

CHAPTER 1: INTRODUCTION

Food fermentation is one of the oldest known uses of biotechnology. Fermented food and beverages dates back many thousands of years and it continues to provide an important part of human diet supplying about 20-40% of food worldwide (Campbell-Platt, 1994). In recent years this method has been widely used to improve food quality, safety, nutritional values and palatability and to develop new food products.

Fermentation is a result of metabolic activity of microorganism which performs complex transformations of organic materials to sustain its growth and proliferation. Useful fermentation by products are produced which enhanced food quality and lengthen the shelf life of fresh fruits, vegetables and milk. Certain fermented food such as soy sauce, pickles, vinegar, bread, yoghurt, and cheese may also contain fermentation by products that have therapeutical properties (Farnworth, 2003).

In recent years consumers are increasingly interested in incorporating healthy foods into their diet (Reid, 2006) and in many circumstances are willing to pay more for food with functional properties. Fermented dairy products, also categorized in functional foods group, are considered to have functional properties because of its enhanced nutritional values and the presence of probiotics (friendly bacteria). A number of health benefits have been claimed for probiotic bacteria such as *Lactobacillus acidophilus*, *Bifidobacterium* spp., and *Lactobacillus casei* (Shah, 2000b). These provide therapeutic benefits such as modification of the immune system, reduction in cholesterol and hypertension, alleviation from lactose intolerance, faster relief from diarrhoea, and restoration of a healthy vaginal microbiota (Reid, 1999; Reid, 2001).

One of the most popular dairy products for the delivery of viable *Lactobacillus* sp. cells is yoghurt (Analie and Bennie, 2001). Viable bacteria in yoghurt are believed to actively enhance health by improving the balance of microflora in the gut (Fuller 1989, Fuller

1992). Due to this, yoghurt by itself has been recognized as a healthy food by virtue of the beneficial action of its viable bacteria that compete with pathogenic bacteria for nutrients and space. Available yoghurt-like products include reduced fat content yoghurts, probiotic yoghurts, yoghurt shakes, drinkable yoghurts, yoghurt mousse, yoghurt ice-cream, etc. (Fizman & Salvador, 1999).

The other beneficial aspect of yoghurt is the presence of bioactive peptides. These peptides which are inactive within the sequence of the parent protein are released by enzymatic proteolysis, for example during gastrointestinal digestion or during food processing (Miesle, 1998). Once they are liberated, those bioactive peptides may act as regulatory compounds with hormone-like activity. Thus, these peptides represent potential health enhancing nutraceuticals for food and pharmaceutical applications (Miesle, 1998). For instance angiotensin converting enzyme-inhibitory (ACE-I) peptides from milk based products have beneficial effects on people with hypertension (Ana *et al*, 2004). In this regard lactic acid bacteria (LAB) plays important roles in the production of formulated food with myriads of function which include preserve foods, preserved with and without functional properties.

The key to market growth is a continuous evaluation and modification of the product to match consumer expectations. Plant materials such as herbs demonstrate a multifunctional physiological property and have been used as value added ingredients in various food products. Herbs and spices are used widely in modern culinary. Apart from natural, effective and fascinating and able to provide reasonable solutions to modern health, frequent consumption of herbs helps to prevent the development of certain diseases. These are illustrated in several epidemiological studies which correlate human consumption of diets rich in fruits and vegetables containing high levels of phytochemicals to lower the risk of getting chronic diseases such as diabetes, cancer and cardiovascular diseases (Craig, 1997). It is interesting to note that these phytochemicals

also affect the metabolic activities of microbes including LAB. Based on this knowledge the objectives of the present studies were to investigate the effects of selected herbs on probiotic growth and metabolism in yoghurts. In particular the ACE inhibitory effects from these yoghurts were measured and compared to evaluate the potential of using herbal yoghurts in the management of hypertension.

CHAPTER 2: LITERATURE REVIEW

2.1 Importance of milk in human nutrition

Milk is being used in many ways for human nutrition from the beginning of animal domestication. A grocery store's inventory on dairy products may range from milk [skim milk 0% fat, 1%, 2%, and full cream milk (approximately 3.5%)], milk powder, whipping cream, sour cream, butter, ghee, ice cream, cheese and yoghurt. Dairy products are commonly found in Middle Eastern, Indian and European cuisine. The consumption of dairy products contributes up to 20% of daily energy intake as well as up to 60% of calcium intake in these populations (Miller, 1989). Raw milk for processing generally comes from cows, but may occasionally comes from other mammals such as goats, sheep, water buffalo, yaks or horses.

The composition of some mammals' milk is shown in Table 2.1. Milk is a nutrient-dense food that provides a high level of essential nutrients. These include lactose (milk sugar), casein micelles (the major protein of milk), fat and fat soluble vitamins (e.g. vitamin A, thiamine, riboflavin, nicotinic acid and vitamin B12), whey proteins, mineral salts (calcium, phosphorus, potassium) and other substances. Thus milk is a good source of high quality protein, phosphorus and an excellent source of calcium.

The pH of fresh milk ranges from 6.5 to 6.7 with an initial acidity of 0.14 to 0.16%. pH and acidity measurements are often used as acceptance tests and quality of milk. These tests are used to monitor processes such as cheese-making and yoghurt-making. Approximately 80% of the milk proteins are caseins which consist of α_s -, β -, κ - and γ -caseins. The casein micelles and the fat globules give milk most of its physical characteristics, and give taste and flavour to dairy products (Mottar and Bassier, 1989). Processing of milk by its nature involves the imposition on a changing colloidal system. This is because the colloidal particles in milk alter their nature and

behaviour. For instance changing pH causes disintegration rearrangement of the micelles and if pH is low enough, new particles of iso-electric casein are formed. Also, heating to high enough temperatures causes the binding of serum proteins to the micellar to breakdown (Mottar and Bassier, 1989).

Table 2.1 Typical nutrient contents (per 100 ml) in various types of milk.

