueckii* subsp.

bulgaricus. The pattern in the first two hours of this graph shows that the 10% sample produced the highest amount of β-galactosidase, followed by the 5% sample, 1% sample, LA control and the LB control. During the next two hours (4th hour), the 10% sample slowed down slightly and the 5% sample became the highest. In the sixth hour, the 1% batch shows the highest amount of β-galactosidase, closely followed by the 5% sample and the LA control. In the eighth hour, the LA control had the highest amount followed by the 1% sample and the LB control and then the 10% and 5% samples have the lowest amount of β-galactosidase. This change in pattern where the lowest inoculations had the highest amount of β-galactosidase could be due to no competition. The LA and LB control are not fighting another bacteria for nutrients and can grow without competition, even the 1% sample that are competing with each other, do not have as much bacteria to fight against, as the inoculation was quite low. The 5% and 10% samples had a high inoculation and they are competing with each other. Even though *Lactobacillus delbrueckii* subsp. *bulgaricus* is not growing at optimum temperature, it still is a robust bacterium and will fight for the nutrients. Another possibility is due to the high inoculation of the 5%, and particularly the 10% sample, as it will run out of nutrients before the other samples. This means that not as much β-galactosidase can be produced, as the bacteria maybe saving energy to survive.

The changes in viable counts of *L. acidophilus* when grown with *Lactobacillus delbrueckii* subsp. *bulgaricus* are shown in Table 19 and Figures 6 and 7. The growth of *L. acidophilus* started off with different inoculations but after eight hours, they had very similar counts, which is possibly due to only a certain amount of nutrients being available, and the 10% sample would have used the nutrients up more quickly, than the 1% sample. The *Lactobacillus delbrueckii* subsp. *bulgaricus* counts followed the same pattern. The initial counts are slightly different, but after eight hours the 1% and 5% samples are very similar and the 10% sample had a lower viable count and had slightly decreased in the eighth hours. The *Lactobacillus delbrueckii* subsp. *bulgaricus* counts were lower than the *L. acidophilus* viable counts, but are still very high and if in yoghurt would still continue to grow and decrease the pH further. It does appear that *Lactobacillus delbrueckii* subsp. *bulgaricus* does decrease slightly in the presence of a high inoculation and this may have been more obvious if the growth

continued for several more hours. This, however, would not be feasible in yoghurt making.

This experiment showed that *L. acidophilus* did not decrease the viable count or lyse *Lactobacillus delbrueckii* subsp. *bulgaricus* enough to slow the production of lactic acid to stop post-acidification.

4.5 Purification of bacteriocin

As the experiment in section 4.4 shows *L. acidophilus* does not inhibit *Lactobacillus delbrueckii* subsp. *bulgaricus* enough to stop its growth. Therefore, it was thought, that if the bacteriocin produced by *L. acidophilus* could be concentrated, purified, and added as a supplement to yoghurt, this would inhibit *Lactobacillus delbrueckii* subsp. *bulgaricus*. The process of ultra-filtration of bacteriocin is described in section 3.8.1. Dave and Shah (1997) have reported that the bacteriocin produced by *L. acidophilus* LA-5 had a molecular weight of approximately 50 kDa. Therefore an ultra-filtration unit, with a molecular weight cut off of 30 kDa was used. This retained the bacteriocin in the retentate and was concentrated approximately 50 times, when the initial solution was either 10 or 15L.

Once the crude bacteriocin was concentrated, the bacteriocin was purified by the method described in Section 3.8. The yield of protein extracted from the concentrate was approximately 0.17% of the total volume filtered.

In this experiment, the well diffusion method (section 3.7) was used to observe inhibition in the media before filtering, in the concentrate and permeate, after purifying, before and after dialysis and after autoclaving. The zones of inhibition can be seen in Figures 8-15. Zones appeared in all samples, except the permeate. This confirms that the molecular weight of the bacteriocin was greater than 30kDa. It also shows that the bacteriocin can sustain autoclaving as a zone still appeared after the sample had been autoclaved (Figure 15).

To make the usage of bacteriocin more practical, attempts were made to dry the bacteriocin into a powdered form. Freeze-drying was attempted several times but without success. The bacteriocin turned into a sticky matter and the last bit of moisture could not be removed even after freeze drying for three days. Drying in a vacuum oven was also tried but without success. A similar sticky matter, found in the freeze drying, was also observed in oven dried samples. It appeared that the protein in some way was trapping moisture.

Morgan *et al.* (2001) developed a method to spray dry lacticin 3147; a bacteriocin produced by *Lactococcus lactis.* This bacteriocin was produced by fermenting *L. lactis* for 24 hours keeping, the pH at 6.5. This fermentate was then pasteurised, evaporated to 40% total solids, and then spray dried. This powder was tested in yoghurt, cottage cheese and soup. The lacticin powder inhibited *Listeria monocytogenes* and *Bacillus cereus* in all three products, when added at a rate of 10%. This method of concentration would be very beneficial to the industry but this equipment was not available for this project. The lacticin 3147 however, is not heat stable and lost considerable amount of activity when autoclaved. The bacteriocin produced by *L. acidophilus* LA-5 did withstand autoclaving, and inhibited *Lactobacillus delbrueckii* subsp. *bulgaricus,* which will be discussed later in this chapter.

4.7 Concentrated bacteriocin grown with *Lactobacillus delbrueckii* subsp. *bulgaricus*

Concentrated bacteriocin was grown with *Lactobacillus delbrueckii* subsp. *bulgaricus* to observe any inhibition. The method used is described in Section 3.9.1. Preliminary trials were conducted first to observe any changes. *Lactobacillus delbrueckii* subsp. *bulgaricus* (1%) was grown with 5% bacteriocin in MRS broth for 8 hours. Viable counts were measured every 2 hours and the results showed (Figure 16) that bacteriocin appeared to inhibit *Lactobacillus delbrueckii* subsp. *bulgaricus.*

The next experiment used different bacteriocin levels, to see which one would inhibit *Lactobacillus delbrueckii* subsp. *bulgaricus* more. The levels used were: 1%, 5% and 10%, and viable counts were measured at 0, 6 and 10 hours. Table 20 and Figure 17

show the results of the three replicates. The results show that certainly the concentrated bacteriocin inhibited *Lactobacillus delbrueckii* subsp. *bulgaricus* more than *L. acidophilus* did, when grown with *Lactobacillus delbrueckii* subsp. *bulgaricus*. Figure 17 shows that there was a 3 to 5 log cycle difference between the control and the experimental batches. The 10% sample did have the lowest viable count after 10 hours with a 5 log cycle difference. The next lowest was the 5% sample which also had a 5 log cycle difference. The 1% sample had a 3 log cycle difference. This experiment showed that *Lactobacillus delbrueckii* subsp. *bulgaricus* is certainly inhibited by concentrated bacteriocin, produced by *L. acidophilus,* and the more bacteriocin added, the more inhibition was observed, which was to be expected.

It was observed that, 1% sample did inhibit *Lactobacillus delbrueckii* subsp. *bulgaricus,* and therefore it was decided to lower the inoculation of bacteriocin, as incorporating 5% and 10% would not be economical in industry.

In the next set of experiments, the inoculation sizes used were: 0.5%, 1% and 2%. The three replicates are shown in Tables 21 to 23. It can be seen from the three tables that the 2% batch did inhibit *Lactobacillus delbrueckii* subsp. *bulgaricus* the most, followed by the 1% and the 0.5% samples. The three replicates are presented rather than one to show that the bacteriocin used lost activity over time. The three experiments were conducted within a week and the bacteriocin was prepared fresh and then stored at 4°C until required. During these 7 days the activity of bacteriocin dropped. The first replicate (Table 21) showed that there was a 4 log cycle difference between the control and the 2% batch, a 2 log cycle difference between the control and the 1% batch and a 1 log cycle difference between the control and the 0.5% batch. In the second replicate (Table 22) the difference between the control was 2 log for the 2% batch, 1 log for the 1% batch and the 0.5% batch was in the same log cycle as the control. The third replicate (Table 23) shows that there was a 1 log cycle difference between the control and all the experimental batches. This shows a marked decrease in activity over the three experiments in only 7 days when the bacteriocin was stored at 4°C.

4.7 Bacteriocin Incorporated in Yoghurt Production

4.7.1 pH and viable counts

Yoghurt was made as described in section 3.4.6. The levels of bacteriocin chosen were 1% and 2% and this was added to the yoghurt at the same time as the bacteria. Samples were taken at the beginning and at the end of fermentation for viable count analysis and the pH was monitored at the start of fermentation, 2 hours after and then every half hour, until a pH of 4.5 was reached. The yoghurt was then stored at 4°C and pH and viable counts were monitored every week for 6 weeks. All samples taken were frozen to measure organic acid concentration.
The results of the viable counts and pH during fermentation and storage are shown in Tables 24, 25 and 26, respectively. The results of organic acid production during fermentation are shown in Tables 27 and 28 and Figures 18 to 24.

The fermentation time of all three batches of yoghurts was 3 hours, which is considered as fast for yoghurt making. There was no difference in time between the batches at all. The pH at the end of fermentation was 4.44 for the control 4.39 for the yoghurt containing 1% bacteriocin and 4.32 for that containing 2% bacteriocin. There is very little difference between these pH values. A possible reason for the slightly lower value in the 2% batch is the time it took to measure all of the replicates and during this short duration the 2% batch continued to ferment and the pH decreased slightly.

The viable counts show that *S. thermophilus* grew about 1 log in all yoghurts, *Lactobacillus delbrueckii* subsp. *bulgaricus* grew 1 log and *L. acidophilus* and *B. longum* stayed in the same log cycle. There appeared to be little difference in the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* between the three batches of yoghurts. The 1% batch had the highest viable counts followed by the control and then the 2% batch. This means that the added bacteriocin did not inhibit *Lactobacillus delbrueckii* subsp. *bulgaricus*.

S. thermophilus grew very well with the 1% having the highest viable count followed by the control and then the 2% batch, which is the same as for the *Lactobacillus delbrueckii* subsp. *bulgaricus* counts. *L. acidophilus* and *B. longum* slightly increased in number. The control yoghurt had the highest number of *L. acidophilus,* then the 1% batch then the 2% batch. The 2% batch had the highest number of *B. longum* followed by the control and the 1% batch. In all batches the probiotic bacteria only grew slightly staying within the same log cycle except for *B. longum* in the 2% batch, which just grew to $1.00x10^6$. The probiotic bacteria did not increase in number further because of the short fermentation time. Probiotic bacteria are known to grow slowly in milk and there was not enough time for the bacteria to increase in number. It has been said that for probiotic bacteria to have any therapeutic effect in the gut the number of these bacteria must be above $1.00x10^6$ CFU/mL of yoghurt. There was only just enough *L. acidophilus* and not quite enough *B. longum* in the yoghurt to cause any therapeutic effect.

During storage the pH dropped considerably in all yoghurts in the first week and in the 2^{nd} week, but went up in week 3 and stayed relatively stable up to week 6. It is unclear why the pH of yoghurt dropped so much in week 2 but it could be due to the high number of *Lactobacillus delbrueckii* subsp. *bulgaricus* present.

S. thermophilus in all the three yoghurts remained constant during storage. The viable counts remained in the 10^8-log cycle and only fluctuated slightly. *Lactobacillus delbrueckii* subsp. *bulgaricus* for all three yoghurts declined in number over the 6 weeks of storage period. In week 1, the counts dropped slightly and in week 2 the counts were still within the 10^7-log cycle but did decrease. In week 3, the counts decreased in the control and in the 2% batch decreased by 1 log. The 1% batch did go up, but only very little. In week 4, the control sample went down by 1 log and the 1% and 2% batches dropped 2 log. This is a fairly substantial decrease. It is possible that the bacteriocin did help to kill the *Lactobacillus delbrueckii* subsp. *bulgaricus* in this week. This may be why there is such a large decrease in counts in this week and also why the control did not decrease as much. In week 5 the control decreased by 3 log as did the 1% batch but the 2% batch decreased by 2 log. In week 6, all yoghurts decreased 1 log and all had counts in the 10^1 log cycle. Therefore, *Lactobacillus delbrueckii* subsp. *bulgaricus* in all three batches decreased from 10^7 to 10^1 in 6

weeks. The decrease in population of *Lactobacillus delbrueckii* subsp. *bulgaricus* as compared to that of *S. thermophilus* was also observed by Kim *et al.* (1993).

The changes in the counts of *L. acidophilus* in yoghurts during manufacture and storage are presented in Tables 24, 25 and 26. In the first week of storage, *L. acidophilus* in the control batch decreased considerably dropping 2 log. It then continued to drop considerably for the rest of the storage period where it only had 47.5 CFU/mL at week 6. The 1% batch had a similar decrease in its *L. acidophilus* population in the first week of storage with a 1 log drop and also continued to drop during the next 5 weeks. The *L. acidophilus* decreased at a slower rate the control batch as it did not drop to 70 CFU/mL until week 4 where as the control fell to 38.3 CFU/mL in week 3. The 2% batch decreased slightly in its first week of storage and then followed a similar pattern to the 1% batch, as it did not drop to 28 CFU/mL until week 3. The incorporation of bacteriocin may have given the *L. acidophilus* a better chance of survival in the yoghurt. The pH of the yoghurts decreased considerably in week 2 and this is possibly why the *L. acidophilus* dropped several log cycles in week 2. However during the rest of the storage period there was no sudden drop in pH and all the yoghurts had very similar pH values so pH may not have been the reason why there was a different rate of *L. acidophilus* decrease in the yoghurts. The other factor that might have affected the viability of *L. acidophilus* could be either antagonism by yoghurt organism. Rybka and Kailasapathy (1995) also observed less viability of *L. acidophilus* in yoghurt with *Lactobacillus delbrueckii* subsp. *bulgaricus*.

In the first week of storage, *B. longum* decreased slightly but remained fairly stable until week 3 when it dropped 1 log cycle in all yoghurts. In week 4 the counts all went up 1 log cycle but dropped again in week 5 and then remained in the same log cycle in week 6. The bifidobacteria seemed to be stable in the yoghurt even though the population was not high enough to cause any therapeutic effect in the gut. Several bifidobacteria strains have shown tolerance to low pH (Lankaputhra *et al.*, 1996b). Martin and Chou (1992) observed that viability of *Bifidobacterium* sp. was species and strain dependent and the viability greatly varied amongst them. The presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* can also increase the viability of bifidobacteria. *Lactobacillus delbrueckii* subsp. *bulgaricus* is known for its proteolytic nature (Shankar and Davies, 1976) and the free amino acids produced by

this organism in yoghurt could be used by other organisms and would have promoted the growth of probiotic bacteria (Dutta *et al.*, 1973; Singh *et al.*, 1980). Most bifidobacteria have been found to be weakly proteolytic and free amino acids are essential for most bifidobacteria (Klaver *et al.*, 1993). Therefore, it is expected that the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* might be beneficial for the growth of bifidobacteria during manufacture of yoghurt.

4.7.2 Organic acid analysis

The analysis of organic acids was performed using the HPLC according to the method described in Section 3.6.3. The changes in organic acid production are presented in Tables 27 and 28 and Figures 18 to 24. Figure 21 shows the concentration of lactic acid produced in yoghurt. During fermentation the concentration of lactic acid increased 10 fold. The 1% yoghurt had the highest concentration at the end of fermentation followed by the control and the 2% yoghurt but the two experimental yoghurts were not significantly different as compared to the control. During storage the lactic acid concentration in the control increased up to week 2 and then decreased slightly. It then went up again in week 4 and continued to increase for the next two weeks.

The lactic acid production in the 1% batch continued to increase during storage until week 3. In week 4 and 5, there was a decrease and then a slight increase in week 6. In the 2% batch, the lactic acid concentration increased in week 1 but then decreased and fluctuated for the rest of the storage period. Although Figure 21 shows that the lactic acid production did fluctuate during storage the experimental yoghurts were significantly higher in week 1 and for the rest of the storage trial there was no significant difference.

Acetic acid (Figure 18) was only detected in the control yoghurt and only during storage. The first week showed a concentration of 15.97μg/g and week 6 had a concentration of 16.76μg/g and there were only small fluctuations in between. The other samples did not show any acetic acid. Acetic acid was produced by bifidobacteria but only after 12 hours. This is why there was no acetic acid detected

during fermentation. Factors that could have influenced the production could be the bacteriocin was affecting the bifidobacteria in some way or the conditions in the control were more favourable to the bacteria than the other two batches.

Butyric acid (Figure 19) was detected at the end of fermentation in all yoghurts. The acid then increased during the storage period. The 1% yoghurt had a significantly higher concentration of butyric acid during storage than the control and the 2% yoghurt was not significantly different. As the results show the starter bacteria produced butyric acid.

Lankaputhra and Shah (1998) studied the levels of acetic, butyric, lactic and pyruvic acids produced by the probiotic bacteria as determined by HPLC. All strains produced these acids with butyric acid being produced by most strains of *L. acidophilus* and bifidobacteria. Lankaputhra and Shah (1998) also studied the antimutagenic activity of organic acids against eight mutagens and promutagens. The study found that butyric acid showed the highest antimutagenic activity against all the 8 mutagens or promutagens. Therefore probiotic bacteria, which produce butyric acid, are more likely to provide antimutagenic properties.

Formic acid (Figure 20) was present in milk at the beginning of fermentation but by the end had decreased considerably. The 2% yoghurt was significantly lower than the control at the end of fermentation. During storage no formic acid was detected. This shows that formic acid must be used as a growth factor for the starter bacteria.

S. thermophilus produced formic acid, which was also reported by Veringa *et al.* (1968) and Bottazzi *et al.* (1971). It was found that the production of formic acid stimulated the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus*. It also can induce the proteolytic activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* in milk so that it became able to hydrolyse β -lactoglobulin, and αs1 and β-casein as compared to only β-casein without the formic acid (Moreira *et al.*, 1997). The stimulatory effect of formic acid remains unnoticed in intensely heated milk because in this milk formic acid had been formed by decomposition of lactose. The production of formic acid by the cocci is, however, essential in industrial practice,

where more moderate heat treatments of yoghurt milk are applied (Walstra *et al.*, 1999).

The changes in orotic acid production in yoghurt are presented in Figure 22. There was a decrease in the concentration of orotic acid during fermentation and then during storage the concentration fluctuated slightly but stayed relatively constant. The 1% yoghurt was significantly lower than the control in weeks 2, 4 and 5 and the 2% yoghurt was significantly lower at the beginning of fermentation and in weeks 2 and 5. Orotic acid is used during fermentation as a growth factor, which can be seen in Figure 22 where there is a decrease in concentration during the three hours of fermentation. Orotic acid is metabolised by the yoghurt starter cultures, most probably by *Lactobacillus delbrueckii* subsp. *bulgaricus* (Tamime and Robinson, 1999). It has been found that orotic acid can be reduced by up to 50% in milk during the manufacture of yoghurt (Tamime and Robinson, 1999; Navder *et al.*, 1990). The reduction was not the same in the yoghurts in this experiment, which is probably due to a rapid fermentation. Orotic acid possessed some significant therapeutic properties, since it plays an important role in the biosynthesis of nucleic acids and the lowering of serum cholesterol (Fernandez-Garcia and McGregor, 1994).

Figure 23 shows the production of propionic acid in yoghurt. There was no propionic acid detected in the milk at the beginning of fermentation, however, the production began during fermentation and continued during storage. The 1% yoghurt was not significantly different to the control but the 2% yoghurt was significantly lower. The yoghurt starter bacteria produce propionic acid as a volatile flavour compound (Tamime and Robinson, 1999).

The production of uric acid is presented in Figure 24. Uric acid is already present in milk and remained fairly constant throughout fermentation and storage for all three yoghurts. This result was also observed by Navder *et al.* (1990) where uric acid content was not significantly altered after fermentation. Uric acid is present in milk as a result of normal bovine biomedical processes (Navder *et al.*, 1990).

From these experiments, it was observed that the bacteriocin has not inhibited *Lactobacillus delbrueckii* subsp. *bulgaricus* in the yoghurt. The viable counts of

Lactobacillus delbrueckii subsp. *bulgaricus* are very similar between the three yoghurts at the end of fermentation and this suggests no inhibition has occurred. The pH and fermentation times are also very similar and this means the bacteriocin has not lysed *Lactobacillus delbrueckii* subsp. *bulgaricus* to reduce the amount of acid produced which would in turn have allowed the pH to remain stable and the fermentation time may have been slower. The lactic acid production is very similar for all three yoghurts suggesting that the *Lactobacillus delbrueckii* subsp. *bulgaricus* is very active. Therefore it is hypothesised that there is some substance that is blocking the bacteriocin in yoghurt and the next experiments pursue this hypothesis.

4.8 Bacteriocin in milk

Lactobacillus delbrueckii subsp. *bulgaricus* (1%) was grown with 1%, 5% and 10% levels of bacteriocin for 8 hours as described in section 3.9.2. Samples were taken every 8 hours to measure viable counts and these results are presented in Figure 25 and Table 29. The results show that the bacteriocin had no effect on the *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteria. All the counts at the 8^{th} hour are very similar and have grown the same as the control with out any inhibition observed.

When these results are compared to the results in Table 20 and Figure 17 when the bacteriocin was grown with *Lactobacillus delbrueckii* subsp. *bulgaricus* in MRS broth there is a difference observed. The *Lactobacillus delbrueckii* subsp. *bulgaricus* when grown in MRS broth had a 3 log cycle drop. This suggests that there is something in the RSM that is blocking the bacteriocin activity.

4.8.1 Bacteriocin in different levels of milk

To determine if it is the milk that caused the loss of activity in bacteriocin, different milk media were made containing 3%, 6% and 12% RSM. *L. acidophilus* (1%) was grown with *Lactobacillus delbrueckii* subsp. *bulgaricus* (1%) for 6 hours. The spot-on lawn technique was used to observe any inhibition against *Lactobacillus delbrueckii* subsp. *bulgaricus*. The results are presented in Table 30. It was observed that the 12% milk batch did not produce any zones and when incubation continued after 18 hours there still was no zone observed. Dave and Shah (1999) found that

bacteriocin was not observed in 12% RSM milk until after 22 hours of incubation. The 6% batch showed a zone after 6 hours of incubation and the 3% batch showed a zone after 2 hours of incubation. These results indicate that there is something present in milk that is interfering with the bacteriocin activity.

In the next experiment 12% RSM and 3% RSM were inoculated with 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and different levels of bacteriocin and grown for 18 hours. The purpose was to see if concentrated bacteriocin would have some effect on *Lactobacillus delbrueckii* subsp. *bulgaricus* in a lower concentration of milk. The results are shown in Table 31. It was observed that the bacteriocin had no effect on the viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus*. The viable counts were in the same log cycle; however, the batch with 10% and 5% bacteriocin grown in the 12% milk had a higher count than the LB control. The only difference that could be seen was the viable counts between the 12% milk and the 3% milk controls. The viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* were 1 log lower in the 3% sample. This was probably due to the lack of nutrients in the milk.

4.8.2 Bacteriocin in different media

It has been confirmed that there is something present in milk that is blocking the activity of bacteriocin. In this experiment casein was removed to see if it is the cause of this decrease in activity. Three percent milk was made and casein was removed using hydrochloric acid as described in Section 3.9.3. The results are presented in Table 32 and Figure 26. It was observed that in the MRS broth the bacteriocin did inhibit the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus*. The batch with no casein but with bacteriocin was higher than its control but was lower than the sample that did contain casein. There does not seem to be any differences between the batch with casein or without, therefore this experiment indicates that casein may not be the substance blocking the bacteriocin.

Table 2: Yoghurt made with two different commercial strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (mild and robust) and *S. thermophilus*.

		During Fermentation					During Storage					
		Initial[1]		Final[2]			Week 1		Week 2		Week 4	
	Bacteria	pH	Count	pH	Count	Fermentation time (hours)	pH	Count	pH	Count	pH	Count
		----Viable Count CFU/mL----					----Viable Count CFU/mL----					
Mild[3]	LB[5]	6.52	$2.40x10^3$	4.49	$1.00x10^5$	9	4.39	$8.00x10^4$	4.35	$3.00x10^4$	4.37	$9.00x10^4$
	ST[6]		$5.50x10^6$		$1.10x10^9$			$1.80x10^9$		$2.02x10^9$		$2.12x10^9$
Robust[4]	LB	6.53	$2.40x10^5$	4.47	$2.63x10^8$	6.5	4.12	$4.53x10^8$	3.99	$3.35x10^8$	4.13	$2.13x10^8$
	ST		$2.30x10^6$		$5.43x10^8$			$4.35x10^8$		$4.93x10^8$		$5.85x10^8$

[1]Initial – Sample taken immediately after inoculation
[2]Final – Sample taken when yoghurt reached pH 4.5, Mild 9 hours and Robust 6.5 hours.

[3]Mild- contains a mild freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*

[4]Robust – contains a robust freeze dried *Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus*

[5]LB – *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515

[6]ST – *Streptococcus thermophilus* 2014

Table 3: The effect on probiotic bacteria (*L. acidophilus* LA-5 and *B. infantis* 1912) during fermentation when grown with two commercial yoghurt strains.

		Initial[1]		Final[2]		
	Bacteria	pH	Counts	pH	Counts	Fermentation time
		----Variable counts CFU/mL----				Hours
Mild[3]	ST[5]	6.46	1.52×10^7	4.49	1.72×10^8	7
	LB[6]		4.26×10^5		5.64×10^6	
	LA[7]		5.26×10^5		6.98×10^6	
	BB[8]		1.33×10^6		1.09×10^7	
Robust[4]	ST	6.46	9.93×10^6	4.47	3.75×10^8	5
	LB		9.26×10^5		9.77×10^7	
	LA		5.50×10^5		3.18×10^6	
	BB		8.71×10^4		8.50×10^5	

[1]Initial- Sample taken immediately after inoculation

[2] Final – Sample was taken when yoghurt reached pH 4.5, Mild 7 hours and Robust 5 hours.

[3]Mild – contains a mild freeze dried *L. bulgaricus* and *S. thermophilus and 1% or fresh L. acidophilus* and *Bifidobacteria*1912

[4]Robust - contains a robust freeze dried *L. bulgaricus* and *S. thermophilus* and 1% each of fresh *L. acidophilus* and *Bifidobacteria* 1912

[5]ST – *Streptococcus thermophilus* 2014

[6] LB – *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515

[7]LA – *Lactobacillus acidophilus* LA-5

[8]BB – *Bifidobacterium infantis* 1912

Table 4: The effect on probiotic bacteria (*L. acidophilus* LA-5 and *B. infantis* 1912) during storage when grown with two commercial yoghurt strains.

		Week 1		Week 2		Week 3		Week 4	
	Bacteria	pH	Counts	pH	Counts	pH	Counts	pH	Counts
					----Viable counts CFU/mL----				
Mild[1]	ST[3]	4.50	$3.00x10^8$	4.47	$2.97x10^8$	4.37	$1.90x10^8$	4.47	$1.83x10^8$
	LB[4]		$6.65x10^6$		$1.57x10^6$		$1.01x10^7$		$2.33x10^6$
	LA[5]		$7.88x10^6$		$4.23x10^6$		$8.83x10^5$		$1.80x10^4$
	BB[6]		$8.75x10^5$		$6.23x10^6$		$2.85x10^7$		$2.20x10^7$
Robust[2]	ST	4.34	$3.05x10^8$	4.32	$2.83x10^8$	4.19	$2.39x10^8$	4.35	$2.36x10^8$
	LB		$1.07x10^8$		$1.65x10^7$		$7.72x10^6$		$1.00x10^5$
	LA		$2.56x10^6$		$5.75x10^5$		$1.71x10^6$		$2.50x10^3$
	BB		$5.95x10^4$		$9.82x10^5$		-		$8.45x10^4$

[1]Mild – contains a mild freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus and 1% or fresh L. acidophilus* and *B. infantis*1912

[2]Robust - contains a robust freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and 1% each of fresh *L. acidophilus* and *B. infantis 1912*

[3]ST – *Streptococcus thermophilus* 2014

[4] LB – *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515

[5]LA – *Lactobacillus acidophilus* LA-5

[6]BB – *Bifidobacterium infantis* 1912

Table 5: The concentration of organic acids in yoghurt when fermented with 2 different commercial yoghurt strains and probiotic bacteria (*L. acidophilus* LA-5 and *B. infantis*).

		During Fermentation			
		Initial[1]		Final[2]	
		Average	St. Dev.	Average	St. Dev.
		----µg/g of yoghurt----			
Mild[3]	Acetic	0.00		0.00	
	Butyric	0.00		0.00	
	Formic	[a]461.35	16.25	[a]1729.63	364.56
	Lactic	[a]428.27	41.85	[a]9534.65	208.10
	Orotic	[a]150.82	8.94	135.25	3.01
	Propionic	0.00	0.00	[a]0.00	0.00
	Uric	33.60	1.33	34.19	0.83
Robust[4]	Acetic	0.00		0.00	
	Butyric	0.00		20.51	5.67
	Formic	427.03	18.29	0.00	0.00
	Lactic	325.13	80.72	10953.12	960.70
	Orotic	138.64	2.48	132.52	11.11
	Propionic	0.00		42.09	7.88
	Uric	33.66	0.77	33.81	0.58

[1]Initial- Sample taken immediately after inoculation

[2] Final – Sample was taken when yoghurt reached pH 4.5. All fermentation ended after 3 hours

[3]Mild – contains a mild freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus and 1% or fresh L. acidophilus* and *B. infantis* 1912

[4]Robust - contains a robust freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and 1% each of fresh *L. acidophilus* and *B. infantis* 1912

[a] Statistically significant ($P<0.05$) as compared to Robust yoghurt.

Table 6: The concentration of organic acids in yoghurt during storage when fermented with 2 different commercial yoghurt strains and probiotic bacteria (*L. acidophilus* LA-5 and *B. infantis*).

		During Storage							
		Week 1		Week 2		Week 3		Week 4	
		Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
		----μg/g of yoghurt----							
Mild[1]	Acetic	0.00		0.00		0.00		0.00	
	Butyric	[a]0.00		[a]0.00		[a]0.00		[a]0.00	
	Formic	[a]1575.20	258.99	[a]1208.74	122.30	[a]943.71	20.49	[a]563.24	325.92
	Lactic	[a]10236.92	113.46	[a]10500.23	67.76	[a]10736.10	180.98	11527.74	830.83
	Orotic	135.30	1.24	133.60	1.98	133.60	2.01	136.20	3.01
	Propionic	[a]0.00		[a]0.00		[a]0.00		[a]0.00	
	Uric	[a]34.64	0.25	34.04	0.20	33.14	0.26	[a]34.60	0.70
Robust[2]	Acetic	0.00		0.00		0.00		0.00	
	Butyric	63.03	8.25	66.39	5.46	66.70	6.34	75.85	2.78
	Formic	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Lactic	12071.54	366.40	12430.60	500.27	12008.19	981.14	12378.51	459.42
	Orotic	139.99	12.75	135.87	12.00	141.83	14.43	134.95	12.76
	Propionic	62.26	2.34	61.29	2.36	62.79	5.47	65.01	4.17
	Uric	34.06	0.21	33.84	0.59	32.70	1.82	33.82	0.27

[2]Robust - contains a robust freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and 1% each of fresh *L. acidophilus* and *B. infantis* 1912

[a] Statistically significant ($P<0.05$) as compared to Robust yoghurt.

[1]Mild - contains a mild freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus and 1% or fresh L. acidophilus* and *B. infantis* 1912

Table 7: The effect on probiotic bacteria (*L. acidophilus* LA-5 and *B. longum* 1941) during fermentation when grown with 2 commercial yoghurt strains.

		Initial[1]		Final[2]		
	Bacteria	pH	Counts	pH	Counts	Fermentation time
		----Viable Counts CFU/mL----				Hours
Mild[3]	ST[5]	6.45	$1.39x10^7$	4.49	$1.58x10^8$	7
	LB[6]		$1.67x10^6$		$9.33x10^6$	
	LA[7]		$1.72x10^5$		$5.30x10^6$	
	BB[8]		$8.90x10^5$		$4.87x10^7$	
Robust[4]	ST	6.30	$9.38x10^6$	4.49	$2.19x10^8$	5
	LB		$1.59x10^5$		$5.52x10^7$	
	LA		$1.35x10^5$		$3.50x10^6$	
	BB		$3.07x10^5$		$6.35x10^5$	

[1]Initial- Sample taken immediately after inoculation

[2] Final – Sample was taken when yoghurt reached pH 4.5, Mild 7 hours and Robust 5 hours.

[3]Mild – contains a mild freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus and 1% or fresh L. acidophilus* and *B. longum*1941

[4]Robust - contains a robust freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and 1% each of fresh *L. acidophilus* and *B. longum* 1941

[5]ST – Streptococcus thermophilus 2014

[6] LB – *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515

[7]LA – *Lactobacillus acidophilus* LA-5

[8]BB – *Bifidobacterium longum* 1941

Table 8: The effect on probiotic bacteria (*L. acidophilus* LA-5 and *B. longum* 1941) during storage when grown with two commercial yoghurt strains.

		Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
	Bacteria	pH	Counts	pH	Counts	pH	Counts	pH	Counts	pH	Counts	pH	Counts
							----Viable Counts CFU/mL----						
Mild[1]	ST[1]	4.46	$1.56x10^8$	4.33	$1.43x10^8$	4.42	$1.39x10^8$	4.25	$1.47x10^8$	4.18	$1.92x10^8$	4.34	$1.78x10^8$
	LB[2]		$5.65x10^6$		$3.55x10^6$		$1.09x10^6$		$5.35x10^2$		$4.01x10^5$		$3.00x10^5$
	LA[3]		-		$1.66x10^6$		$9.70x10^5$		$2.38x10^6$		$3.05x10^4$		$1.21x10^3$
	BB[4]		$3.33x10^4$		$4.57x10^7$		$4.10x10^5$		$6.42x10^6$		$4.85x10^5$		$1.65x10^5$
Robust[2]	ST	4.40	$2.47x10^8$	4.26	$1.75x10^8$	4.32	$1.83x10^8$	4.17	$1.65x10^8$	4.10	$2.59x10^8$	4.29	$2.11x10^8$
	LB		$4.28x10^7$		$3.80x10^6$		$2.10x10^6$		$5.30x10^2$		$1.98x10^4$		$8.70x10^3$
	LA		-		$7.00x10^4$		$1.80x10^4$		$1.50x10^3$		$1.00x10^3$		$7.80x10^1$
	BB		$3.00x10^3$		$1.17x10^2$		$3.30x10^4$		$1.40x10^3$		$1.25x10^4$		$1.01x10^5$

[1]Mild - contains a mild freeze dried *L. bulgaricus* and *S. thermophilus and 1% or fresh L. acidophilus* and *B. longum*1941
[2]Robust - contains a robust freeze dried *L. bulgaricus* and *S. thermophilus* and 1% each of fresh *L. acidophilus* and *B. longum 1941*
[3]ST - *Streptococcus thermophilus* 2014
[4] LB – *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515
[5]LA - *Lactobacillus acidophilus* LA-5
[6]BB – *Bifidobacterium longum* 1941

Table 9: The concentration of organic acids in yoghurt when fermented with 2 different Commercial yoghurt strains and probiotic bacteria (*L. acidophilus* LA-5 and *B. longum* 1941).

		During Fermentation			
		Initial[1]		Final[2]	
		Average	St.Dev.	Average	St.Dev.
		----μg/g of yoghurt----			
Mild[3]	Acetic	0.00		0.00	
	Butyric	0.00		0.00	
	Formic	[a]503.14	15.53	[a]1035.52	173.80
	Lactic	[a]606.86	88.17	[a]9584.85	158.46
	Orotic	154.46	18.20	[a]129.58	0.69
	Propionic	0.00		[a]0.00	
	Uric	33.58	1.20	[a]33.04	0.92
Robust[4]	Acetic	0.00		0.00	
	Butyric	0.00		20.63	4.29
	Formic	477.73	5.21	0.00	
	Lactic	511.52	27.61	11019.00	431.89
	Orotic	146.54	6.88	136.16	2.29
	Propionic	0.00		27.37	2.49
	Uric	35.03	1.8	35.50	0.21

[1]Initial- Sample taken immediately after inoculation

[2] Final – Sample was taken when yoghurt reached pH 4.5. All fermentation ended after 3 hours

[3]Mild – contains a mild freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus and 1% or fresh L. acidophilus* and *B. longum* 1941

[4]Robust - contains a robust freeze dried *L. bulgaricus* and *S. thermophilus* and 1% each of fresh *L. acidophilus* and *B. longum* 1941

[a] Statistically significant ($P<0.05$) as compared to Robust yoghurt.

Table 10: The organic acid concentration in yoghurt during storage when fermented with 2 different Commercial yoghurt strains and probiotic bacteria (*L. acidophilus* LA-5 and *B. longum* 1941).

		During Storage											
		Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
		Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.
		----μg/g of yoghurt----											
Mild[1]	Acetic	0.00		0.00		0.00		0.00		0.00		0.00	
	Butyric	[a]0.00		[a]0.00		[a]0.00		[a]0.00		[a]0.00		[a]0.00	
	Formic	[a]1093.15	181.59	[a]860.46	146.35	[a]848.15	65.77	[a]31.42	3.95	[a]30.42	3.32	[a]27.28	6.01
	Lactic	[a]10363.55	92.37	[a]10722.01	28.81	[a]11219.10	40.25	12329.71	143.01	12381.44	157.96	12321.69	191.77
	Orotic	[a]130.40	1.33	[a]130.99	1.35	[a]129.75	0.66	148.90	8.63	156.07	2.47	154.61	3.75
	Propionic	[a]0.00		[a]0.00		[a]0.00		[a]0.00		[a]0.00		[a]0.00	
	Uric	34.23	0.49	34.47	0.09	[a]33.77	0.51	[a]24.57	0.17	31.95	0.54	[a]32.69	0.94
Robust[2]	Acetic	0.00		0.00		0.00		0.00		0.00		0.00	
	Butyric	34.80	21.78	42.22	15.19	60.34	3.56	74.72	3.98	79.73	4.51	90.72	2.84
	Formic	0.00		0.00		0.00		0.00		0.00		0.00	
	Lactic	11143.62	478.33	11933.38	465.66	12297.70	155.81	12389.52	174.00	12851.23	209.05	13037.46	1092.51
	Orotic	137.64	1.43	151.31	10.83	147.62	13.53	150.13	7.68	156.33	2.28	152.83	3.99
	Propionic	32.43	8.39	32.91	6.03	37.06	11.59	30.41	4.15	34.42	4.44	33.69	9.07
	Uric	33.30	2.00	35.09	0.98	35.00	0.79	21.06	2.66	30.04	3.36	29.49	2.47

[1]Mild – contains a mild freeze dried *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus and 1% or fresh L. acidophilus* and *B. longum* 1941
[2]Robust - contains a robust freeze dried *L. bulgaricus* and *S. thermophilus* and 1% each of fresh *L. acidophilus* and *B. longum* 1941

[a] Statistically significant ($P<0.05$) as compared to Robust yoghurt.

Table 11: Concentration of organic acids in commercial yoghurts.

	Lactic		Uric		Orotic		Propionic		Butyric		Formic	Acetic
	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	Average
	----µg/g of yoghurt----											
Yoplait[1]	17598.62	146.72	37.20	0.42	189.55	17.29	0.00		573.11	113.18	0.00	0.00
Vaalia[2]	15647.15	72.42	38.34	0.35	174.67	14.56	0.00		897.88	2.14	0.00	0.00
Ski[3]	15503.47	191.29	39.94	0.77	178.62	4.65	48.61	33.33	898.97	0.23	0.00	0.00

[1]Yoplait - Yoplait Lite Creamy Vanilla (use-by-date 16/6/02)
[2]Vaalia - Vaalia French Vanilla (use-by-date 5/6/02)
[3]Ski – Ski No Fat Vanilla Crème (use-by-date 21/5/02)
Experiment completed on the 17/5/02

Table 12: The effect of sonicating *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515 cultures has on fermentation time and the survival of *S. thermophilus* 2014, *L. acidophilus* LA-5 and *B. infantis* 1912 during fermentation and storage.

		During Fermentation					During Storage							
		Initial[1]		Final[2]			Week 1		Week 2		Week 3		Week 4	
	Bacteria	pH	Counts[3]	pH	Counts	Fermentation time	pH	Counts	pH	Counts	pH	Counts	pH	Counts
		----Variable counts CFU/mL----					----Variable counts CFU/mL----							
Control[4]	ST[9]	6.16	$8.82x10^7$	4.46	$9.80x10^8$	3 h 20min	4.07	$9.05x10^8$	4.00	$6.65x10^8$	3.96	$8.25x10^8$	3.87	$8.67x10^8$
	LB[6]		$8.33x10^7$		$2.22x10^8$			$4.33x10^8$		$4.02x10^8$		$3.78x10^8$		$2.83x10^8$
	LA[7]		$4.40x10^7$		$7.55x10^7$			$6.80x10^7$		$3.52x10^7$		$4.82x10^7$		$5.35x10^7$
	BB[8]		$3.85x10^7$		$8.03x10^7$			$4.17x10^7$		$3.35x10^7$		$3.23x10^7$		$4.44x10^7$
Sonicated[5]	ST	6.29	$7.20x10^7$	4.46	$7.70x10^8$	4 h 25min	4.24	$9.47x10^8$	4.18	$6.30x10^8$	4.08	$7.38x10^8$	4.03	$8.38x10^8$
	LB		$6.88x10^7$		$1.93x10^8$			$2.16x10^8$		$1.80x10^8$		$1.36x10^8$		$6.82x10^7$
	LA		$3.60x10^7$		$7.66x10^7$			$8.30x10^7$		$6.15x10^7$		$6.68x10^7$		$6.80x10^7$
	BB		$3.60x10^7$		$7.28x10^7$			$7.05x10^7$		$6.35x10^7$		$6.07x10^7$		$4.90x10^7$

[1] Initial – Sample taken immediately after inoculation

[2] Final - Sample was taken when yoghurt reached pH 4.5, Control 3h 20min and Sonicated 4h and 25min

[3] Counts - Viable count (CFU/mL)

[4] Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B.* infantis1912

[5] Sonicated - Contains 1% of *L. bulgaricus* that has been sonicated for 4 min, *S. thermophilus, L. acidophilus* and *Bifidobacteria* 1912

[6] LB - *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515

[7] LA - *Lactobacillus acidophilus* LA-5

[8] BB – *Bifidobacterium infantis* 1912

[9] ST – *Streptococcus thermophilus* 2014

Table 13: The effect of sonicating *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515 cultures has on production of organic acids in yoghurt during fermentation by probiotic and yoghurt bacteria.