Nutrients (unit)	Buffalo's milk	Cow's milk	Goat's milk	Human milk
Protein (gm)	4.3	3.2	3.3	1.1
Fat (gm)	6.5	4.1	4.5	3.4
Carbohydrate (gm)	5	4.4	4.6	7.4
Energy (kcal)	117	67	72	65
Calcium (mg)	210	120	170	28
Phosphorous (mg)	130	90	120	11
Iron (mg)	0.2	0.2	0.3	NA
Thiamine (mg)	0.04	0.05	0.05	0.02
Riboflavin (mg)	0.1	0.19	0.04	0.02
Vitamin C (mg)	1	2	1	3
Vitamin B12 (mcg)	0.14	0.14	0.05	0.02

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2.2 Fermented milk products

Fermented food has a long history of safe usage and is found in diets throughout the world. Fermentation is broadly defined as a biochemical changes in organic substances that are caused by the action of microorganisms or enzymes to produce organic acid, alcohol, carbon dioxide and energy in the form of ATP (adenosine triphosphate). Fermentation is applicable for many purposes, among others to extend the shelf life by protection and preservation of foods, producing desirable taste and flavour,

enhancement of nutritional value, producing required physicochemical properties, improvement of food safety and food security (Caplice & Fitzgerald, 1999).

The optimum fermentation conditions (temperature, pH, moisture, nutrient and oxygen) depend on the type of microorganisms used. Familiar fermentation includes using yeast in bread-making, production of alcoholic beverages and conversion of corn into fuel ethanol. Lactic acid bacteria (LAB) carry out fermentations leading to the production of yoghurt, cheese, sausage, sauerkraut, wine or beer and pickles. They form a major part of the diet of people around the world. Some composition of milk products are shown in Table 2.2. Fermented milk products, including yoghurt and cheese, are formed when bacteria break down lactose to produce lactic acid, which sours the milk.

2.2.1 Milk fermentation and biochemical changes

Microbial fermentation in food fermentation involves the breakdown of sugar and protein which results in the production of a large array of organic compounds that contribute to the flavour, preservation and outer appearance of the food product (Hugenholtz *et al.*, 1999). Milk fermentation is initiated by *lactobacilli* and *streptococci* bacteria which use nutrients in milk for their growth and alter the nutritional composition and physical appearance of milk (Loones, 1989).

Lactose is used by lactic acid bacteria (LAB) as the principal source of carbon for growth and energy. It is initially hydrolysed by lactase into galactose and glucose (Greenberg & Mahoney, 1982) followed by subsequent glucose conversion to D- or L-lactic acid via the glycolytic, Embden-Meyerhof-Parnas pathway (Hemme *et al.*, 1980). The lactic acid fermentation consists of two major pathways that include homolactic fermentation which produces lactic acid and heterolactic fermentation which produce equimolar amount of lactic acid, carbon dioxide and ethanol (Vakil and Shahani, 1970).

Table 2.2 Comparison of nutrient composition in various milk products

Nutrients	Unit	Whole milk 3.25% fat 1 cup	Chocolate milk (whole milk) 1 cup	Yoghurt whole milk 1cup	Cheddar cheese 1 oz.	Butter milk 1 oz.
Energy	Kcal	149.92	208.38	136.64	114.13	99
Water	G	214.7	205.75	208.81	10.42	220.82
Protein	G	8.03	7.93	14.04	7.06	8.11
Fat	G	8.15	8.48	0.44	9.4	2.16
Carbohydrate by difference	G	11.37	25.85	18.86	0.36	11.74
Fibre ,total dietary	G	0	2	0	0	0
Ash	G	1.76	2	1.89	1.11	2.18
Minerals						
Calcium	mg	291.34	280.25	295.72	204.49	285.18
Magnesium	mg	32.79	32.58	28.3	7.88	26.83
Iron	mg	0.12	0.6	0.12	0.19	0.12
Phosphorus	mg	227.69	251.25	233.51	145.18	218.54
Potassium	mg	369.66	417.25	378.77	27.9	370.69
Vitamins						
Vitamin C	mg	2.29	2.28	1.3	0	2.4
Thiamine	mg	0.09	0.09	0.07	0.01	0.08
Niacin	mg	0.21	0.31	0.02	0.18	0.14
Riboflavin	mg	0.4	0.41	0.35	0.11	0.38
Vitamin B6	mg	0.1	0.1	0.08	0.02	0.08
Vitamin B12	mg	0.87	0.84	0.91	0.23	0.54
Vitamin A	IU	307.44	302.5	301.35	300.23	80.85
Fatty acids						
Saturated	G	5.07	5.26	5.14	5.98	1.34
Monosaturated	G	2.36	2.48	2.19	2.66	0.05
Polysaturated	G	0.3	0.31	0.23	0.27	0.05
Cholesterol	mg	33.18	30.5	31.12	29.74	8.58
Amino acids						
Tryptophan	G	0.11	0.11	0.05	0.09	0.09
Threonine	G	0.36	0.36	0.35	0.25	0.39
Leucine	G	0.79	0.78	0.86	0.68	0.81
Lysine	G	0.64	0.63	0.76	0.59	0.68
Valine	G	0.54	0.53	0.7	0.47	0.6
Arginine	G	0.29	0.29	0.26	0.27	0.31
Proline	G	0.78	0.77	1.01	0.8	0.82
Tyrosine	G	0.39	0.38	0.34	0.43	0.34
Serine	G	0.44	0.43	0.41	0.53	0.42

Source: Newer Knowledge of Dairy Foods / Appendix (From USDA, ARS, USDA Nutrient Database for Standard, Reference,Release 12)

Volatile fatty acids, ethanol, acetaldehyde, acetoin and butanone are yielded through fermentation. The lactic acid, formed in the reduction of pH of milk, resulted in a pleasant soury taste.