		During Fermentation			
		Initial[1]		Final[2]	
		Average	St.Dev.	Average	St.Dev.
		----µg/g of yoghurt----			
Control[3]	Acetic	0.00		0.00	
	Butyric	50.02	5.78	139.64	18.03
	Formic	95.70	7.11	0.00	
	Lactic	9778.65	464.11	13314.05	615.87
	Orotic	122.77	14.17	100.54	18.52
	Propionic	19.51	9.59	77.30	2.45
	Uric	31.65	2.61	39.87	3.60
Sonicated[4]	Acetic	0.00		0.00	
	Butyric	[a]28.76	3.90	[a]73.99	25.76
	Formic	[a]86.03	1.71	0.00	
	Lactic	[a]8006.09	1337.33	[a]12160.25	691.09
	Orotic	121.78	14.82	114.85	15.92
	Propionic	[a]0.00		[a]37.09	3.78
	Uric	30.11	3.66	[a]35.06	3.06

[1]Initial – Sample taken immediately after inoculation

[2] Final – Sample taken when yoghurt reached pH 4.5, Control 3h 20 min, Sonicated 4h 25 min

[3]Control – yoghurt made with 1% of *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *L. acidophilus* and *B. infantis* 1912

[4]Sonicated - yoghurt made with 1% *L. delbrueckii* subsp. *bulgaricus* sonicated 4 minutes with 1% of *S. thermophilus*, *L. acidophilus* and *B. infantis* 1912

[a] Statistically significant ($P<0.05$) as compared to control.

Table 14: The effect of sonicating *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515 cultures has on organic acid content in yoghurt during storage.

		During Storage							
		Week 1		Week 2		Week 3		Week 4	
		Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.
		----µg/g of yoghurt----							
Control[1]	Acetic	0.00		0.00		0.00		0.00	
	Butyric	154.95	13.72	157.59	21.39	152.72	14.99	167.57	32.46
	Formic	0.00		0.00		0.00		0.00	
	Lactic	17364.70	1649.81	17516.78	496.80	17011.52	686.31	16814.18	934.13
	Orotic	97.46	11.63	92.65	10.09	93.48	8.81	89.88	2.86
	Propionic	102.56	5.51	97.65	18.68	87.16	3.24	97.41	19.07
	Uric	40.67	3.87	33.17	3.26	38.32	3.22	45.10	10.53
Sonicated[2]	Acetic	0.00		0.00		0.00		0.00	
	Butyric	[a]80.56	15.99	[a]123.49	14.68	[a]107.54	23.94	[a]106.58	22.77
	Formic	0.00		0.00		0.00		0.00	
	Lactic	[a]15145.65	1409.96	[a]15430.89	470.30	[a]15425.11	553.34	[a]14548.56	731.20
	Orotic	114.92	16.09	[a]109.53	1.37	[a]112.06	15.19	[a]106.06	0.69
	Propionic	[a]49.80	2.32	[a]49.01	0.43	[a]47.09	3.90	[a]50.52	7.26
	Uric	39.29	3.59	[a]27.72	1.50	[a]32.88	2.70	36.17	3.18

[1]Control – yoghurt made with 1% of *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *L. acidophilus* and *B. infantis* 1912

[2]Sonicated - yoghurt made with 1% *L. delbrueckii* subsp. *bulgaricus* sonicated for 4 minutes with 1% of *S. thermophilus*, *L. acidophilus* and *B. infantis* 1912

[a]Stastistically significant ($P<0.05$) as compared to control.

Table 15. Average zone of inhibition produced by *L. acidophilus* (LA-5, LA-2404, LA-2405, LA-2406) against target organism *L. delbrueckii* subsp. *bulgaricus* (LB-2501 and 2515)

Strain	Zone of inhibition (mm)[1]			
	LA-5	LA-2404	LA-2405	LA-2406
LB 2515[2]	**14.00 ± 2.18**	13.83 ± 1.65	9.13 ± 0.88	13.08 ± 0.59
LB 2501[3]	13.33 ± 0.47	13.00 ± 0.59	9.14 ± 0.20	11.67 ± 0.47

[1]Zone of inhibition includes 6mm well diameter
[2] *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515
[3] *Lactobacillus delbrueckii* subsp. *bulgaricus* 2501

Table 16. Sensitivity of bacteriocin produced by *L. acidophilus* LA-5 against *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515 a target organism to various enzymes and pH

Treatment	Zone of inhibition[1] (mm)
Control	13.00 ± 0.00
pH[2]	13.33 ± 0.58
Catalase	11.67 ± 0.58
Papain	nil
Proteinase K	nil
Crude Protease	nil

[1]Zone of inhibition includes 6mm well diameter

[2] pH neutralised to 6.0

Table 17. Antagonism between yoghurt and probiotic bacteria

	Producer organism				
Target organism	LA-5	LB-2515	ST-2014	BB-1912	BB-1941
	----------*Zone of inhibition*[1] *(mm)*----------				
LA-5[3]	NA[2]	16.67 ± 0.29	nil	18.67 ± 2.23	17.58 ± 0.38
LB-2515[4]	16.67 ± 1.23	NA	nil	21.67 ± 1.13	18.08 ± 1.38
ST-2014[5]	8.00 ± 0.00	nil	NA	19.75 ± 3.19	19.83 ± 0.63
BB-1912[6]	nil	8.67 ± 1.15	8.00 ± 0.00	NA	NA
BB-1941[7]	8.00 ± 0.00	8.00 ± 0.00	8.33 ± 0.58	NA	NA

[1]zone includes 6mm well diameter
[2]NA- not applicable
[3]*Lactobacillus acidophilus* LA-5
[4]*Lactobacillus delbrueckii* subsp. *bulgaricus* 2515
[5]*Streptococcus thermophilus* 2014
[6]*Bifidobacterium infantis* 1912
[7]*Bifidobacterium longum* 1941

Table 18. Nature of inhibitory substance produced by yoghurt and probiotic bacteria to various enzymes and pH[1]

Lactic acid bacteria		Zone of inhibition[2] (mm)					
Producer organism	Target organism	Control	pH[3]	Catalase	Papain	Proteinase K	Crude Protease
La-5[4]	Lb2515	9.67 ± 0.58	8.33 ± 0.58	7.00	nil	nil	nil
Lb2515[5]	La-5	8.00	8.00	8.50 ± 0.58	8.33 ± 0.58	8.00	8.00
St2014[6]	La-5	8.00 ± 1.00	9.33 ± 1.15	9.00 ± 1.00	9.00	8.33 ± 0.58	9.33 ± 0.58
BB1912[7]	La-5	8.33 ± 0.58	8.33 ± 0.58	7.67 ± 0.58	8.00	8.33 ± 0.58	8.00
BB1941[8]	La-5	8.33 ± 0.58	8.00	8.33 ± 0.58	8.33 ± 0.58	8.33 ± 0.58	8.33 ± 0.58

[1]No zones of inhibition were observed by other probiotic and yoghurt bacteria listed in Table 20.

[2]zone includes 6mm well diameter

[3]pH neutralised to 6.0

[4]*Lactobacillus acidophilus* LA-5

[5]*Lactobacillus delbrueckii* subsp. *bulgaricus* 2515

[6]*Streptococcus thermophilus* 2014

[7]*Bifidobacterium infantis* 1912

[7]*Bifidobacterium longum* 1941

Table 19. Changes in viable counts when *Lactobacillus delbrueckii* subsp. *bulgaricus* is grown with different inoculum sizes of *L. acidophilus*.

		Time (hours)				
LA Inoculation		0	2	4	6	8
		------CFU/mL------				
1%[1]	LA[4]	2.12×10^7	4.35×10^7	7.60×10^8	7.83×10^8	8.68×10^8
	LB[5]	7.55×10^6	1.72×10^7	2.17×10^8	3.74×10^8	3.31×10^8
5%[2]	LA	1.20×10^8	1.83×10^8	1.00×10^9	7.90×10^8	8.15×10^8
	LB	3.86×10^7	9.84×10^7	3.06×10^8	3.51×10^8	3.38×10^8
10%[3]	LA	1.82×10^8	3.90×10^8	8.58×10^8	6.28×10^8	8.45×10^8
	LB	6.71×10^7	1.36×10^8	2.71×10^8	2.93×10^8	2.61×10^8

[1]1% - Sample containing 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus* and 1% *L. acidophilus*
[2]5% - Sample containing 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 5% *L. acidophilus*
[3]10% - Sample containing 1%*Lactobacillus delbrueckii* subsp. *bulgaricus* and 10% *L. acidophilus*
[4]LA- *Lactobacillus acidophilus*
[5]LB – *Lactobacillus delbrueckii* subsp. *bulgaricus*

Table 20. Changes in viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* when grown with different levels of concentrated bacteriocin.

	Time (hours)		
	0	6	10
		----CFU/mL----	
Control[1]	4.04×10^5	1.46×10^7	8.85×10^7
1%[2]	3.66×10^5	1.57×10^5	1.71×10^4
5%[3]	2.96×10^5	5.84×10^2	8.19×10^2
10%[4]	4.53×10^5	4.39×10^2	2.78×10^2

[1]Control - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus*
[2]1% - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 1% bacteriocin
[3]5% - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 5% bacteriocin
[4]10% - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 10% bacteriocin

Table 21. The effect on viable counts when growing *Lactobacillus delbrueckii* subsp. *bulgaricus* with different levels of bacteriocin (1st Replicate)

	Time (hours)				
	0	2	4	6	8
	----CFU/mL----				
Control[1]	$2.85x10^5$	$4.35x10^5$	$8.30x10^5$	$5.40x10^6$	$1.40x10^7$
0.5%[2]	$3.50x10^5$	$8.55x10^4$	$1.36x10^5$	$7.10x10^5$	$2.90x10^6$
1%[3]	$1.64x10^5$	$1.67x10^4$	$1.64x10^4$	$2.76x10^4$	$1.06x10^5$
2%[4]	$1.41x10^5$	$6.35x10^3$	$2.20x10^3$	$4.05x10^3$	$5.05x10^3$

[1]Control - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

[2]0.5% - 0.5% bacteriocin and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

[3]1% - 1% bacteriocin and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

[4]2% - 2% bacteriocin and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

Table 22. The effect on viable counts when growing *Lactobacillus delbrueckii* subsp. *bulgaricus* with different levels of bacteriocin (2nd Replicate)

	Time (hours)				
	0	2	4	6	8
	----CFU/mL----				
Control[1]	$5.90x10^5$	$5.45x10^5$	$9.50x10^5$	$7.55x10^6$	$2.00x10^7$
0.5%[2]	$5.35x10^5$	$3.36x10^5$	$4.45x10^5$	$2.82x10^6$	$1.02x10^7$
1%[3]	$5.10x10^5$	$2.78x10^5$	$4.10x10^5$	$2.51x10^6$	$6.72x10^6$
2%[4]	$4.45x10^5$	$1.09x10^5$	$9.50x10^4$	$2.54x10^5$	$5.38x10^5$

[1]Control - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

[2]0.5% - 0.5% bacteriocin and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

[3]1% - 1% bacteriocin and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

[4]2% - 2% bacteriocin and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

Table 23. The effect on viable counts when growing *Lactobacillus delbrueckii* subsp. *bulgaricus* with different levels of bacteriocin (3rd Replicate)

	Time (hours)				
	0	2	4	6	8
	----CFU/mL----				
Control[1]	$4.54x10^5$	$8.30x10^5$	$5.70x10^6$	$3.45x10^7$	$1.33x10^8$
0.5%[2]	$7.25x10^5$	$2.60x10^5$	$2.16x10^6$	$1.26x10^7$	$3.75x10^7$
1%[3]	$6.15x10^5$	$2.66x10^5$	$1.23x10^6$	$5.90x10^6$	$1.87x10^7$
2%[4]	$6.35x10^5$	$1.37x10^5$	$5.50x10^5$	$4.00x10^6$	$1.40x10^7$

[1]Control - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

[2]0.5% - 0.5% bacteriocin and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

[3]1% - 1% bacteriocin and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

[4]2% - 2% bacteriocin and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* inoculated in MRS broth

Table 24. Effects of incorporation of bacteriocin on survival of *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *L. acidophilus* and bifidobacteria during fermentation of yoghurt

		Initial[1]		Final[2]		
	Bacteria	pH	Count	pH	Count	Fermentation time (hours)
			----CFU/mL----			
Control[3]	ST[6]	6.07	$3.38x10^7$	4.44	$7.07x10^8$	3
	LB[7]		$4.98x10^6$		$7.75x10^7$	
	LA[8]		$2.78x10^6$		$7.40x10^6$	
	BB[9]		$8.65x10^5$		$8.68x10^5$	
1%[4]	ST	5.98	$3.68x10^7$	4.39	$7.18x10^8$	3
	LB		$4.90x10^6$		$8.72x10^7$	
	LA		$2.70x10^6$		$6.62x10^6$	
	BB		$7.95x10^5$		$8.60x10^5$	
2%[5]	ST	6.04	$4.23x10^7$	4.32	$5.20x10^8$	3
	LB		$4.48x10^6$		$5.82x10^7$	
	LA		$2.93x10^6$		$4.65x10^6$	
	BB		$8.20x10^5$		$1.00x10^6$	

[1]Initial- Sample taken immediately after inoculation

[2] Final – Sample was taken when yoghurt reached pH 4.5. All fermentation ended after 3 hours

[3]Control - contains 1% each of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium longum*

[4]1% - contains 1% bacteriocin and 1% each of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *B. longum*

[5]2% - contains 2% bacteriocin and 1% each of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *B. longum*

[6]ST – *Streptococcus thermophilus* 2014

[7]LB – *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515

[8]LA – *Lactobacillus acidophilus* LA-5

[9]BB – *Bifidobacterium longum*1941

Table 25. Effects of incorporation of bacteriocin on survival of *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *L. acidophilus* and *B. longum* during weeks 1-3 of storage.

		Week 1		Week 2		Week 3	
	Bacteria	pH	Count	pH	Count	pH	Count
			----CFU/mL----				
Control[1]	ST[4]	4.22	8.02×10^8	4.09	4.70×10^8	4.17	6.82×10^8
	LB[5]		6.38×10^7		1.76×10^7		9.35×10^6
	LA[6]		2.67×10^4		3.25×10^2		3.83×10^1
	BB[7]		6.92×10^5		5.53×10^5		3.33×10^4
1%[2]	ST	4.21	6.90×10^8	4.12	4.60×10^8	4.13	5.53×10^8
	LB		5.22×10^7		2.06×10^7		2.59×10^7
	LA		9.10×10^5		3.95×10^4		3.23×10^3
	BB		7.53×10^5		4.72×10^5		2.33×10^4
2%[3]	ST	4.24	5.97×10^8	4.09	4.18×10^8	4.16	4.10×10^8
	LB		3.98×10^7		2.27×10^7		3.83×10^6
	LA		1.28×10^6		4.37×10^4		1.58×10^3
	BB		5.65×10^5		3.73×10^5		1.46×10^4

[1]Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B. longum*

[2]1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B. longum*

[3]2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B. longum*

[4]ST – *Streptococcus thermophilus* 2014

[5]LB – *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515

[6]LA – *Lactobacillus acidophilus* LA-5

[7]BB – *B. longum* 1941

Table 26. Effects of incorporation of bacteriocin on survival of *Lactobacillus delbrueckii* subsp. *bulgaricus, S. thermophilus, L. acidophilus* and *B. longum* during weeks 4-6 of storage.

		Week 4		Week 5		Week 6	
	Bacteria	pH	Count	pH	Count	pH	Count
			----CFU/mL----				
Control[1]	ST[4]	4.17	$6.53x10^8$	4.18	$7.38x10^8$	4.14	$6.43x10^8$
	LB[5]		$4.22x10^5$		$2.58x10^2$		15.80
	LA[6]		2.33		9.70		47.50
	BB[7]		$2.38x10^5$		$1.96x10^4$		$1.44x10^4$
1%[2]	ST	4.12	$6.00x10^8$	4.15	$6.87x10^8$	4.12	$5.63x10^8$
	LB		$1.96x10^5$		$4.48x10^2$		24.80
	LA		$7.00x10^1$		59.70		32.80
	BB		$2.25x10^5$		$1.79x10^4$		$1.04x10^4$
2%[3]	ST	4.14	$6.07x10^8$	4.15	$7.42x10^8$	4.13	$4.75x10^8$
	LB		$1.30x10^4$		$1.87x10^2$		44.50
	LA		40.25		64.70		48.00
	BB		$2.20x10^5$		$1.80x10^4$		$1.01x10^4$

[1]Control – contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B. longum*
[2]1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B. longum*
[3]2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B. longum*
[4]ST – *Streptococcus thermophilus* 2014
[5]LB – *Lactobacillus delbrueck*ii subsp. *bulgaricus* 2515
[6]LA – Lactobacillus acidophilus LA-5
[7]BB – *B. longum* 1941

Table 27. The effects of incorporating bacteriocin in yoghurt on production of organic acid during fermentation.

		Initial[1]		Final[2]	
		Average	St. Dev.	Average	St. Dev.
		----------µg/g of yoghurt----------			
Control[3]	Acetic	0.00		0.00	
	Butyric	0.00		40.57	9.09
	Formic	468.16	193.68	92.63	62.11
	Lactic	979.61	224.70	10395.41	912.74
	Orotic	176.81	16.70	136.05	18.12
	Propionic	0.00		50.65	15.82
	Uric	47.21	2.40	43.57	2.67
1%[4]	Acetic	0.00		0.00	
	Butyric	0.00		[a]66.56	12.12
	Formic	455.06	13.05	37.55	0.26
	Lactic	1091.87	159.55	10755.14	1240.81
	Orotic	155.26	18.08	132.26	1.64
	Propionic	0.00		65.68	22.64
	Uric	[a]43.15	1.36	44.20	1.45
2%[5]	Acetic	0.00		0.00	
	Butyric	0.00		30.76	6.69
	Formic	485.60	22.68	[a]16.22	0.11
	Lactic	1096.25	121.37	10135.47	135.01
	Orotic	[a]142.41	3.83	131.62	2.27
	Propionic	0.00		45.53	8.93
	Uric	[a]41.68	1.21	42.93	0.17

[1]Initial- sample taken immediately after inoculation

[2] Final – sample taken when yoghurt reached pH 4.5. All fermentation completed after 3 hours

[3]Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B. longum 1941*

[4]1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B. longum 1941*

[5]2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *B. longum 1941*

[a] Statistically significant ($P<0.05$) as compared to the control

Table 28. The effects of incorporating bacteriocin in yoghurt on production of organic acids during storage.

		Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
		Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
		----µg/g of yoghurt----											
Control[3]	Acetic	15.97	3.51	18.45	4.49	19.27	4.28	18.52	3.12	16.88	2.85	16.76	3.94
	Butyric	62.05	8.57	74.61	10.85	70.97	8.72	78.02	12.03	77.46	14.14	77.17	8.07
	Formic	0.00		0.00		0.00		0.00		0.00		0.00	
	Lactic	11551.14	554.80	12353.85	92.77	12005.96	1080.65	12314.28	1368.38	12724.72	609.31	12732.78	336.85
	Orotic	135.23	14.50	147.41	17.00	142.27	27.42	151.37	24.22	149.84	14.89	143.66	19.55
	Propionic	69.85	12.19	75.82	13.68	76.29	1.96	80.53	12.56	85.31	5.27	86.20	2.67
	Uric	43.76	2.76	44.19	3.32	42.19	4.71	42.62	5.72	43.88	1.85	44.24	2.28
1%[4]	Acetic	[a]0.00		[a]0.00		[a]0.00		[a]0.00		[a]0.00		[a]0.00	
	Butyric	[a]83.56	15.05	[a]96.75	17.01	[a]94.52	13.41	[a]101.74	16.90	[a]101.93	14.14	[a]106.55	8.07
	Formic	0.00		0.00		0.00		0.00		0.00		0.00	
	Lactic	[a]11732.25	779.51	12447.59	686.12	13225.35	765.80	12773.56	513.75	12379.55	499.91	12815.05	560.54
	Orotic	124.32	4.78	[a]125.85	4.08	138.75	13.48	[a]123.95	4.48	[a]122.82	3.78	127.61	4.41
	Propionic	80.09	13.77	84.18	17.77	89.91	14.32	87.20	15.07	89.11	4.61	[a]92.82	6.26
	Uric	43.44	1.63	43.56	0.94	43.95	4.55	41.92	2.94	41.55	2.04	41.87	2.18
2%[5]	Acetic	[a]0.00		[a]0.00		0.00		0.00		0.00		0.00	
	Butyric	69.28	11.16	74.49	17.71	76.91	15.35	80.89	18.00	80.66	19.69	93.78	26.58
	Formic	0.00		0.00		0.00		0.00		0.00		0.00	
	Lactic	[a]12108.09	170.36	11928.79	623.68	12475.04	368.05	12324.88	296.53	12659.84	130.61	12526.09	425.86
	Orotic	132.76	1.87	[a]127.25	4.99	130.17	3.54	126.47	3.37	[a]129.77	2.60	128.50	1.28
	Propionic	61.31	7.02	[a]55.54	10.31	[a]61.36	9.82	[a]60.67	6.96	[a]62.22	9.47	[a]67.48	7.25
	Uric	44.80	0.78	42.94	2.07	42.58	0.36	40.47	2.46	[a]41.64	0.36	[a]38.90	4.58

[1]Initial- sample taken immediately after inoculation

[2] Final – sample taken when yoghurt reached pH 4.5. All fermentation completed after 3 hours

[3]Control – contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

[4]1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

[5]2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

[a] Statistically significant ($P<0.05$) as compared to the control

Table 29: 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* grown with different inoculation of bacteriocin in 12% reconstituted skim milk.

	0	2	4	6	8
			----------CFU/mL----------		
LB[1]	$5.03x10^5$	$4.23x10^5$	$1.73x10^7$	$2.13x10^7$	$3.70x10^7$
1%[2]	$6.05x10^5$	$4.73x10^5$	$2.35x10^7$	$3.27x10^7$	$3.91x10^7$
5%[3]	$3.43x10^5$	$2.90x10^5$	$1.72x10^7$	$3.36x10^7$	$3.13x10^7$
10%[4]	$3.08x10^5$	$2.80x10^5$	$1.96x10^7$	$3.39x10^7$	$2.80x10^7$

[1]LB - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus*
[2]1% - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 1% bacteriocin
[3]5% - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 5% bacteriocin
[4]10% - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 10% bacteriocin

Table 30: Effects of bacteriocin produced from *Lactobacillus acidophilus*- LA-5 on *Lactobacillus delbrueckii* subsp. *bulgaricus* -2515 after growing in different levels of milk.

	Zone of Inhibition (mm)[1]		
Time (hours)	3%[2]	6%[3]	12%[4]
0	0	0	0
2	10.33	0	0
4	12.67	0	0
6	10.00	8.33	0
18	NA[5]	NA	0

[1]Zone includes 6 mm well diameter
[2]3% - milk contains 3% reconstituted skim milk powder
[3]6% - milk contains 6% reconstituted skim milk powder
[4]12% - milk contains12% reconstituted skim milk powder
[5]NA – not applicable

Table 31: The effects of bacteriocin on the viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* when grown in 12% and 3% skim milk.

Milk Level		Time (hours)	
		0	18
		----CFU/mL----	
12%[1]	LB[3]	37.00×10^4	33.50×10^6
	1%[4]	40.00×10^4	53.00×10^6
	5%[5]	39.00×10^4	11.700×10^7
	10%[6]	40.00×10^4	22.90×10^7
3%[2]	LB	46.00×10^4	34.00×10^5
	1%	34.50×10^4	115.50×10^5
	5%	39.00×10^4	57.50×10^5
	10%	42.00×10^4	49.00×10^5

[1]12%- milk containing 12% reconstituted skim milk powder
[2]3%- milk containing 3% reconstituted skim milk powder
[3]LB- sample contains 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* only
[4]1% - sample contains 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 1% bacteriocin
[5]5% - sample contains 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 5% bacteriocin
[6]10% - sample contains 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 10% bacteriocin

Table 32: The effect bacteriocin has on viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* when grown in different media.

Media	Time (hours)			
	0	2	4	6
	----------CFU/mL----------			
3%[1]	$1.08x10^5$	$2.75x10^5$	$4.05x10^6$	$6.45x10^6$
3%B[2]	$1.12x10^5$	$2.85x10^5$	$4.13x10^6$	$8.03x10^6$
NC[3]	$2.09x10^5$	$4.03x10^5$	$2.75x10^6$	$2.77x10^6$
NC-B[4]	$1.13x10^5$	$2.72x10^5$	$3.08x10^6$	$5.05x10^6$
MRS[5]	$6.20x10^5$	$6.53x10^5$	$9.13x10^5$	$9.90x10^6$
MRS-B[6]	$5.85x10^5$	$6.75x10^5$	$4.75x10^5$	$4.90x10^6$

[1]3%- 1% Lactobacillus delbrueckii subsp. bulgaricus in 3% RSM
[2]3%B- 1% Lactobacillus delbrueckii subsp. bulgaricus in 3% RSM and 1% bacteriocin
[3]NC- 1% Lactobacillus delbrueckii subsp. bulgaricus in 3% RSM with casein removed
[4]NC-B – 1% Lactobacillus delbrueckii subsp. bulgaricus in 3% RSM with casein removed and 1% bacteriocin
[5]MRS- 1% Lactobacillus delbrueckii subsp. bulgaricus in MRS broth
[6]MRS- 1% Lactobacillus delbrueckii subsp. bulgaricus in MRS broth and 1% bacteriocin

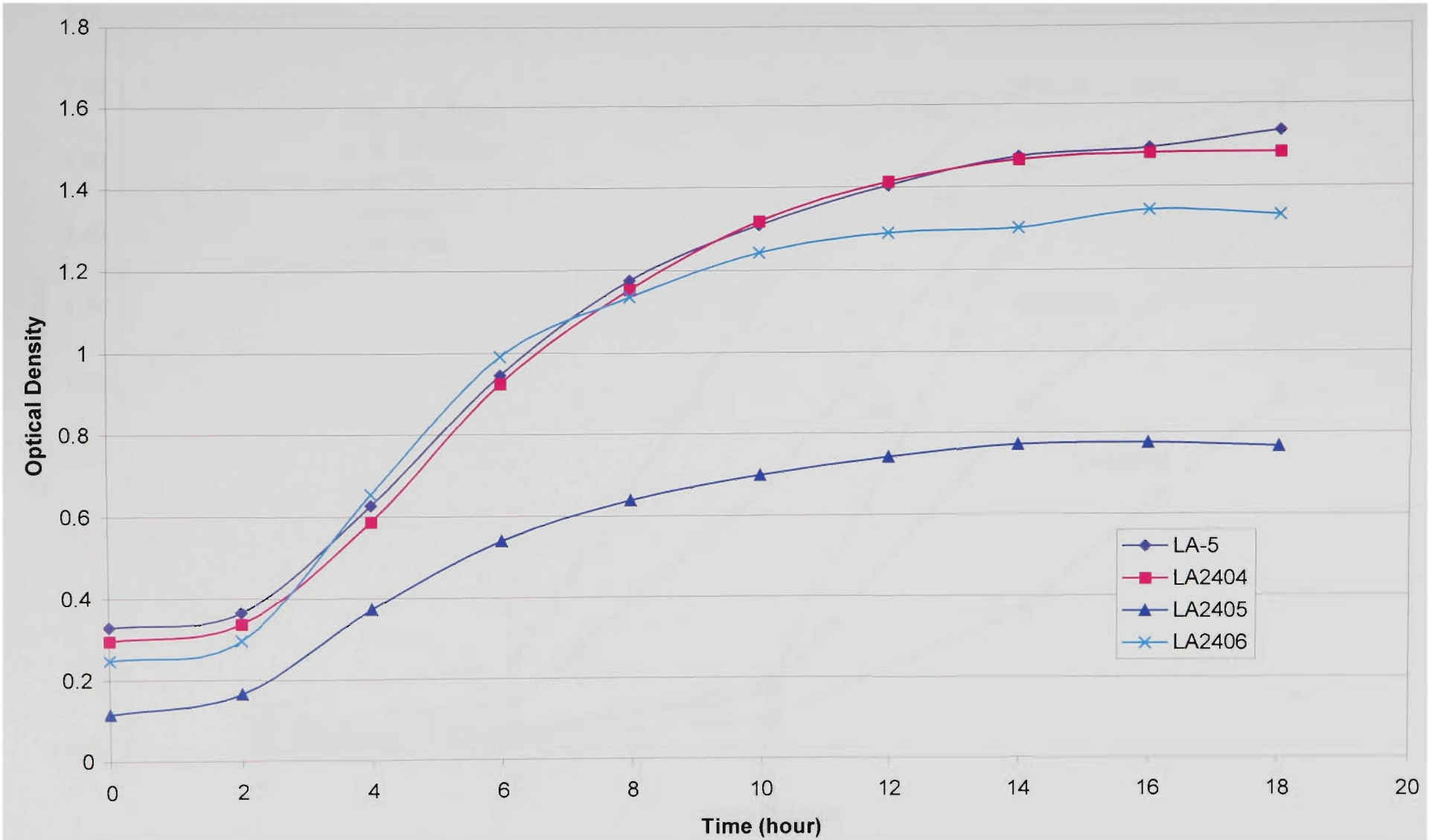

Figure 3. Growth curve of 4 strains of *L. acidophilus* over 18 hours as measured by optical density.
(LA-5 = *Lactobacillus acidophilus* LA-5, LA2404 = *Lactobacillus acidophilus* 2404, LA2405 = *Lactobacillus acidophilus* 2405, LA2406 = *Lactobacillus acidophilus* 2406)

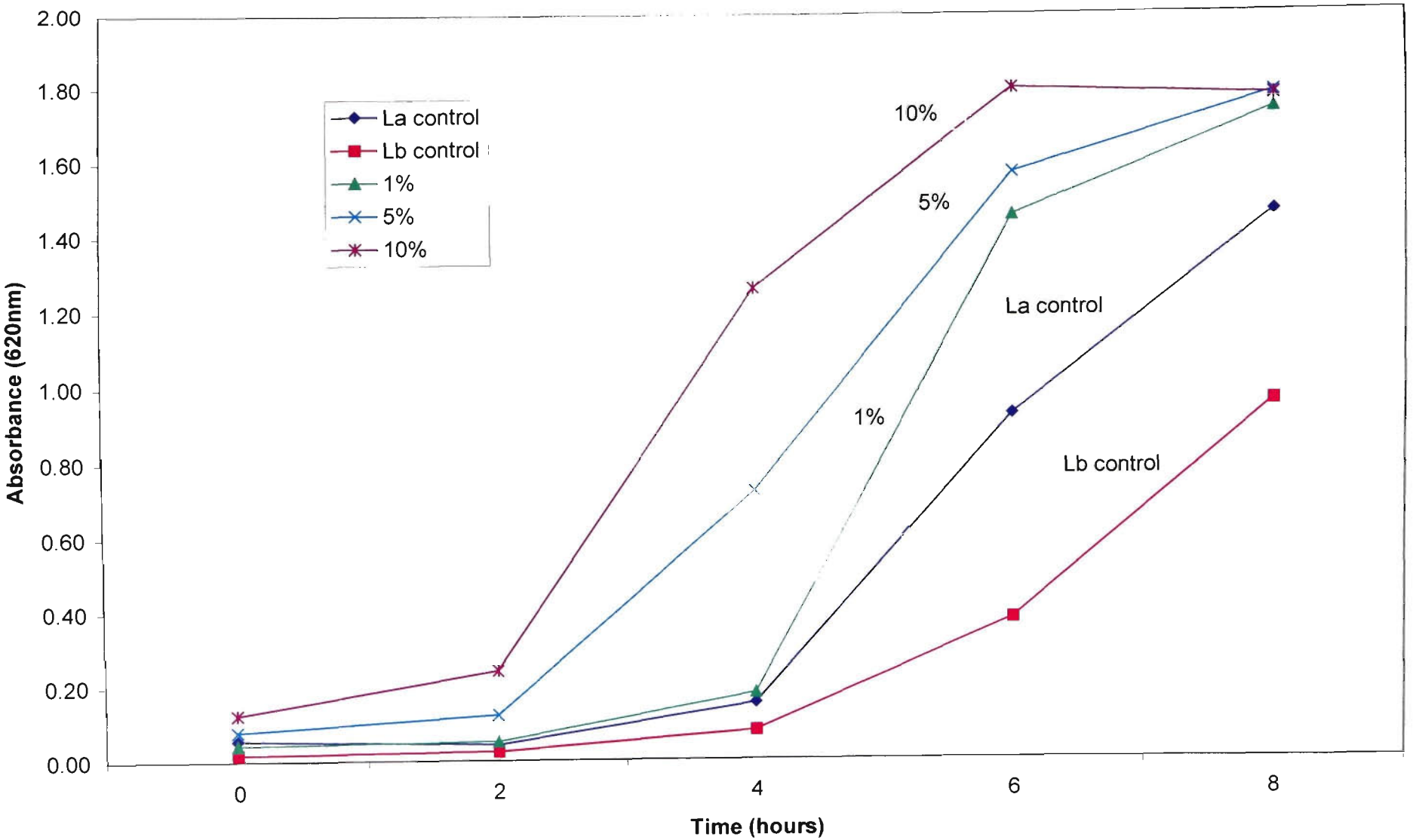

Figure 4. Changes in cell density of *L. acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* when grown together and separately. La control- 1% *L. acidophilus* only, LB control – 1%*Lactobacillus delbrueckii* subsp. *bulgaricus* only, 1%- 1% of *L. acidophilus* and 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*, 5% - 5% of *L. acidophilus* and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus*, 10%- 10% *L. acidophilus* and 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*.

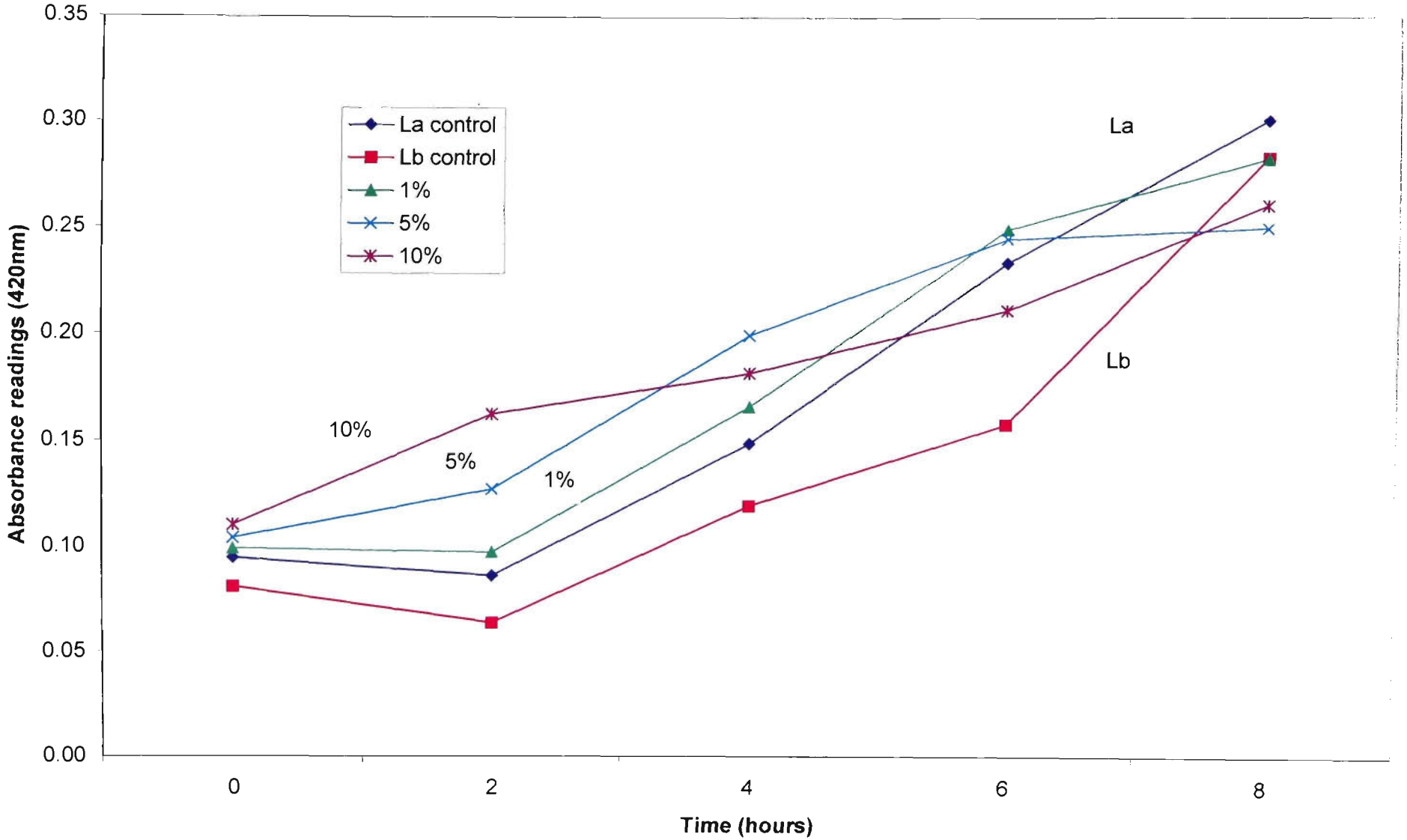

Figure 5. Changes in β-galactosidase concentration when *Lactobacillus delbrueckii* subsp. *bulgaricus* is grown with different inoculation sizes of *L. acidophilus*. La control- 1% *L. acidophilus* only, LB control – 1%*Lactobacillus delbrueckii* subsp. *bulgaricus* only, 1%- 1% of *L. acidophilus* and 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*, 5% - 5% of *L. acidophilus* and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus*, 10%- 10% *L. acidophilus* and 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*

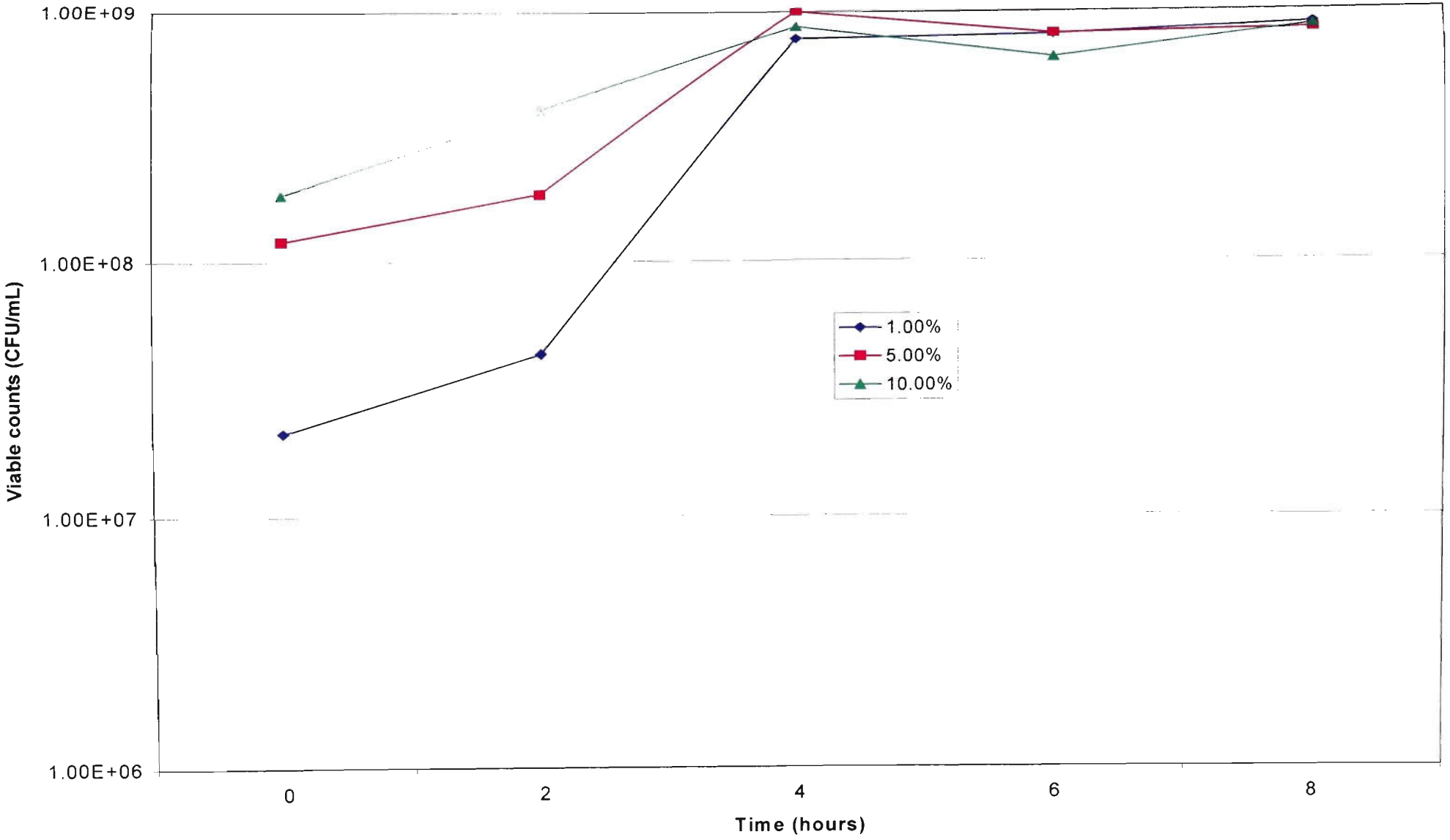

Figure 6. Changes in viable counts of *L. acidophilus* when grown with *Lactobacillus delbrueckii* subsp. *bulgaricus*. 1%- 1% of *L. acidophilus* and 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*, 5% - 5% of *L. acidophilus* and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus*, 10%- 10% *L. acidophilus* and 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*