LAB has very limited capacity to synthesize amino acids by using inorganic nitrogen sources. Therefore they are dependent on preformed amino acids, the requirement for amino acids differs among the species of these bacteria in the growth medium as nitrogen source (Williams *et al.*, 2002). Protein is degraded by proteolysis and increases the peptide and free amino acid content of fermented milk products (Livia 1982). Lipids are sparingly hydrolysed by LAB lipases which are more active towards lower but not higher molecular weight triglycerides (Yvone *et al.*, 2003; Nihal *et al.*, 1986). Although lipases are present in *S. thermophilus* & *L. delbrueckii* subsp. *bulgaricus*, they have little effect on free fatty acid content of fermented milk products (Fernandes *et al.*, 1991). LAB require minerals and vitamins for growth (as mineral catalysis and mediators in the enzymatic reaction respectively) but their requirement is small and would not significantly alter the total content of fermented milk products (Fernandes & Shahani, 1989a). The bioavailability of some of the minerals may be changed due to pH changes caused by fermentation.

Numerous scientific papers and review articles (Hughes and Hoover, 1991; Kurmann and Rasic, 1991; Modler *et al.*, 1990) have reported the health benefits associated with the consumption of fermented dairy products. Some of the proposed health benefits are thought to be conferred by live bacteria contained in the products. For instance the higher free amino acid content in fermented milk is possibly due to partial hydrolysis of the milk proteins by LAB (Friend & Shahani, 1984).

Foods containing probiotic bacteria which were originally used as a mean to enhance storage life in much of the undeveloped countries are currently categorized as 'functional foods'. Such products are now gaining widespread popularity and acceptance throughout the developed world. A number of health benefits for product containing live probiotic bacteria have been claimed including alleviation of symptoms of lactose intolerance, treatment of diarrhoea, anti-carcinogenic properties (Daniel *et*

al.,2005), antimutagenic activity (Nadathur and Bakalinsky,1995), reduction in blood cholesterol and improvement in immunity. The importance of probiotics in humane health is considered further in the following section.

2.2.2 Probiotics

Probiotics (derived from the Greek word meaning “for life”) are live microbes which influence the well-being of their host through their effect on the intestinal microflora (Guarner and Schaafsma, 1998). It was also called “a live microbial food ingredient that is beneficial to health” (Salminen *et al.*, 1998). Probiotic improves intestinal microbial balance and reduction in these bacteria which are naturally found in the human small intestine and large intestine increases the presence of potentially pathogenic microbes.

Many probiotics are members of the genera of *Lactobacillus* and *Bifidobacteria* (Macfarlane and Cummings, 1999). At present approximately ten to fifteen bacterial strains have passed extensive investigations for some of the probiotic criteria. Some probiotic strain with scientific documentation include: *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Bbifidobacterium lactis* ,*Bifidobacterium longum* and *Lactobacillus gasseri* (Kneifel *et al.*, 2000). Over the past decade, considerable interest has developed in the use of probiotic organisms in food, pharmaceutical and feed products (Crittenden *et al.*, 2005).

Dairy products including yoghurt and cheese, due to the presence of lactose and peptides, are preferred medium for probiotics or health promoting bacteria. They provide the ideal food system for the delivery of these beneficial bacteria to the human gut, given the suitable environment to promote growth and support viability of these cultures (Ricke & Pillai, 1999; Schiffrin & Blum 2002). In fact fermented dairy

products are increasingly consumed as functional foods in recent years because of the probiotics as well as highly digestible fermentation products (FitzGerald and Meisel, 2003). Functional food contains a proper balance of nutrients and non nutrients such as dietary fibre and various bioactive compounds as well as probiotics which aid in the preventing and treatment of diseases. They have several health specific advantages and nutritive values such as preventing diet related diseases, coronary heart diseases, obesity, hypertension, certain type of cancer, gastrointestinal diseases and osteoporosis (Guarner & Malagelad, 2003).

From health point of view probiotic bacteria (*Lactobacillus acidophilus* and bifidobacteria) are widely used as dietary adjuncts as these organisms are normal inhabitants of the intestine (Kurmann and Rasic, 1991; Misra and Kuila, 1991; Havenaar and Huis in't Veld, 1992; Kanbe, 1992; Mital and Garg, 1992). One of the most important properties of probiotic bacteria is their ability to survive passage through a gastrointestinal tract and persist for a sufficient time in the gut so they can provide beneficial health effects (Huang & Adams, 2004). However, a minimum number of probiotic bacteria is required (10^5 to 10^6 CFUg⁻¹) at the time of consumption of a product (Schuler- Malyoth and Muller, 1968; Robinson, 1987; Kurmann & Rasic, 1991). It is also important to consider the composition of microbial species consumed because intestinal microorganisms can exert either beneficial or deleterious effects on the host.

2.2.3 Health benefits of probiotics

Several reported health benefits of probiotic bacteria are reduced duration of diarrhea (Kneifel, 2000), antagonistic effects against pathogenic microorganisms (Schiffrin and Blum, 2002), improved lactose digestion (Mital and Garg, 1992; Klaver and Meer, 1993) regulation of intestinal motility, reduced activities of cancer-related

enzymes (Marteau *et al.*, 1990), improved calcium resorption and provision of water soluble vitamins (Crittenden *et al.*, 2005; Jackson and Savaiano, 2001). The action of probiotics on intestinal flora results in vital benefits, including protection against pathogens, development of the immune system (Isolauri *et al.*, 2002) and positive effects on colonic health and host nutrition (Falk *et al.*, 1998; Naidu *et al.*, 1999; Umesaki & Setoyama, 2000). There is also evidence to suggest that certain species/strain of probiotics are anti-carcinogenic (Daniel *et al.*, 2005). Other important properties that have been attributed to probiotics include prevention and treatment of gastrointestinal disorders (Lewis & Freedman, 1998), reduction of food intolerance (Dunne *et al.*, 2001), modulation of the host immune responses (Isolauri *et al.*, 2001), and prevention of cancer and cardiovascular diseases (reduction of serum cholesterol and lipids) (Wallowski *et al.*, 1999).

Multiple species or high numbers of probiotic organisms may be required to be administered simultaneously to achieve /colonization, as shown in the treatment of pouchitis (Reid *et al.*, 2003) and in reducing the risk of urogenital infections (Reid & Burton, 2002). It is becoming more apparent that the more these intestinal-friendly bacteria are present in the colon, the lower are the chances of acquiring colon diseases.