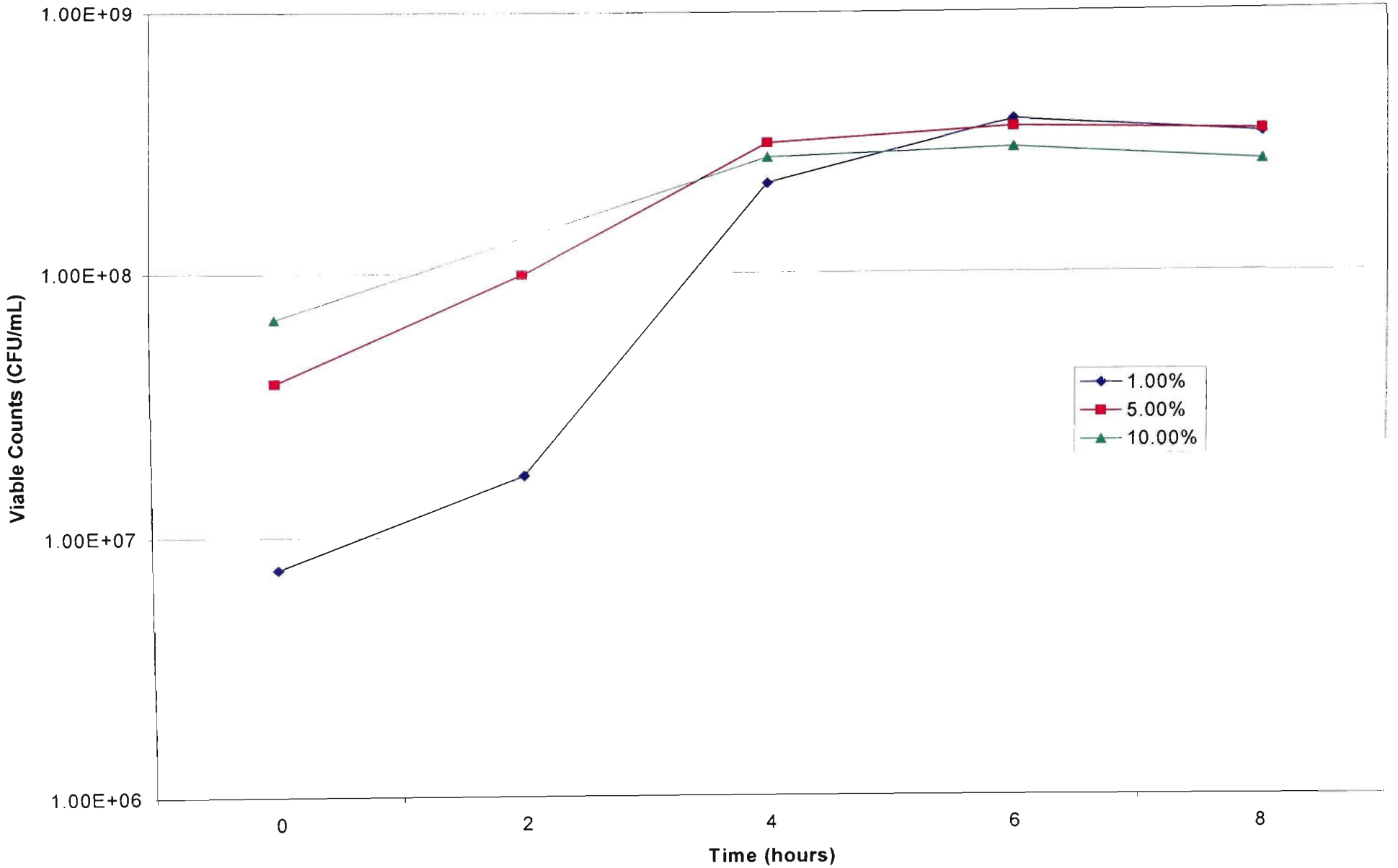

Figure 7. Changes in viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* when grown with different inoculum sizes of *L. acidophilus*. 1%- 1% of *L. acidophilus* and 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*, 5% - 5% of *L. acidophilus* and 1% *Lactobacillus delbrueckii* subsp. *bulgaricus*, 10%- 10% *L. acidophilus* and 1% of *Lactobacillus delbrueckii* subsp. *bulgaricus*

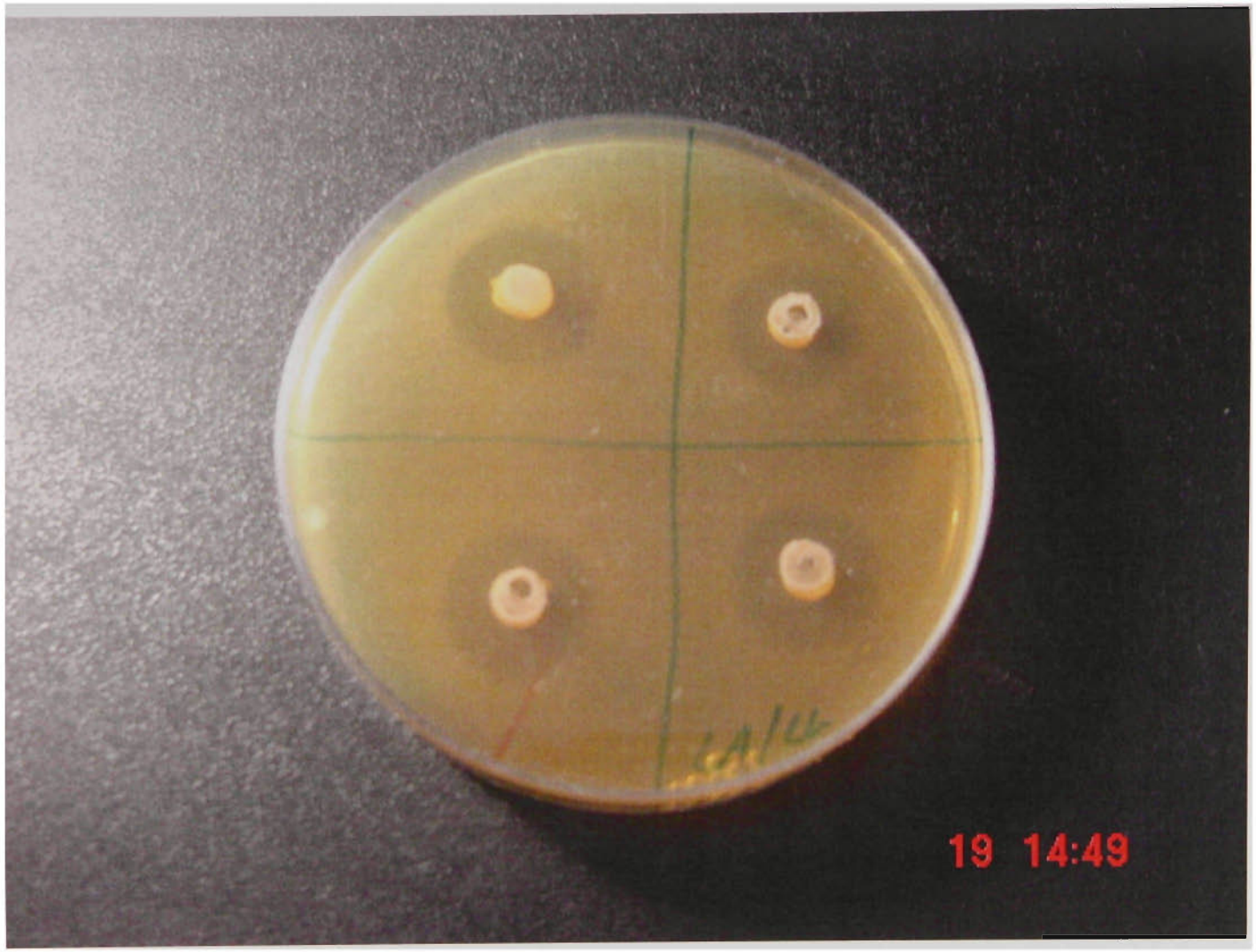

Figure 8: Zones of inhibition of *L. acidophilus* against *L. delbrueckii* subsp. *bulgaricus*.

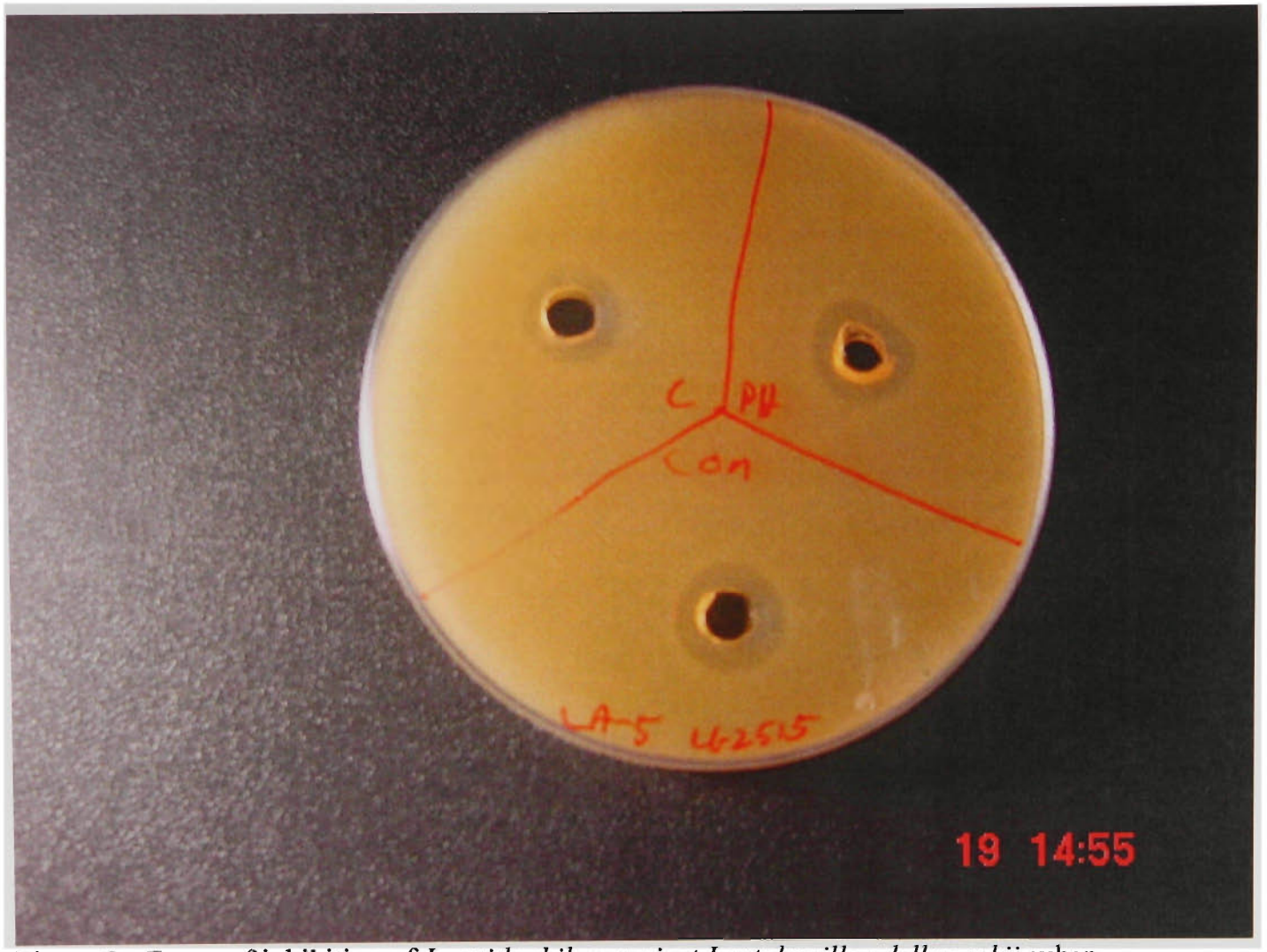

Figure 9 : Zones of inhibition of *L. acidophilus against Lactobacillus delbrueckii* subsp. *bulgaricus* before filtration, cells removed, neutralised to pH 6.0 and treated with catalase. Con- cells removed, pH- neutralised to pH 6.0, C- treated with catalase.

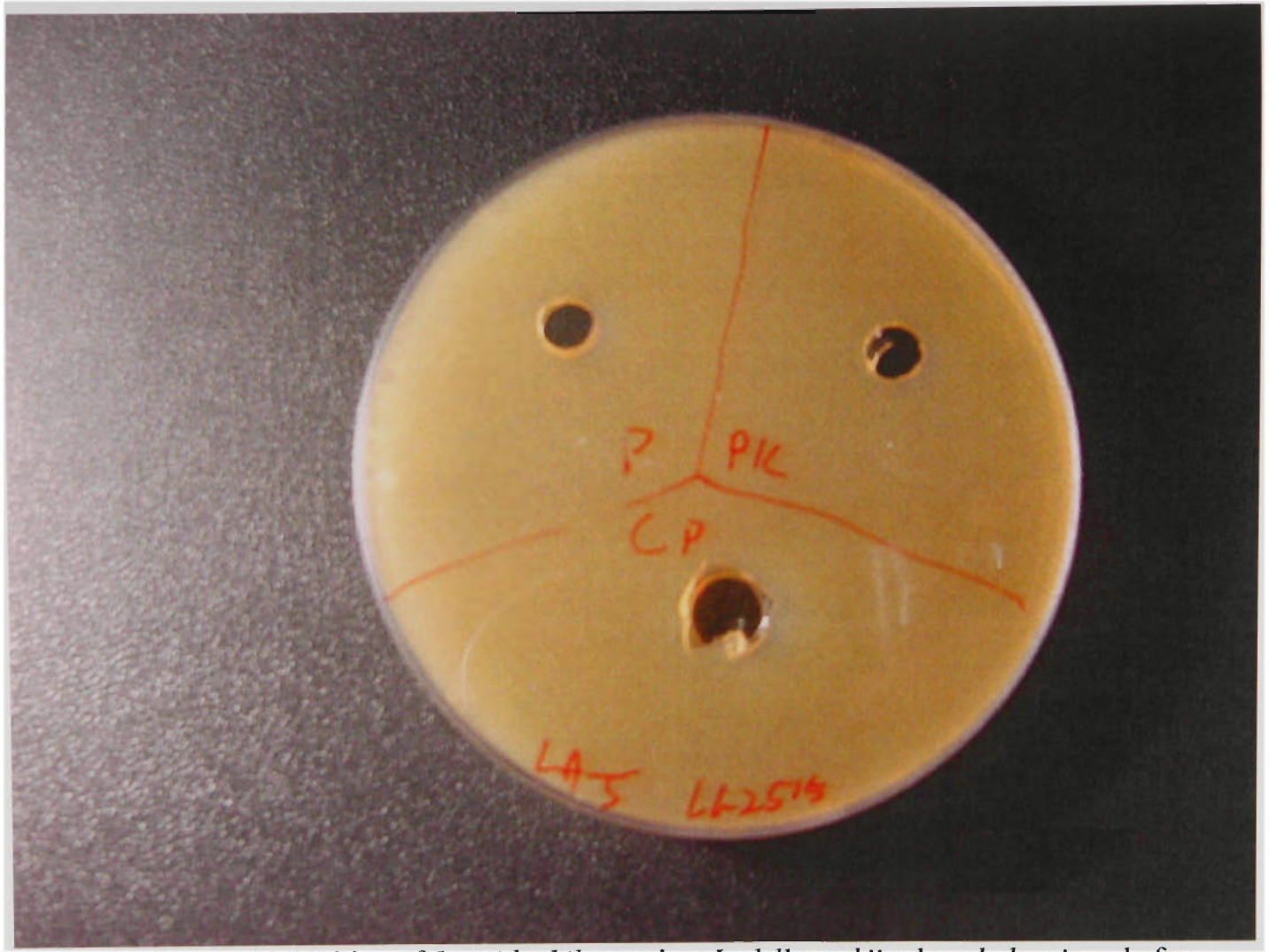

Figure 10: Zones of inhibition of *L. acidophilus* against *L. delbrueckii* subsp. *bulgaricus,* before filtration, treated with proteolytic enzymes. P- Papain, PK- Proteinase K, CP- Crude protease

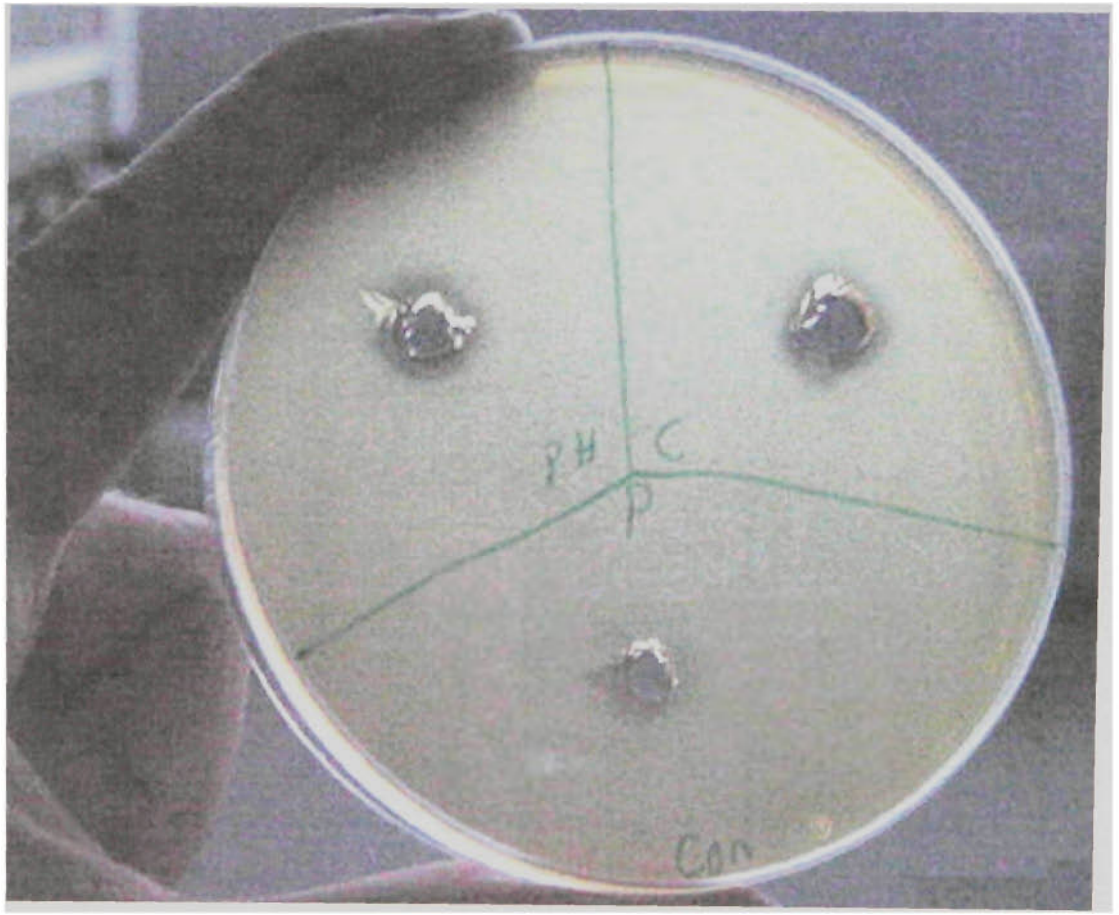

Figure 11: Zones of inhibition of the concentrate obtained by *L. acidophilus* against *L. delbrueckii* subsp. *bulgaricus* after passing through a 30kDa MWCO membrane. pH- pH adjusted 6.0, c- broth treated with catalase, P-broth treated with papain enzyme.