2.2.4 Lactic acid bacteria:

Lactic acid bacteria (LAB) are widely used recently in food technology, microbiology and biotechnology and hygiene with respect to the production of fermented food. LAB are used as industrial microorganisms in beverage, meat product, sugar industry, souring of pickles, olives and dairy products. LAB (*Lactobacilli sp.*, the *Leuconostocs*, the *Streptococci* and the *Pediococci*) are also responsible for fermentation of milk products. They alter flavour, texture, and appearance of foods, enhance nutritional values, retard spoilage, reduce contamination and are widely used in

dairy processes because in addition to lactic acid production they also produce volatile compound such as acetaldehyde, diacetyl and alcohol (Law, 1981).

LAB are fastidious micro-organisms and their growth is often restricted in milk because of its paucity in essential nutrients. The large population of LAB in fermented milk products like yoghurt competes strongly with microbial contaminants for available nutrient and thus enhances product safety. Some LAB produce antimicrobial peptides, known as bacteriocins which may target certain pathogens (Havenaar and JHJ, 1992). Two clear examples of health-promoting activities by LAB are removal of lactose and production of L-alanine, and the production of vitamins (Hugenholtz *et al.*, 1999). Although lactic acid bacteria are usually considered to be only weakly proteolytic (Dutta *et al.*, 1971), they do cause significant degree of proteolysis in multiple fermented dairy products including yoghurts (Klaenhammer, 1985; Law and Haandrikman, 1997).

2.2.5 Yoghurt culture bacteria

The thermophilic LAB, *Streptococcus thermophilus* & *Lactobacillus delbrueckii* subsp. *bulgaricus* are used together as important starter microorganisms in the production of yoghurt and some kind of cheeses. Because both bacteria are able to grow alone in milk, this indirect positive interaction is called proto-cooperation (Fredrickson, 1977). This positive relationship often has a beneficial effect on bacterial growth and on the production of lactic acid and aromatic compounds. Lactic acid production results in the lowering of pH and this makes it unsuitable for growth of spoilage or pathogenic microorganisms (Donkor *et al.*, 2007).

The proteolytic activity of the two yoghurt bacteria is moderate but is very significant and leads to symbiotic growth of the two organisms, and production of flavour compounds. *L. bulgaricus* is known to be the more proteolytic (Rapp, 1969) of

the two bacterial strains used for yoghurt production. *L. bulgaricus* has the ability to hydrolyse caseins whereas *S. thermophilus* has only limited proteinase activity (Tamime and Deeth, 1980)

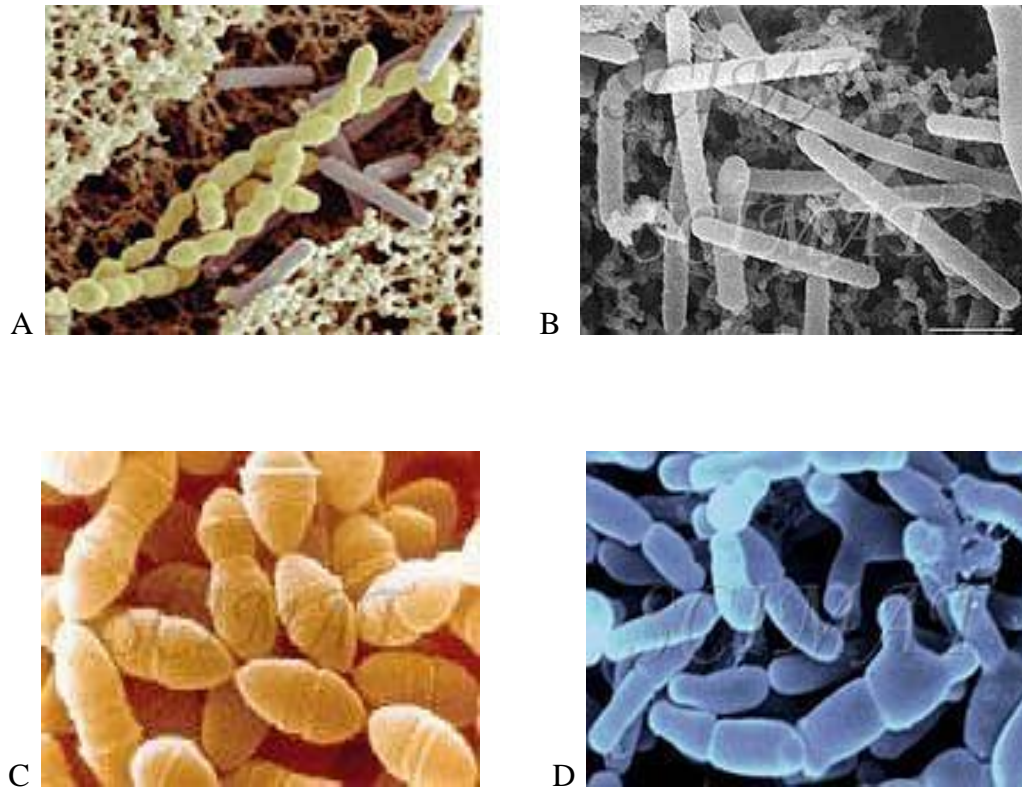


Figure 2.1 Lactic acid bacteria: (A) Yoghurt bacteria (*Streptococcus thermophilus* & *Lactobacillus delbrueckii* subsp. *bulgaricus*), (B) *Lactobacillus acidophilus*, (C) *Streptococcus thermophilus*, (D) *Bifidobacterium bifidum*

2.3 Yoghurt

Yoghurt is a very popular flavourful and healthful food and one of a family of cultured dairy products in all over the world. This product is dependent upon the fact that casein (the major protein of milk) is insoluble at its isoelectric point (pH 4.6, where the net charge of the casein is 0). Lactic acid bacteria produce lactic acid which reduces the pH from the natural pH of milk (pH 6.5-6.6) to pH 4.6 and lower. Yoghurt is produced by lactic acid bacteria that grow best at about 40° C. Since early times it has been an important food item in the Middle Eastern Mediterranean coast. Commercial production of yoghurt increased rapidly in Europe after Metchinkoff's (1908) findings

that consumption of sour milk prolongs life. The typical yoghurt flavour is caused by lactic acid, which imparts an acidic and refreshing taste, and a mixture of various carbonyl compounds like acetone, diacetyl, and acetaldehyde, the latter of which is considered the major flavour component (Law, 1981; Ott *et al.*, 1999; Tamime and Deeth, 1980).

Yoghurt or yoghurt-like products have also been used as the most popular vehicle for incorporation of probiotic organisms (Dave & Shah, 1997). The LAB must survive in the gastrointestinal tract to provide beneficial properties. When viable LAB are consumed through fermented milk, the dairy constituents offer excellent buffering capacity. Furthermore since LAB are in yoghurt (pH 4 - 4.5) the cells may be conditioned to low pH environment and survivability may be high in gastric juice which has low pH.

2.3.1 Health benefits of yoghurt

Healthy reasons to eat yoghurt are accumulating especially with the continuing research findings on the consumption of yoghurt and prevention of diseases formation. These are briefly described in the following:

- 1) Many people who cannot tolerate milk either because of protein allergy or lactose intolerance can enjoy yoghurt. The culturing process makes yoghurt more digestible than milk (Bertrand-Harb *et al.*, 2003).
- 2) The friendly bacteria in yoghurt reduces the conversion of bile into carcinogenic bile acids and this seems to deactivate harmful substances (such as nitrates and nitrites before they are converted to nitrosamines) before they can become carcinogenic (Commane *et al.*, 2005).
- 3) Consumption of yoghurt during antibiotic prescription will minimize the effects of the antibiotic removal of friendly bacteria in the intestines. The live bacterial cultures in

yoghurt can help replenish the intestines with helpful bacteria before the harmful ones take over (Macfarlane and Cummings, 1999).

4) Yoghurt can decrease yeast infection and it has prevention of growth of pathogenic bacteria (Gilliland, 1989).

5) Yoghurt is a rich source of calcium (Smith *et al.*, 1985). Because the live-active cultures in yoghurt increase the absorption of calcium, serving of yoghurt gets more calcium into the body than the same volume of milk. Smith *et al.*, (1985) and Rusoff (1987) verified that bioavailability of calcium in fermented milk is high and readily absorbed. Daily intake of yoghurt may also either reduce the risk of osteoporosis because it increases calcium assimilation in body or help lactase deficient individuals take steps to prevent osteoporosis (Wynckel *et al.*, 1991).

6) Yoghurt is an excellent source of protein (McGee, 2005). Besides being a rich source of proteins, the limited proteolysis of the milk proteins during fermentation makes these proteins easier to digest. For this reason proteins in yoghurt are often called “pre-digested protein” and have beneficial uses for certain people who lack the digestive enzyme due to disease states (Savaiano and Levitt, 1984).

7) Fermented milk products are excellent dietary minerals, particularly calcium, phosphorus, magnesium and zinc (Rusof, 1987).

8) Several LAB are capable of synthesising B-vitamins (Nilson *et al.*, 1965) and their concentration in fermented milk is generally high (Shahani & Chandan, 1979).

9) There are a few studies that have shown that yoghurt can reduce the blood cholesterol. This is because the live cultures in yoghurt can assimilate the cholesterol or because yoghurt binds bile acids (which has also been shown to lower cholesterol), or both (Liong & Shah, 2006).

10) Yoghurt and various dairy contain LAB are believed to confer a variety of important nutritional and therapeutical benefits to consumers including antimutagenic, anticancer

and anti carcinogenic activity (Rao *et al.*, 1986; Rao *et al.*, 1999; Fernandes *et al.*, 1987).

11) It is well known that whey proteins, especially β -lactoglobulin (BLG) and to a lesser extent α -lactalbumin (ALAC), are allergenic (Wal, 1998). Hydrolysis of these proteins by lactic bacteria may decrease this allergenicity.

12) Certain whey peptides are known to have biological activity such as opioid and bactericidal activity (Schlimme & Meisel, 1995).

13) Several peptides arising from proteolysis of milk proteins have been cited as exerting biological activity (Meydani and Ha, 2000) and influence calcium absorption and have pharmacological effects on the central nervous system, cardiovascular system, and digestive system including immuno-modulating properties (Meisel & Schlimme, 1990).

2.3.2 Syneresis

Syneresis in yoghurt is a physical separation of water from the curd of milk. The bonding type of water and mobility of water molecules are relevant to yoghurt manufacturing process with regard to sensory evaluation, stability, texture and food processing (Amatayakul *et al.*, 2006). The unique microstructure of yoghurt means that all the liquid (whey) is immobilized within its body. No consumer would like to buy yoghurt from which whey separates easily. This would be a sign that the yoghurt is susceptible to 'syneresis', and that there was something wrong in the yoghurt manufacture. If, for example, the milk is not heated at about 90°C for a time long enough (about 15 min), larger pores may develop in the yoghurt body in some areas and larger clusters of casein micelles may develop in other areas. The whey then starts showing in the containers during storage.

To be on the safe side, some yoghurt manufacturers use small additions of various ‘thickening agents’ such as starch gel, various plant gums or pectin to the milk to improve the retention of water in yoghurt. Water may also be retained in the yoghurt by increasing the amount of milk solids but in this case the reduction of the pore sizes changes the overall mouthfeel of the yoghurt and is not desirable (Cerning & Marshall, 1999). The casein strand can be broken apart by shearing, and the size of the aggregates decrease as the rate of shearing increases (Afonso and Joao, 1999).

Syneresis of the acid-induced casein network in yoghurt occurs during storage (Van Vliet and Walstra, 1994), and is related to the i) amount of total solid and casein content in milk, ii) incubation temperature, iii) rate of acidification and iv) presence of stabilizers that interact with the casein network. The firmness of yoghurt is affected by homogenization, pH, denaturizing of β -lactoglobulin (whey proteins) and adjacency to casein micelles (Lucey et al., 1998). Yoghurt is usually prepared from homogenized milk to improve stability. This process coats the increased surface of fat globules with casein, enabling the fat globules to participate as a copolymer with casein to strengthen the gel network and reduce syneresis (Keogh and O’Kennedy, 1998).