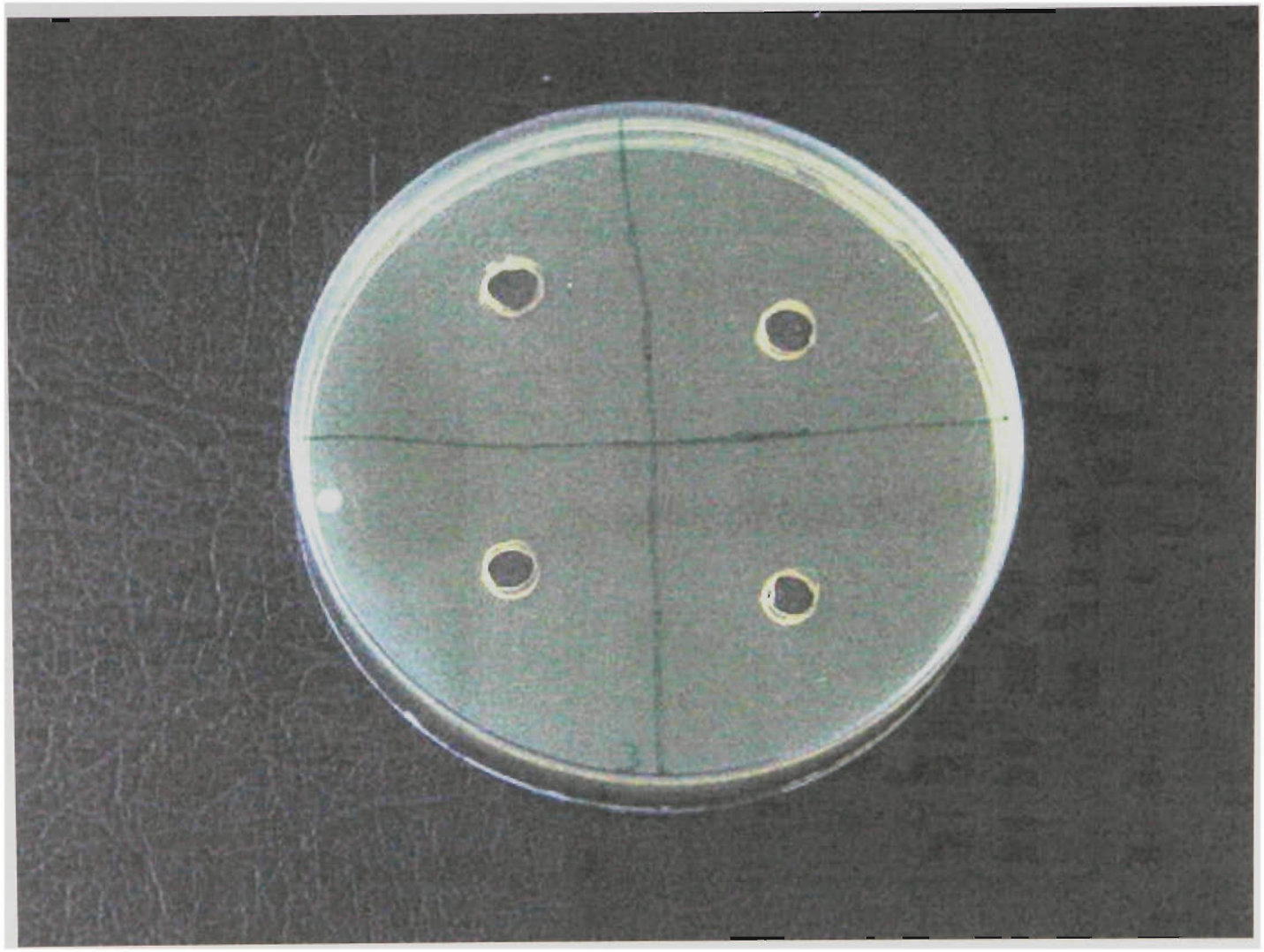

Figure 12: Zones of inhibition of the permeate obtained by *L. acidophilus* against *L delbrueckii* subsp. *bulgaricus* after passing through a 30kDa MWCO membrane

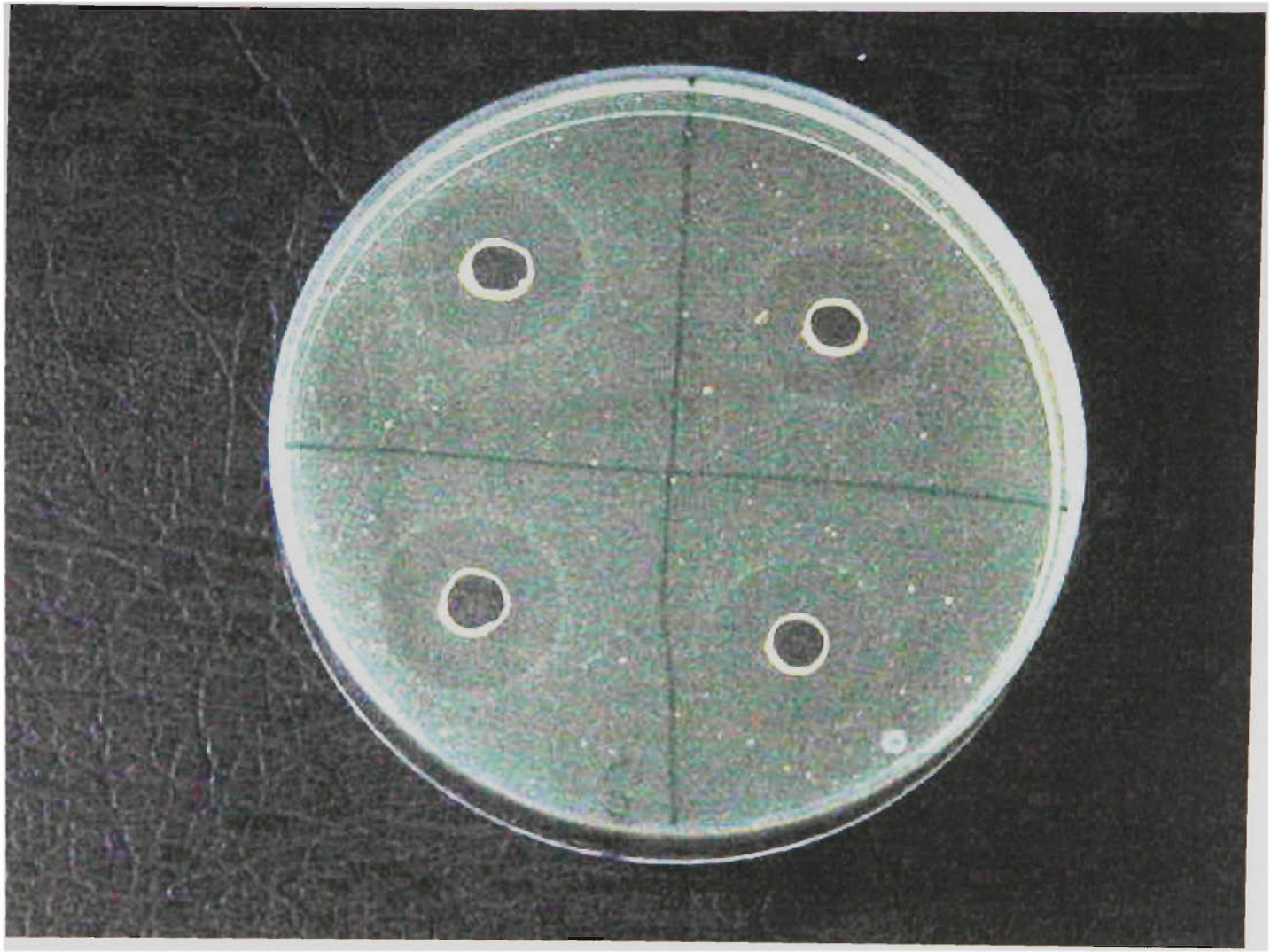

Figure 13: Zones of inhibition of the purified bacteriocin suspended in sodium carbonate before dialysis against *L. delbrueckii* subsp. *bulgaricus*.

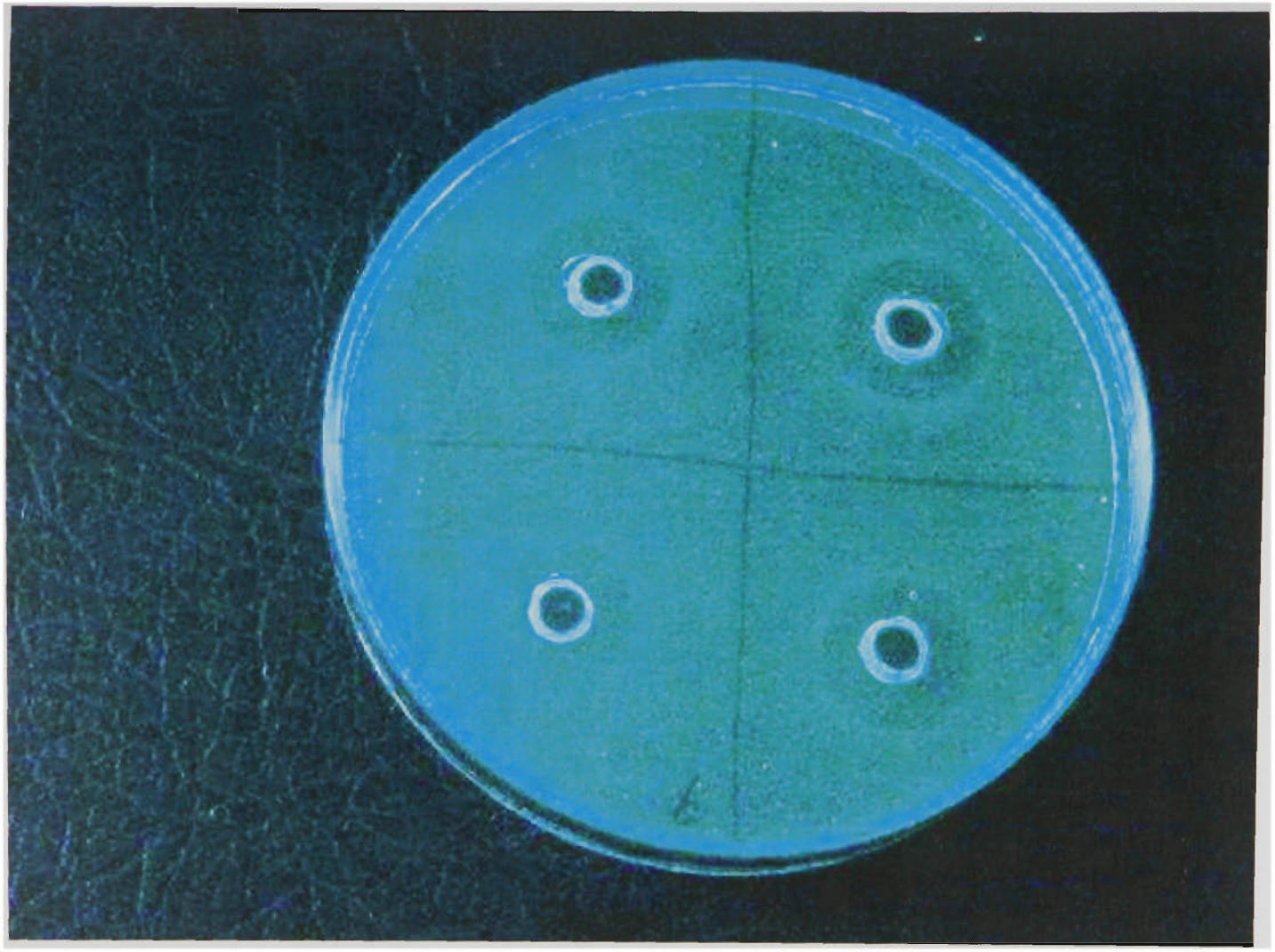

Figure 14: Zones of inhibition of the purified bacteriocin solution after dialysis against *L. delbrueckii* subsp. *bulgaricus*.

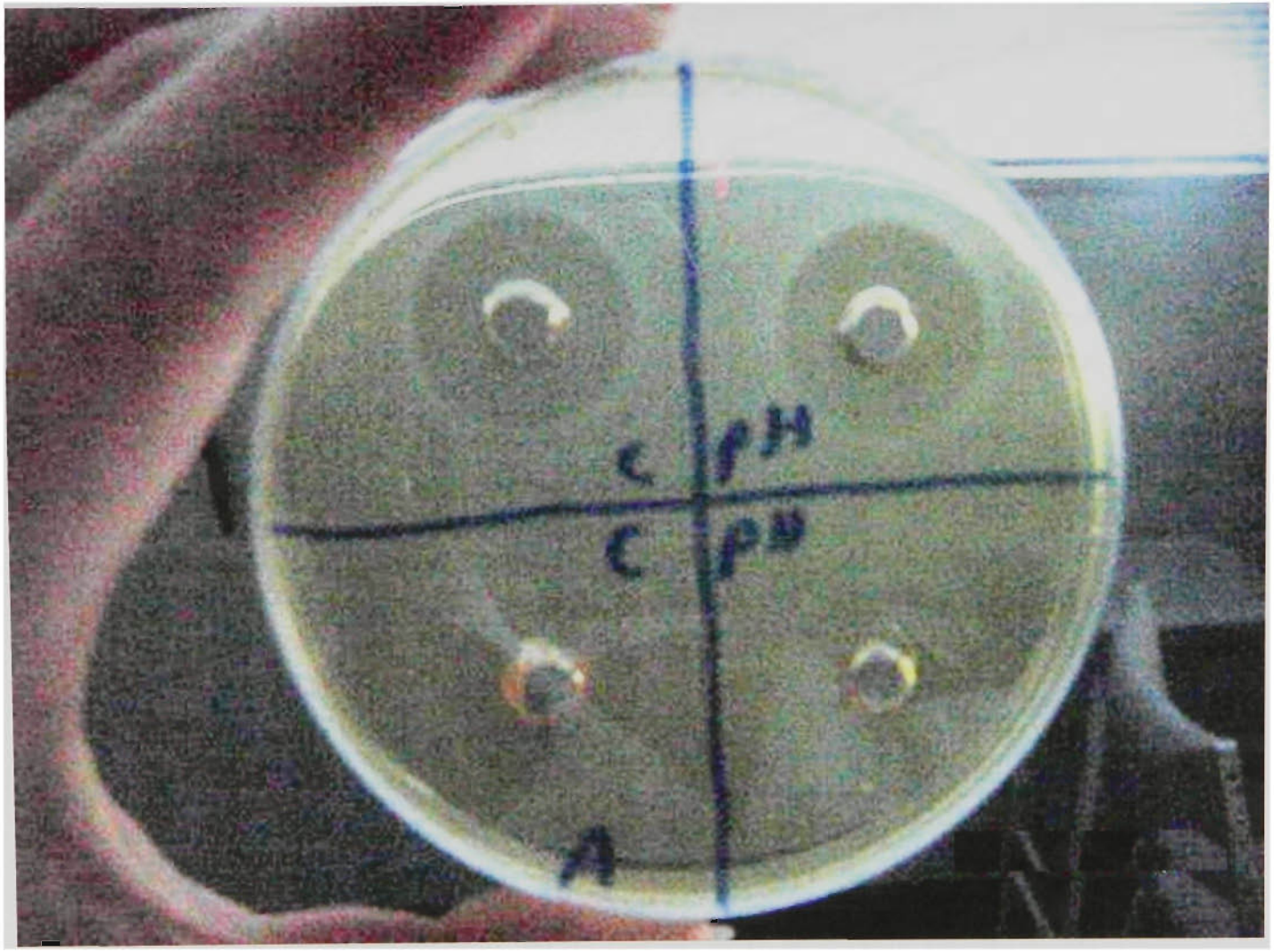

Figure 15: Zones of inhibition of autoclaved purified bacteriocin solution against *L. delbrueckii* subsp. *bulgaricus*.
pH-pH adjusted to 6.0, C- solution treated with catalase.

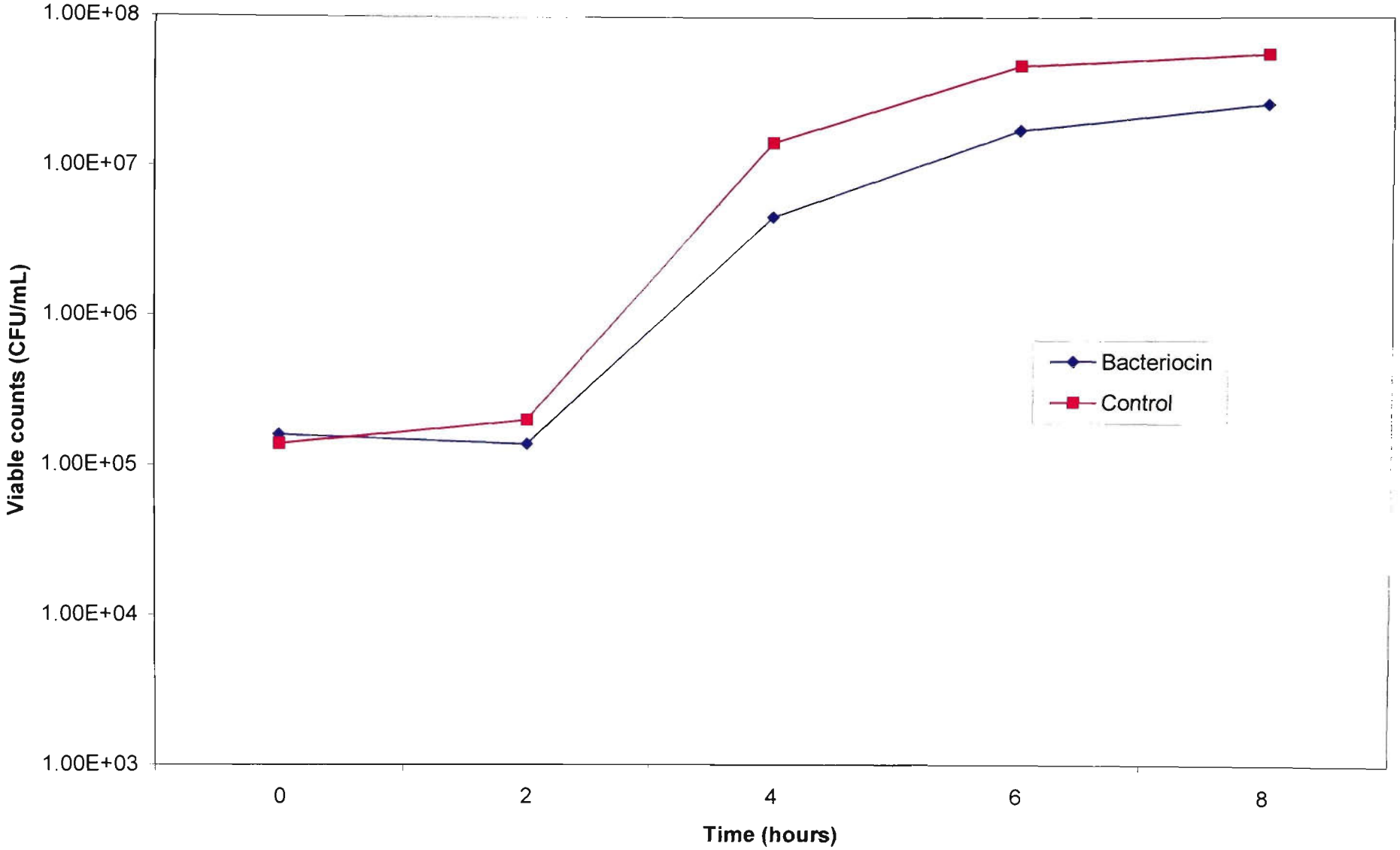

Figure 16. Effects of purified bacteriocin (5%) on the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Bacteriocin- 1%*Lactobacillus delbrueckii* subsp. *bulgaricus* and 5% bacteriocin, Control- 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* only.

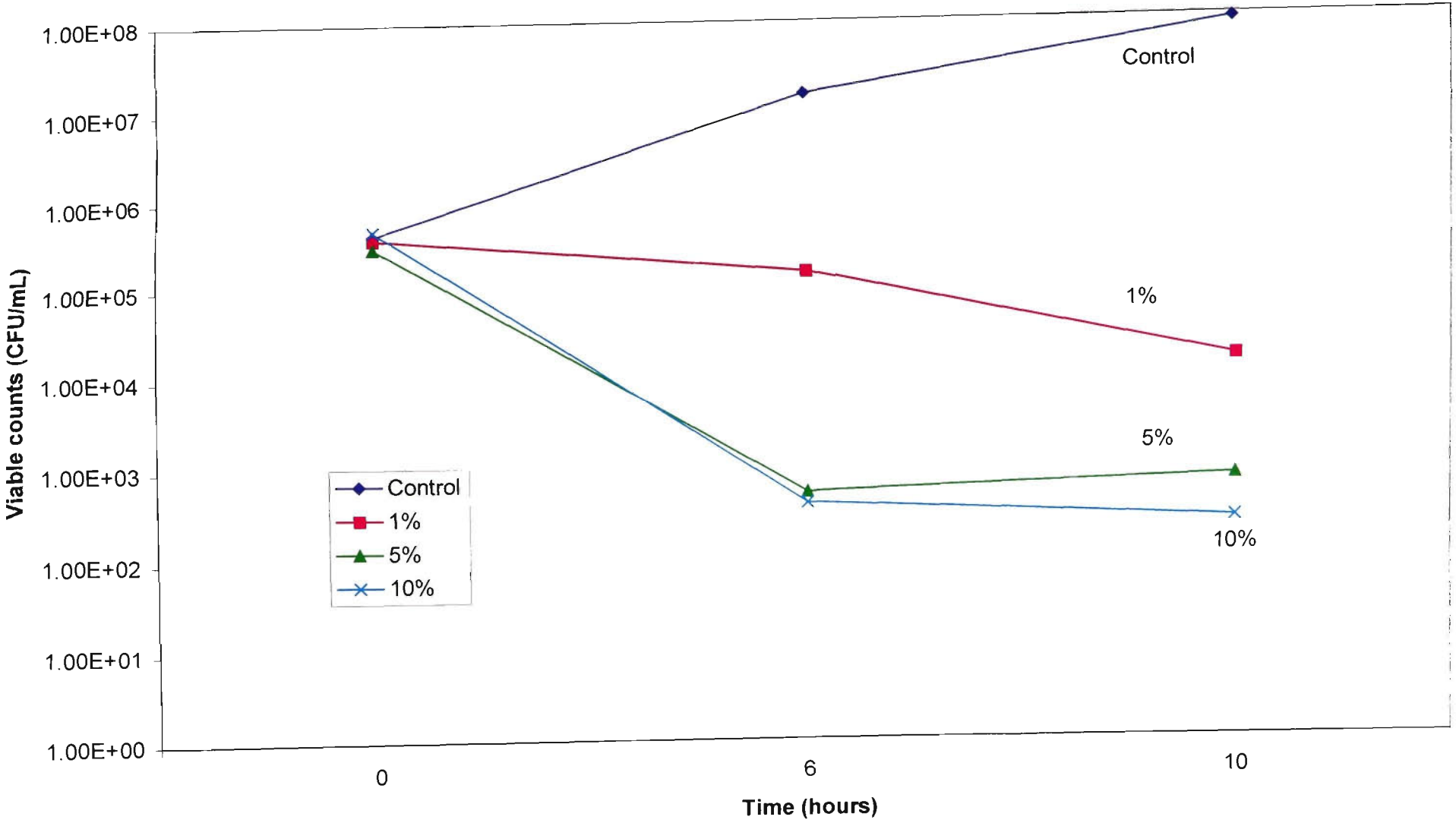

Figure 17. Changes in viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* when grown with different levels of concentrated bacteriocin. Control- 1% *Lactobacillus delbrueckii* subsp. *bulgaricus*, 1%-1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 1% bacteriocin, 5%- 1%*Lactobacillus delbrueckii* subsp. *bulgaricus* and 5% bacteriocin, 10%- 1%*Lactobacillus delbrueckii* subsp. *bulgaricus* and 10% bacteriocin.