2.3.3 Yoghurt appearance

Appearance and physical characteristics are important quality parameters of yoghurt. Good quality yoghurt should be thick and smooth with no signs of syneresis. Set yoghurt with a high level of syneresis on the surface may be regarded as a low quality product, even though this is a natural phenomenon. Conventionally, syneresis is reduced by increasing the total solids of yoghurt mix to around 14% (w/w) with dry dairy ingredients (Tamime & Deeth, 1980) or by using stabilizers. Dry dairy ingredients such as skim milk powder, whey protein isolate, whey protein concentrate, sodium (Na)-caseinate or calcium (Ca)-caseinate are commonly used to increase the solids

content of the yoghurt mix. Nevertheless, fortification with these ingredients affects production costs. The use of stabilizers including gelatine, modified starches, or gums may affect the consumer perception of yoghurt. The use of stabilizers is also prohibited in some European countries (De Vuyst & Degeest, 1999).

2.4 Milk proteins

Approximately 3.0-3.5% of normal bovine milk is made up of protein; the concentration and composition of which can change during lactation (Phadungath, 2005a). Originally, milk proteins were believed to be a simple homogeneous protein, but about a century or more ago, milk proteins were divided into two broad classes (Fox and McSweeney, 1998). The first fraction, casein, constitutes about 80% of the protein in bovine milk (Brunner, 1977) and is phosphoproteins, precipitated at pH 4.6 (isoelectric pH) at 30°C. The second fraction which is whey protein or serum protein or non casein nitrogen makes up about 20% of protein, is soluble under those conditions (Dalglish, 1982; Fox and McSweeney, 1998). The two main components of serum protein in bovine milk are α -lactalbumin and β -lactoglobulin, and the rest are (blood) serum albumin, immunoglobulins, protease- peptones, and trace amount of enzymes and proteins with specific metabolic functions, such as lysozyme and lactoferrin (Brunner, 1977, Walstra *et al.*, 1999) and glycoproteins (Walstra *et al.*, 1999).

Rollema (1992) defined the casein micelles as the major part of the milk proteins, together with calcium phosphate, occurs in the form of large colloidal particles. Casein is made up of many components, and the main types are α s1- casein, α s2-casein, β -casein, and κ -casein (Walstra *et al.*, 1999) as defined and validated by analysis of amino acid sequences. There are trace amounts of γ -casein occurring naturally on account of limited proteolysis of β -casein by plasmin (Swaigood, 1992).

2.4.1 Changes in milk protein structure during fermentation

The central process in conversion of milk to yoghurt is agglomeration of casein micelles into a three dimensional network structure. Casein micelles disperse evenly in the milk and are separated from each other by a distance of three micelle diameters in fresh milk, they are subject to Brownian motion and thus they do not settle at the bottom of the container. In fresh or boiled milk, the surfaces of the casein micelles are non-reactive. When the temperature of the milk reaches 85°C, one particular micellar protein (κ -casein) at the surface of the casein micelles reacts with one particular whey protein (β -lactoglobulin). This interaction produces minute 'bumps' on the casein micelle surfaces (frame A in Figure 2.2 on the left). When yoghurt bacteria metabolize lactose and produce lactic acid, the milk starts to coagulate and casein micelles are destabilized. However the β -lactoglobulin- κ -casein complex prevents other casein micelles from getting attached at these sites.

In the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Streptococcus salivarius* subsp. *thermophilus* additional lactic acid is produced and this increases the acidity of the milk and destabilizes the micelles. After certain acidity is reached, the micelles stick together and the milk can be observed by naked eye to 'coagulate' or 'curdle'.



A

B

C

Figure 2.2 : A: Interaction of casein micelles with whey protein after heating;
B: Casein micelles before heating; C: Coagulated milk

Source: Dr.Miloš Kaláb -medicinal food news.

The surfaces of the heated casein micelles are partially blocked, so only a few micelles can interact. This leads to the formation of short branched micellar chains. The milk changed into a gel when the coagulation is complete. Under an electron microscope, the gel looks like a sponge with small pores filled with the whey (Figure C: scanning electron microscopy). Milk that has not been heated consists of casein micelles with smooth surfaces (frame B, on the right). This milk is used to make cheese. Casein micelle surfaces interact with other casein micelles and form large micellar clusters from which whey separates easily. The casein micelles become compacted to form curd which is then processed into one of the cheeses varieties. Cheeses have markedly lower water content than yoghurt.

Table 2.3 The milk proteins, their Isoionic points and molecular weights

Proteins	% of the milk proteins	Isoionic point	Molecular weight (Dalton)
<i>Caseins</i>			
α_s -caseins	45-55	4.92-5.35	22068-22723
κ -caseins		5.37	19005-19037
β -caseins	23-35	5.20-5.85	23939-24089
γ -caseins		5.80-6.0	11556-22629
<i>Whey proteins</i>			
β -lactoglobulin		5.35-5.41	18275-18362
BSA	0.7-1.3	5.13	66500-69000
α -lactalbumin		4.20-4.5	14146-14174
Immunoglobulins	1.9-3.3	5.5-8.3	150000-106
Proteose peptones		3.30-3.7	4100-40800

2.4.2 Proteolysis in fermented milk

Lactic acid bacteria (LAB) are characterised by their high demand for essential growth factors such as peptides and amino acids. However, milk does not contain sufficient free amino acids and peptides to allow the growth of LAB (Zourari et al., 1992; Abu-Tarboush, 1996). Therefore, these LAB possess a complex system of proteinases and peptidases, which enable them to use milk casein as a source of amino acids and nitrogen. The first step in casein degradation is mediated by cell wall located proteases, which cleave casein to oligopeptides. Further degradation to smaller peptides and amino acids that can pass through the cell membrane is performed by peptidases (Wohlrab & Bockelmann, 1992).

2.5 Rennin -Angiotensin System

The rennin system has been considered as a hormonal regulator of blood pressure together with sodium, potassium and water balance. The primary element of this mechanism is rennin which is released by juxtaglomerular cells in the kidney

(Gavras & Brunner, 2001). Renin acts on angiotensinogen, an α -globulin substrate which is performed in the liver, to form the inactive decapeptide angiotensin-I. Angiotensin-I is converted to biologically active angiotensin II that is a potent vasoconstrictor of vascular smooth muscle adrenal cortex which in turn promotes salt and water retention by the kidneys and increase blood volume and blood pressure with a consequent improvent of renal perfusion. It also inhibits rennin release from the kidney (Gavras & Brunner, 2001).

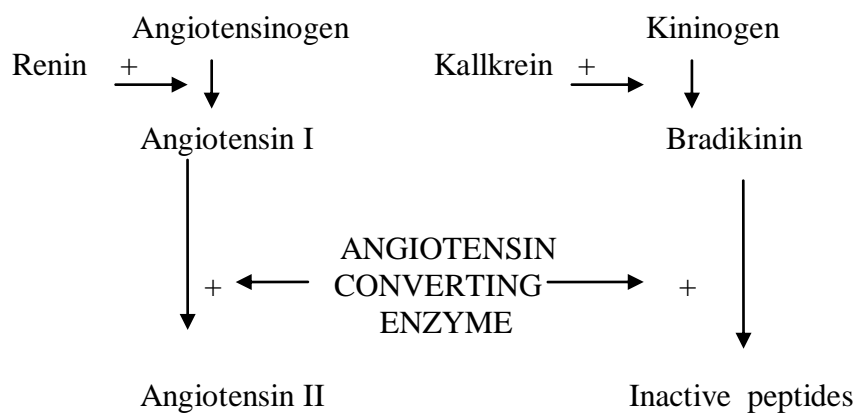


Figure 2.3 The renin-angiotensin system (Starr & Whalley, 1994)

2.5.1 Angiotensin-I converting enzyme

Angiotensin-I converting enzyme (ACE) plays a major role in the regulation of blood pressure (Vermeirssen, 2002). ACE is a glycoprotein which has oligosaccharide chain attached to polypeptide, found in lung, endothelial cells, plasma and associated with the rennin-angiotensin system, which regulates the peripheral blood pressure. The enzyme is also known as kininase II because it is able to degrade bradikinin (a vasodilatory peptide) to increase blood pressure. ACE can increase blood pressure by converting angiotensin I to angiotensin II (Petrillo *et al.*, 1982). ACE has been classically associated with the renin-angiotensin system which regulates peripheral blood pressure,

where it catalyzes both the production of the vasoconstrictor angiotensin-II and the inactivation of the vasodilator bradykinin (Zhao *et al.*, 2008).

2.5.2 ACE-Inhibition

Inhibition of ACE prevents the conversion of angiotensin I to II. ACE inhibitors act as a blocker of enzymes needed to form a substance that narrows blood vessels or the activity of an enzyme that causes constriction or narrowing the blood vessels. Consequently blood vessels relax and widen, make it easier for blood to flow through the vessels, which reduce blood pressure. ACE inhibitor will also increase the release of water and salt (sodium) to the urine, which lowers blood pressure. Therefore the inhibition of ACE activity exerts an antihypertensive effect through a decrease of angiotensin II and increase of bradikinin (Zhao *et al.*, 2008). Due to the multifunctional properties of this enzyme, inhibition may further have an effect on different regulatory system involved in immuno-defense and nervous system activities (Fitz-Gerald & Meisel, 2003). ACE inhibition which results mainly in an antihypertensive effect may thus also influence different regulatory systems involved in modulating blood pressure, immune defense and nervous system activity (Hebert, 1999).

Synthetic ACE inhibitors such as captopril and enalapril are common drugs established alongside β -blockers, thiazide diuretics, calcium antagonists and α -1 antagonists as first line agents in the treatment of hypertension (Choo *et al.*, 1999). However the side effects such as cough and angioneutric edema associated with clinically used ACE inhibitors have been addressed (Israili and Hall, 1992). Thus the screening and the development of new ACE inhibitors would be beneficial in the treatment of hypertension. Recently many studies (Meisel, 1998) have screened bioactive or biogenic substances derived from food such as milk, fish, gelatine and plants inhibitory effects on ACE. Milk proteins are precursors of many different biologically active peptides. These peptides are inactive within the sequence of the precursor

proteins but can be released into active forms by enzymatic proteolysis during intestinal digestion or during food processing (Gobbetti *et al.*, 2000). Since peptidase activity is intracellular in lactic acid bacteria, it has been suggested that lactic acid bacteria probably contribute only after cell lysis, which is considered a rare event in fermented milk due to the short fermentation time (Meisel & Bockelmann, 1999).

2.5.3 Bioactive peptides with ACE-inhibiting properties

Bioactive peptides are derivative products resulted from proteolysis of several food proteins. They are considered to be dietary components which are suggested as an aid in maintaining good health beyond basic nutrition. Milk naturally contains bioactive peptides by virtue of the presence of lysozyme, lactoferrin, immunoglobulins, growth factors and hormones which are secreted in their active form by mammary glands (Table 2.4; Schanbacher *et al.*, 1997). These biogenic peptides formed during gastrointestinal transit or food processing (Meisel, 1998) may act as an anti-hypertension, antibacterial, antigastric and enhancing utilization properties. In addition, degradation of casein by proteases leads to formation of peptides during fermentation of milk.

Laffiner *et al.* (1996) found that lactic acid bacteria are able to release peptides with immunomodulatory activity from β -casein (β -CN) in milk. This has been the basis of the use of different types of cheese as sources of anti ACE peptides (Smacchi and Gobbetti, 1998; Nakamura *et al.*, 1995a; Table 2.4). Among the bioactive peptides from milk proteins, much interest has been on those that inhibit ACE. Structure correlation between different peptides inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptides sequence of the substance (Chen *et al.*, 2007). A list of bioactive peptides found in dairy products is given in Table 2.4.