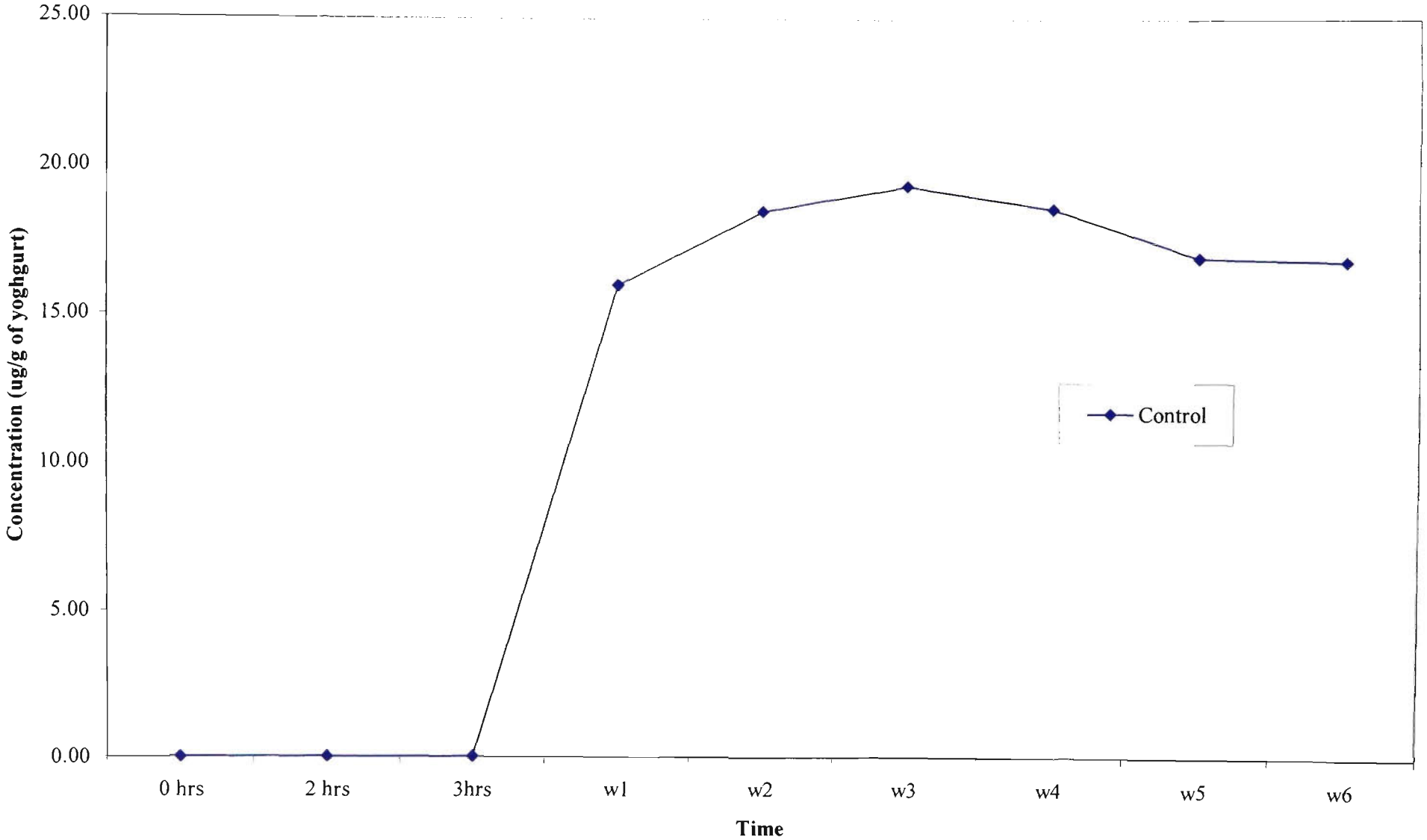

Figure 18: Effects on acetic acid production when bacteriocin is incorporated in yoghurt

Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

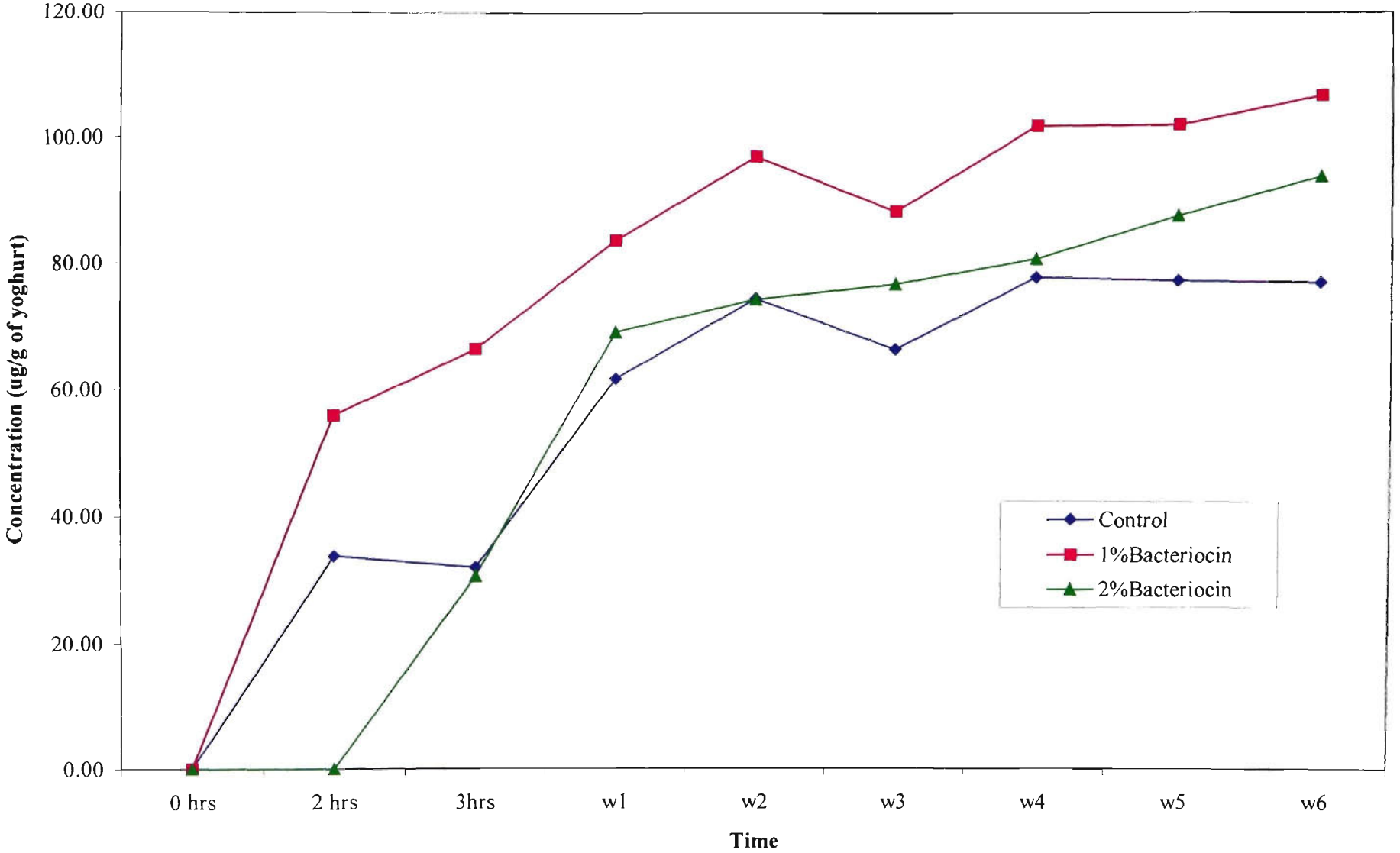

Figure 19: Effects on butyric acid production when bacteriocin is incorporated in yoghurt

Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

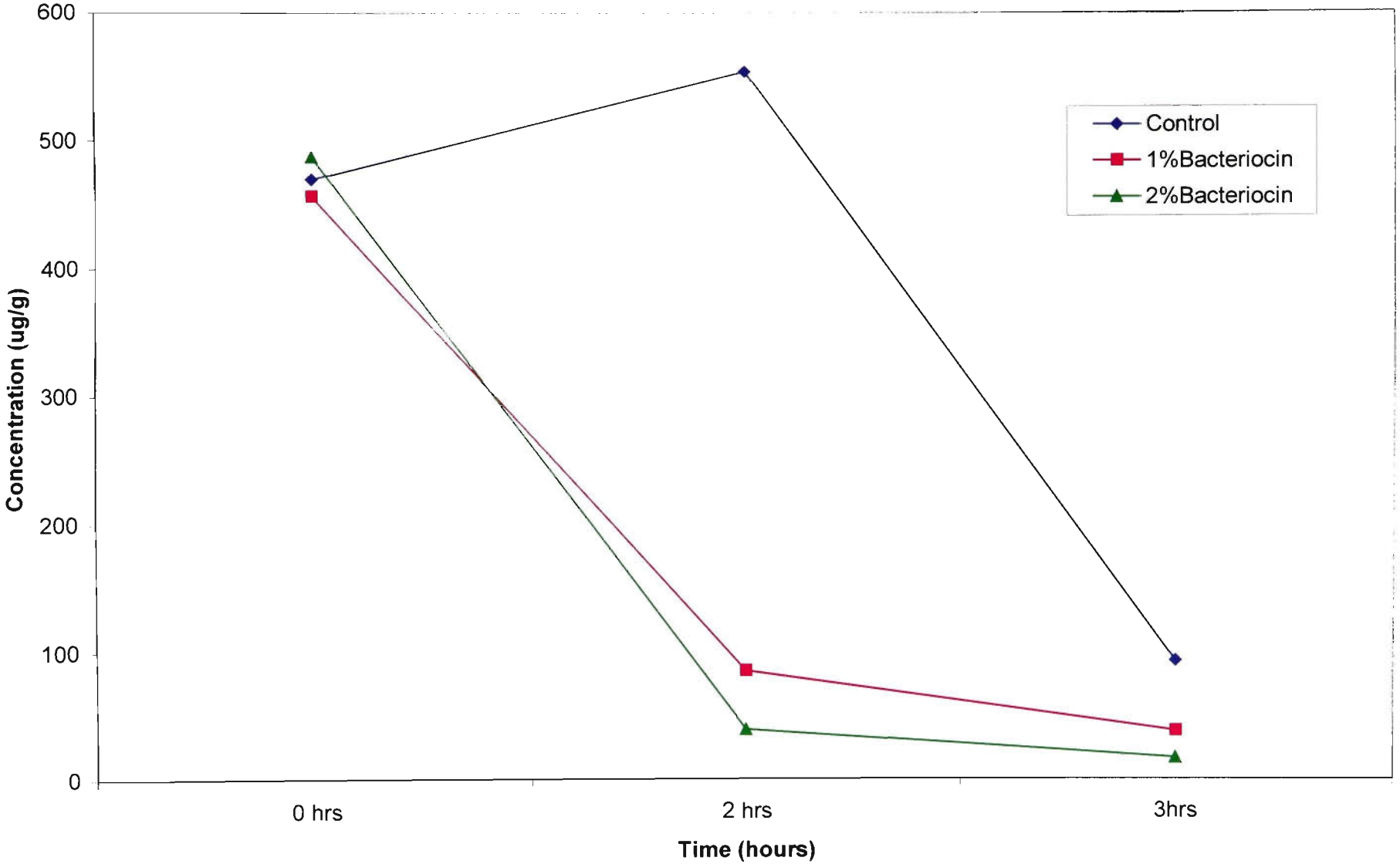

Figure 20: Effects on formic acid production when bacteriocin is incorporated in yoghurt

Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

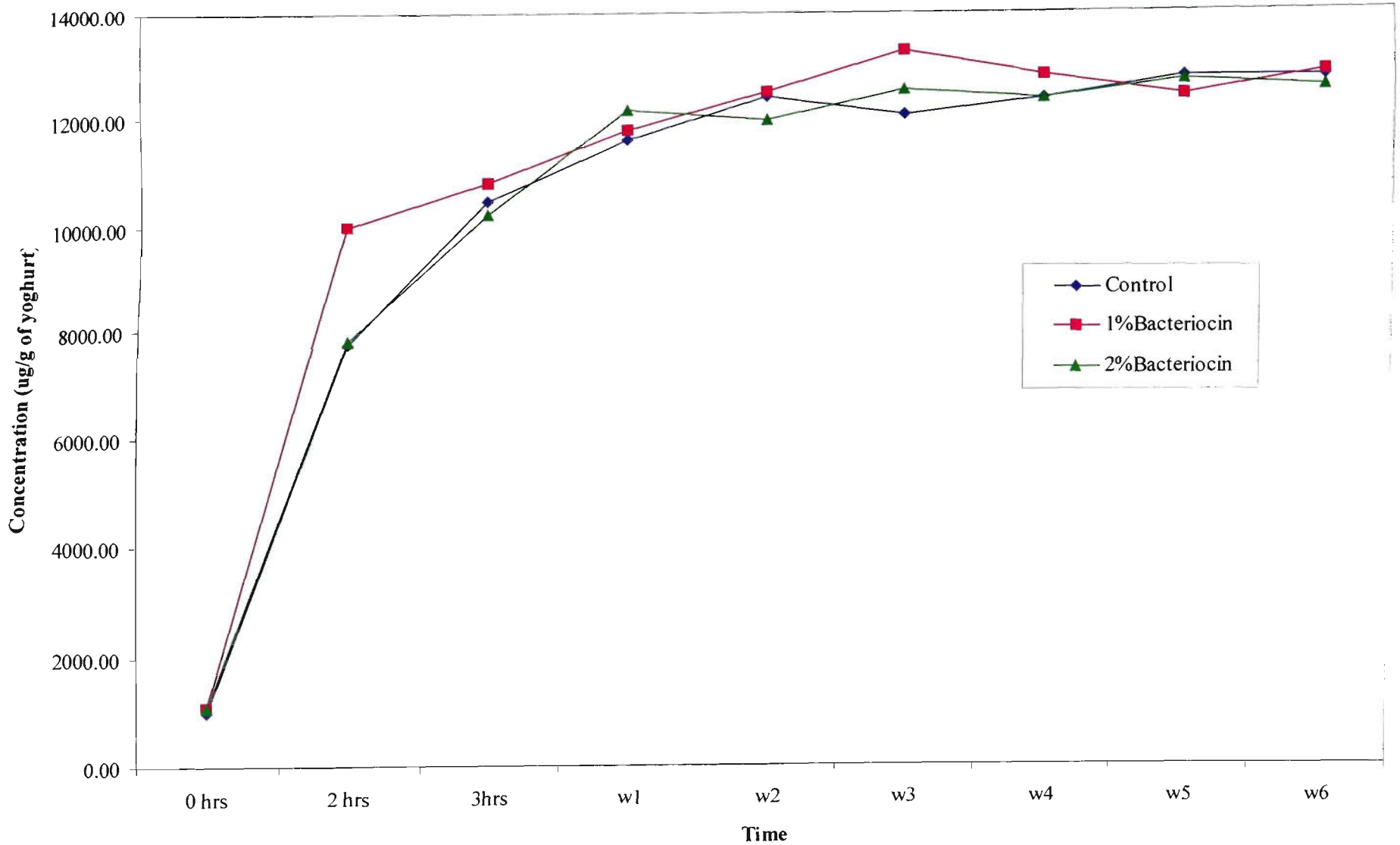

Figure 21: Effects on lactic acid production when bacteriocin is incorporated in yoghurt

Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

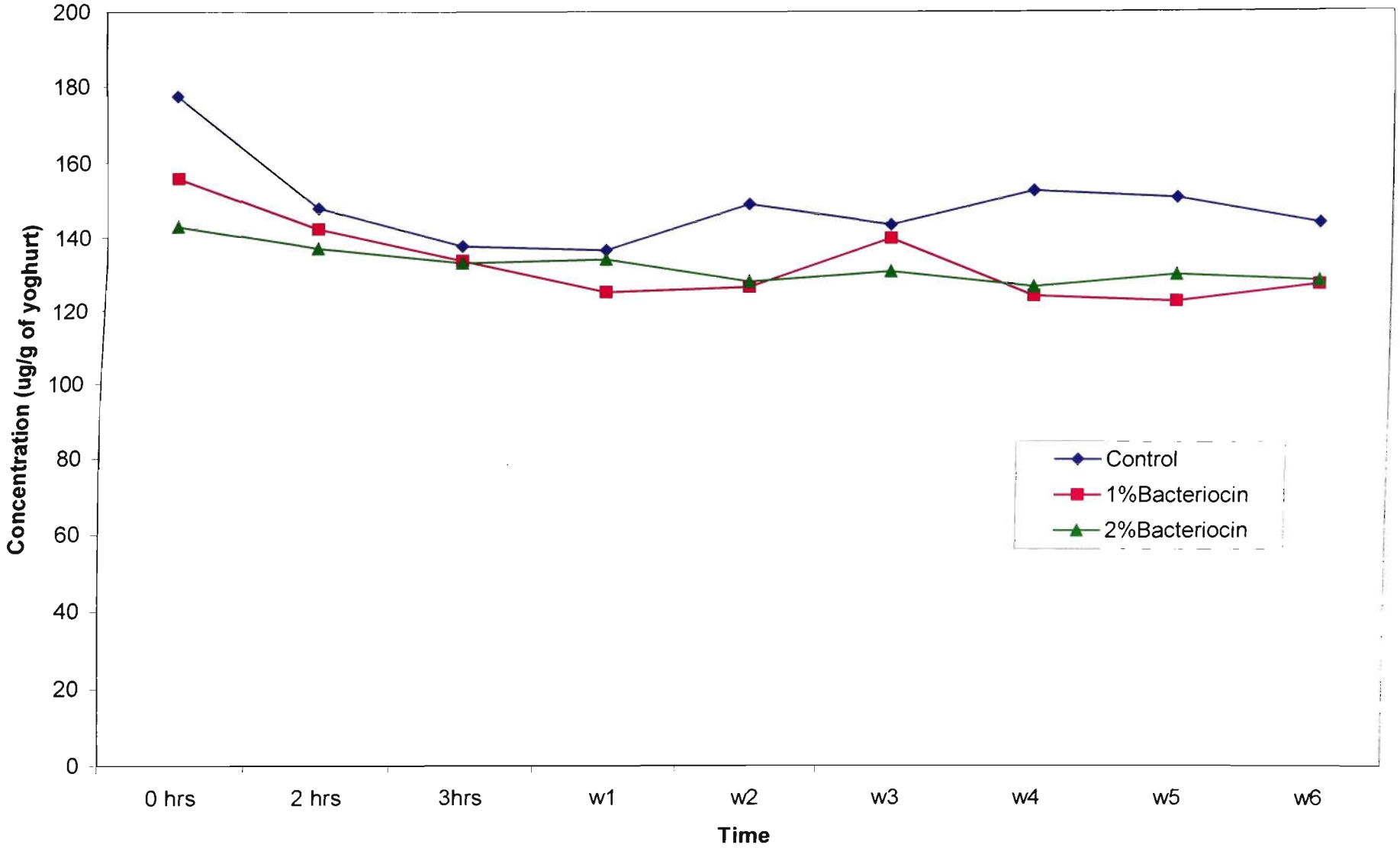

Figure 22: Effects on orotic acid production when bacteriocin is incorporated in yoghurt

Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

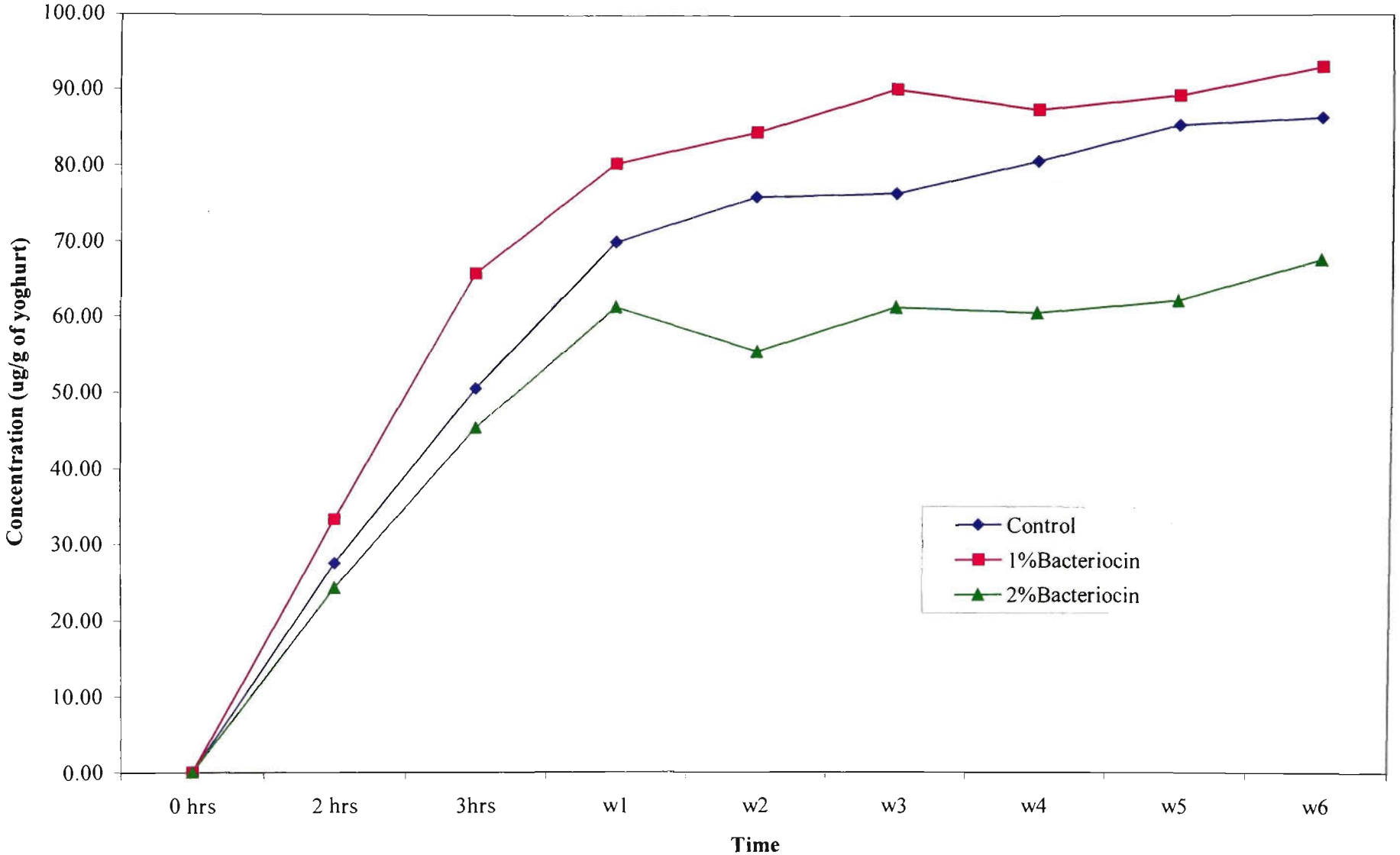

Figure 23: Effects on production of propionic acid when bacteriocin is incorporated in yoghurt

Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

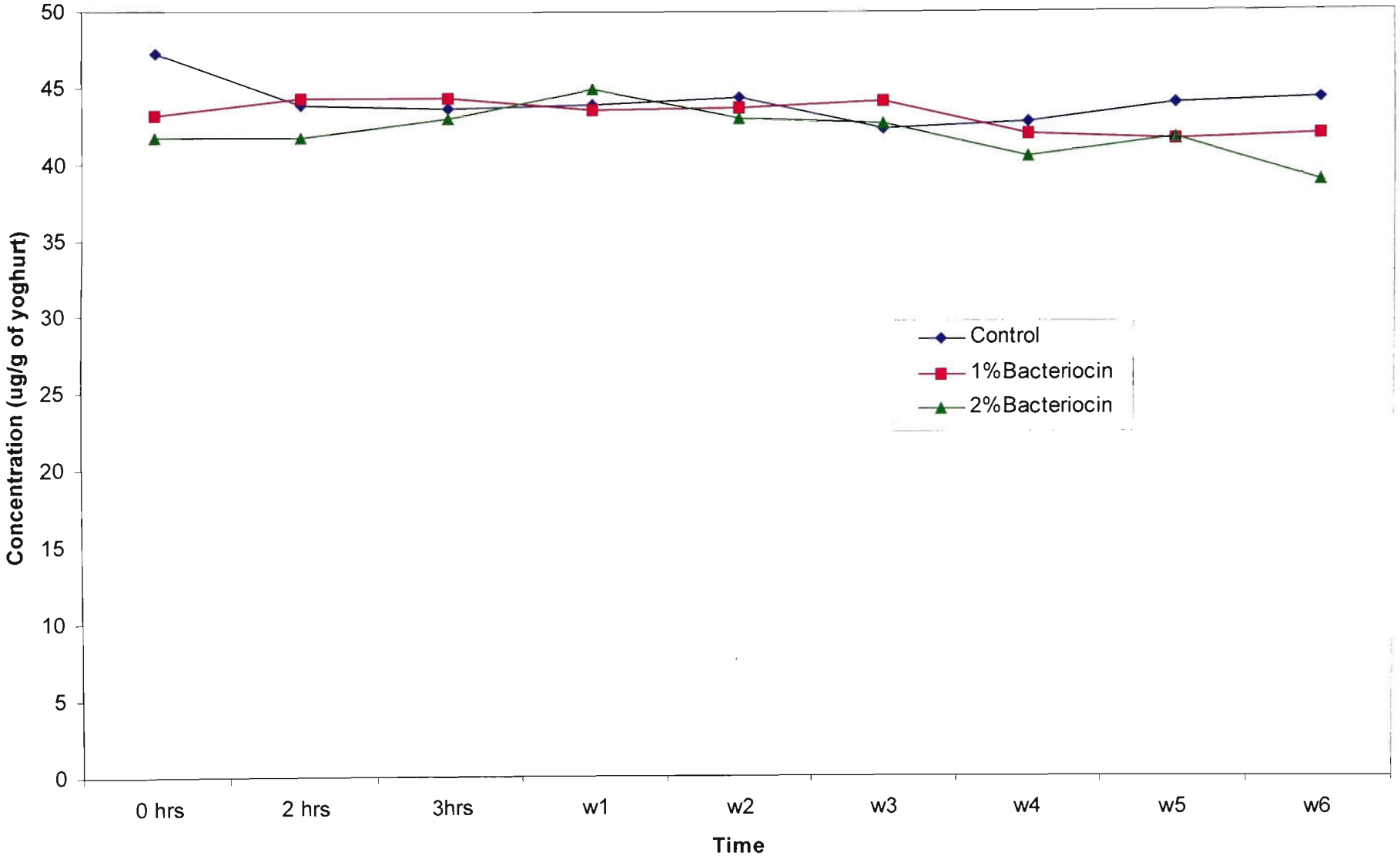

Figure 24: Effects on uric acid production when bacteriocin is incorporated in yoghurt

Control - contains 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

1% - contains 1% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 1941*

2% - contains 2% bacteriocin and 1% each of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* and *Bifidobacterium longum 194*

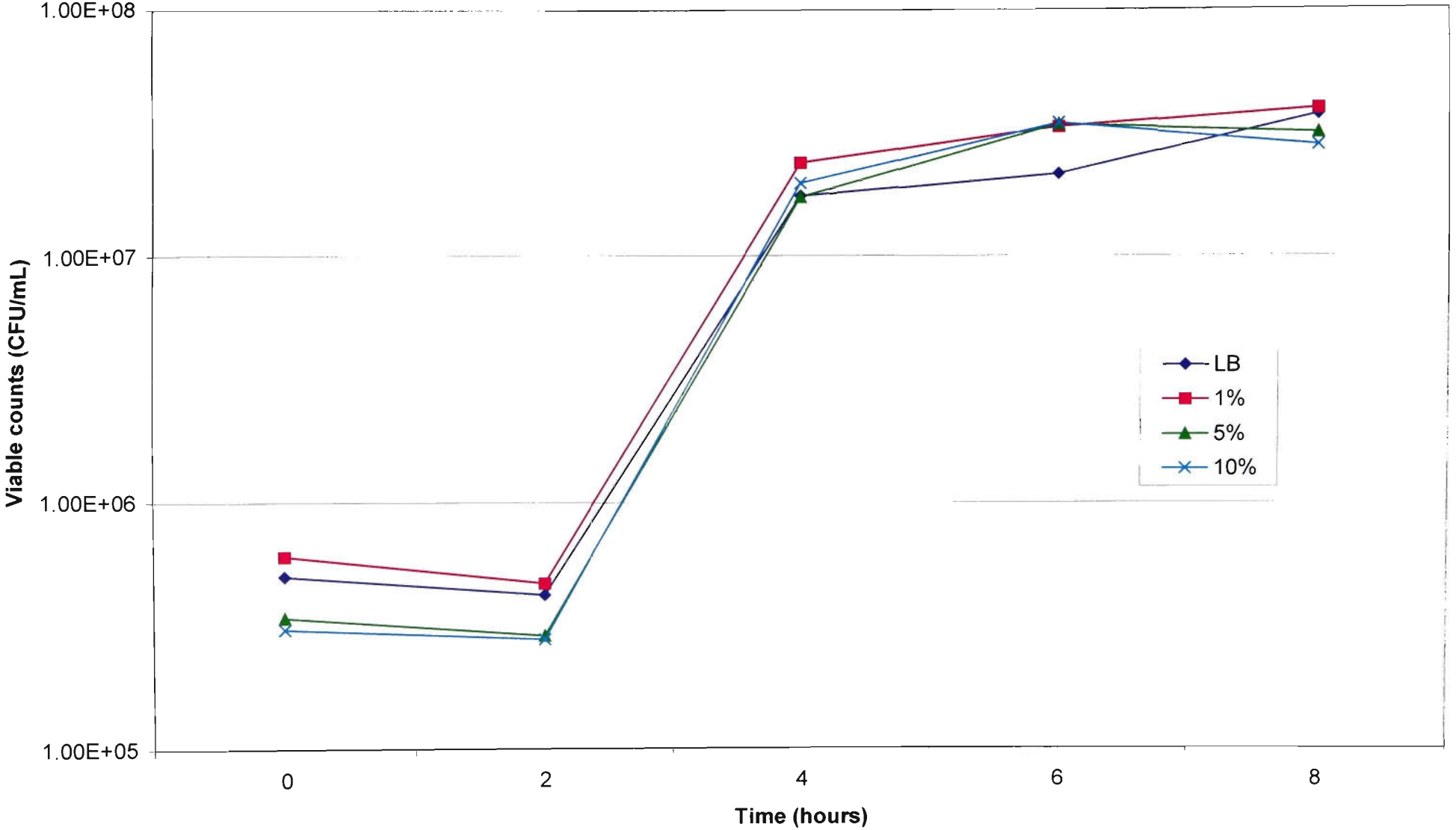

Figure 25: 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* grown with different levels of bacteriocin in 12% RSM.

LB - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus*
1% - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 1% bacteriocin
5% - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 5% bacteriocin
10% - 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* and 10% bacteriocin

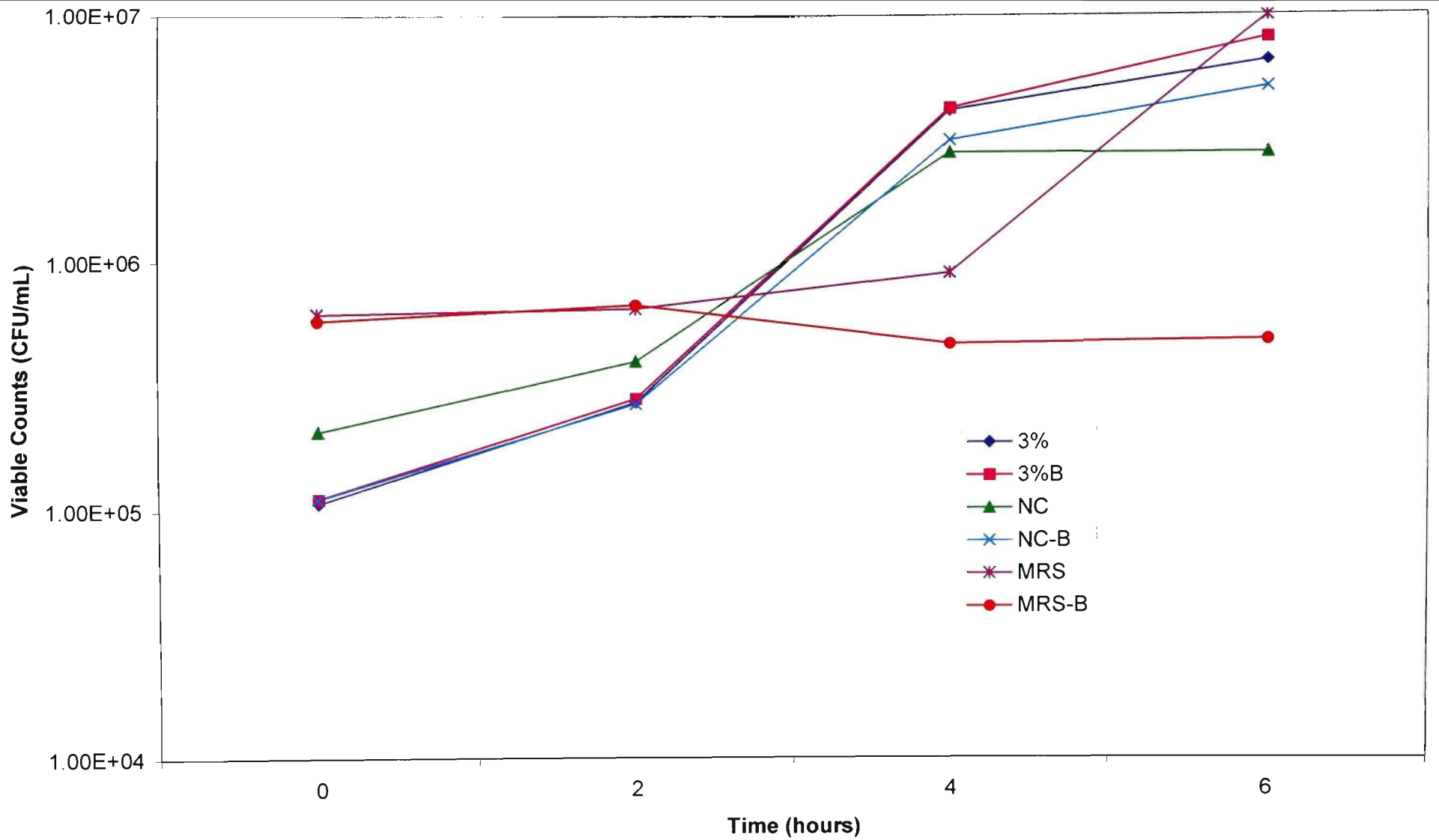

Figure 26: The effect bacteriocin has on viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* when grown in different media.

3%- 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* in 3% RSM
3%B- 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* in 3% RSM and 1% bacteriocin
NC- 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* in 3% RSM with casein removed
NC-B – 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* in 3% RSM with casein removed and 1% bacteriocin
MRS- 1% *Lactobacillus delbrueckii* subsp. *bulgaricus* in MRS broth
MRS-B-1% *Lactobacillus delbrueckii* subsp. *bulgaricus* in MRS broth and 1% bacteriocin

5.0 CONCLUSION

This project investigated ways of lysing *Lactobacillus delbrueckii* subsp. *bulgaricus* to release growth factors. This was to promote the growth of probiotic bacteria, to reduce fermentation time for yoghurt making and to control post-acidification.

Sonication of the *Lactobacillus delbrueckii* subsp. *bulgaricus* could possibly improve the viability of probiotic bacteria in yoghurt and also control post-acidification. The levels of both *L. acidophilus* and bifidobacteria remained higher than the recommended viable count required for health benefits during 4 weeks of storage. However, it would be expensive and impractical to sonicate *Lactobacillus delbrueckii* subsp. *bulgaricus* in a manufacturing plant.

L. acidophilus is known to produce bacteriocin against *Lactobacillus delbrueckii* subsp. *bulgaricus* and if this could be utilised to lyse *Lactobacillus delbrueckii* subsp. *bulgaricus* and release growth factors for probiotic bacteria then this could be a more practical way of improving the viability of probiotic bacteria and controlling post-acidification.

Different strains of *L. acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were grown against each other on agar plates to determine which strain would be most suitable. It was determined that *L. acidophilus*-LA-5 produced an antimicrobial substance against *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515 and these strains were used in the proceeding experiments. The antimicrobial substance was confirmed to be a bacteriocin based on the treatment with NaOH, catalase and proteolytic enzymes.

To test the activity of the bacteriocin producing strain of *L. acidophilus* against *Lactobacillus delbrueckii* subsp. *bulgaricus* 2515, three different inoculation levels of *L. acidophilus* were grown with *Lactobacillus delbrueckii* subsp. *bulgaricus*. The levels used were 1%, 5% and 10%. The Lactobacillus delbrueckii subsp. bulgaricus in the 10% sample had the lowest viable count and had started to decrease at the 8^{th} hour of fermentation but the count was still very high. This experiment showed that

Lactobacillus delbrueckii subsp. *bulgaricus* does decrease slightly in the presence of a high inoculation of *L. acidophilus* and this may have been more obvious if the fermentation continued for several more hours. This, however, would not be feasible in yoghurt making therefore *L. acidophilus* does not decrease the viable count or lyse *Lactobacillus delbrueckii* subsp. *bulgaricus* enough to slow the production of lactic acid to stop post-acidification.

In order to test the effectiveness of bacteriocin produced by *L. acidophilus*, the organism was grown for 18 hours in MRS broth and then centrifuged, neutralised and concentrated. Protein was then extracted from the concentrate and dissolved in sodium carbonate. This was then added to *Lactobacillus delbrueckii* subsp. *bulgaricus* at different inoculation levels and grown for 10 hours. The inoculation sizes used were 1%, 5% and 10%, and viable counts were measured at 0, 6 and 10 hours. The results show that the concentrated bacteriocin inhibited *Lactobacillus delbrueckii* subsp. *bulgaricus* more than *L. acidophilus* did. The 10% batch had the lowest viable count *Lactobacillus delbrueckii* subsp. *bulgaricus* after 10 hours with a 5 log cycle difference as compared to the control. This experiment showed that *Lactobacillus delbrueckii* subsp. *bulgaricus* was inhibited by concentrated bacteriocin produced by *L. acidophilus,* and the more bacteriocin added, the more inhibition was observed, which was to be expected.

It was observed that, 1% batch inhibited L*actobacillus delbrueckii* subsp. *bulgaricus,* and therefore it was decided to lower the inoculation of bacteriocin, as incorporating 5% and 10% would not be economical for industry. The inoculation sizes used in the next experiment were 0.5%, 1% and 2%. The 2% batch inhibited *Lactobacillus delbrueckii* subsp. *bulgaricus* the most. However, the bacteriocin lost activity over time. This could pose a problem for manufacturing, as the bacteriocin may not be reliable and would have to be used fresh for each batch of yoghurt.

The bacteriocin was then used at different rates in yoghurt with probiotic bacteria. Bacteriocin was added at a rate of 1% and 2%. From these experiments, it was observed that the bacteriocin did not inhibit *Lactobacillus delbrueckii* subsp. *bulgaricus* in the yoghurt. The viable counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* were very similar between the control and experimental yoghurts at the

end of fermentation and this suggested that no inhibition occurred. The pH and fermentation times are also very similar and this indicated the bacteriocin had not lysed *Lactobacillus delbrueckii* subsp. *bulgaricus,* as the fermentation time may have been slower. Therefore, it was thought that there was some substance that was blocking the activity of bacteriocin in yoghurt.

Lactobacillus delbrueckii subsp. *bulgaricus* (1%) was grown with 1%, 5% and 10% levels of bacteriocin for 8 hours in 12% RSM. The results show that the bacteriocin had no effect on the *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteria. All the counts at the 8^{th} hour were very similar and grew the same as the control with out any inhibition observed.

To determine if it was the milk that caused the loss of activity in bacteriocin, different milk media were made containing 3%, 6% and 12% RSM. It was observed that the 12% milk sample did not produce any zones whereas the 3% and 6% batches did. This suggested that there was something in milk that was blocking the activity of bacteriocin, possibly the protein (bacteriocin) interacted with casein.

Casein was removed to see if it was the cause of the blocking of the bacteriocin. Three percent milk was made and casein was removed. There did not appear to be any difference between the sample with casein or without, but it was observed that in the MRS broth the bacteriocin inhibited *L. delbrueckii* subsp. *bulgaricus*. Therefore this experiment indicated that casein was not the substance blocking the bacteriocin.

This study has shown that the use of *Lactobacillus delbrueckii* subsp. *bulgaricus* does increase the fermentation time of yoghurt and would be very beneficial to manufacturers. It does, however, increase post-acidification. Sonication could be one way of controlling this but would be expensive and impractical. This study has shown that bacteriocin produced by *L. acidophilus* inhibited the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* and could be a useful way of improving yoghurt. When the bacteriocin is concentrated and purified it is very active against *Lactobacillus delbrueckii* subsp. *bulgaricus* when it is grown in MRS broth. However, in milk there appears to be a protective substance blocking the bacteriocin. This substance was not casein as shown by experiments.

The use of bacteriocin in yoghurt could be beneficial but there are issues that need to be addressed. The bacteriocin can lyse *Lactobacillus delbrueckii* subsp. *bulgaricus* and would certainly help probiotic bacteria, as there would be a better environment for them to survive. Post-acidification would also be controlled, as *Lactobacillus delbrueckii* subsp. *bulgaricus* would not be able to produce as much lactic acid. The fermentation time would be shorter due to the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* and this would be very important to yoghurt manufacturers. The investigation into bacteriocin should be continued, as it will be an important supplement to manufacturers.

6.0 FUTURE DIRECTION

This project aimed to determine if bacteriocin produced by *L. acidophilus* could lyse *Lactobacillus delbrueckii* subsp. *bulgaricus* and use this technique in yoghurt making. *Lactobacillus delbrueckii* subsp. *bulgaricus* can produce necessary growth factors for probiotic bacteria to utilise and improve viability of probiotic bacteria.

This project determined that bacteriocin inhibited *Lactobacillus delbrueckii* subsp. *bulgaricus* when grown in MRS broth, but not in 12% RSM. There is something in milk that blocked the activity of bacteriocin. It is possible that a protein is blocking its inhibitory activity. Future projects should aim at looking at determining as to what is blocking the activity of bacteriocin. The structure of bacteriocin should be looked at. There could be a bonding site on the bacteriocin and it may be possible to block this site before adding to milk.

The addition of bacteriocin should also be investigated. It would be impractical to add the bacteriocin as a liquid as it has been found that it loses activity over time. Stability trials of bacteriocin should be conducted and other methods of application should be explored. Freeze drying was attempted without success as the product became sticky and would not dry completely.

Morgan *et al.* (2001) developed a method to spray dry lacticin 3147; a bacteriocin produced by *Lactococcus lactis.* This powder was tested in yoghurt, cottage cheese and soup; however, lacticin 3147 is not heat stable and lost considerable amount of activity when autoclaved. This method of concentration would be very beneficial to the industry and would be suitable for bacteriocin produced by *L. acidophilus* LA-5 as it does withstand autoclaving temperatures.

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