Table 2.4 Bioactive peptides identified from milk products

Product	Origin	Biofunctional role	Reference
Cheddar cheese	α 1- and β -casein fragments	Several phosphopeptides with a range of properties including the ability to bind and solubilise minerals	Singh et al. (1997)
Italian cheeses: Mozzarella, Crescenza, Italico, Gorgonzola	β -CN f (58–72)	ACE inhibitory	Smacchi and Gobetti (1998)
Yoghurt type products	α 1-, β - and κ -CN fragments	ACE inhibitory	Yamamoto et al. (1999)
Gouda cheese	α 1-CN f (1–9), β -CN f (60–68)	ACE inhibitory	Saito et al. (2000)
Festivo cheese	α 1-CN f (1–9), f (1–7), f (1–6)	ACE inhibitory	Ryhänen et al. (2001)
Emmental cheese	α 1- and β -casein fragments	Immunostimulatory, mineral binding and solubilising	Gagnaire et al. (2001)
Manchego cheese	Ovine α 1-, α 2- and β -casein fragments	ACE inhibitory	Gomez-Ruiz et al. (2002)
Sour milk	β -CN f (74–76, f (84–86), κ -CN f (108–111)	ACE inhibitory/ Antihypertensive	Nakamura et al. (1995a)
Dahi	Ser-Lys-Val-Tyr-Pro	ACE inhibitory	Ashar and Chand (2004)

(Emily Haque and Rattan Chand www.dairyscience.info)

2.6 Herbs and benefits of herbs

Herbs and spices are used to add flavours and tastes to foods. However, their uses may be beyond these because most herbs and spices used in culinary purposes have a long list of potential positive biological effects on human health. In fact, herbs and spices are prepared from plants, whose traditional uses stems from old medicinal remedies for preventing and treating human disease for thousands of years.

Numerous epidemiological studies have correlated human consumption of diets rich in fruits and vegetables containing high levels of phytochemicals to lower risk for specific chronic diseases such as diabetes, cancer and cardiovascular diseases (Davidson & Naidu, 2000). Phytochemicals are non-nutritive compounds found in plants that have potential disease-inhibiting capabilities. Nuts, whole grains, fruits, and vegetables contain an abundance of phenolic compounds, terpenoids, pigments, and other natural antioxidants associated with protection from and treatment of disease such as heart disease and hypertension as well as other medical conditions. Citrus, in addition to providing an ample supply of vitamin C, folic acid, potassium, and pectin, contains a host of active phytochemicals. The phytochemicals in grains reduce the risk of cardiovascular disease and cancer (Craig, 1997).

Phytochemicals give plants their color and fragrance and may also protect the plant against certain fungal or bacterial infections. In the present studies the effect of selected herbs on the nutritional and therapeutical values of fermented milk was investigated. These herbs are elaborated in the following sections.

2.6.1 *Angelica sinensis*

The root of *Angelica sinensis* (Oliv.) Diels, (Dong quai , angélica china) native to China, is a well known oriental herb belonging to family Umbeliferae/ Apiacea, second to ginseng in the Chinese herbal pharmacopoeia. Because of its broad pharmacological effects, it has been used for thousands of years in traditional Chinese medicinal prescriptions (Li 1997). It was first found in Chinese literature in *Collection of Commentaries on the Materia Medica* (500 B.C.) (Duke, 1985)



Figure 2.5. *Angelica sinensis* root

2.6.1.1 Description

This fragrant, perennial herb has a glabrous, smooth, purplish stem, with light linear striations. It grows 0.5 - 1 m high. The lower leaves are tripinnate, with upper leaves being pinnate; leaflets are oval, dentate-incised; 3-11 cm long, sheathed; bracts are rudimentary. The inflorescence is formed in 10-14 umbels, with 12 - 36 flowers per umbel. The flowers are white with five petals, blooming in June to July, with the fruit appears July till August.

2.6.1.2 Function and benefits

A. sinensis is classified as a tonic herb in China. It is said to be one of the most important herbs for strengthening the blood. This is the most commonly used herb in the

orient to regulate menstrual function and to tone the female organs. It can also be used by males to tone their blood and is very efficient in teenage acne. Anti-tumor action of *A.sinensis* has been observed in several studies (Cao *et al.*, 2006).

Modern pharmacological research indicated that these herbs could be used to treat female disease, apoplexy, hypertension, coronary heart disease and thromboangitis obliterans (Augustin *et al.*, 1995). It has been used as an antispasmodic, a blood purifier and to help to control blood pressure and rheumatism (Sung *et al.*, 1982). *A. sinensis* is rich in vitamins including A, B12 and E and minerals that help to build more blood cells and help to control anemia (Noe, 1997). *A. sinensis* is often used in the treatment of menstrual cramps, or dysmenorrhoea and post menstrual syndrome (PMS), hot flashes, and other menopausal symptoms.

Phytochemicals in *A.sinensis* root has 0.4 - 0.7% volatile oils, which include butylidene phthalide, n-valerophenone-O-carboxylic acid, dihydrophthalic anhydride, carvacrol, safrole, isosafrol, sesquiterpenes, cadinene, dodecanol, tetradecanol, n-butylphalid, ferulic acid, succinic acid, nicotinic acid, a number of coumarins and furocoumarins, uracil, adenine, ligustilide, folinic acid, beta-sitosterol, vitamin E, vitamin B12 (0.25 -40 mcg/100g), beta-carotene, palmitic acid, angelic acid, angelol, myristic acid, angelicone (Chu, 1986; Bensky & Gamble, 1986; Hsu and Chen, 1986).

In recent years, plant polysaccharides have been regarded as an important class of biological response modifiers. A wide range of polysaccharides, including pectic polysaccharides isolated from medicinal plants, have been reported to exhibit a variety of biological activity (Yongwen and Yamada, 1996; Haruki *et al.*, 1990). Ye *et al.*, 2001 observed that a crude extract from *A.sinensis* (ASCE) which mainly consisted of polysaccharides, significantly promoted migration and proliferation of normal gastric epithelial cells. Later studies (Ye *et al.*, 2003) showed that ASCE promoted ulcer healing as well as protection on ulcer of gastric.

2.6.2 *Codonopsis pilosula*

Codonopsis pilosula or its common name Dang Shen is a member of family *Campalunaceae*. It is a sweet, warm, soothing herb that is taken as an energy tonic. It is often cooked with rice until it is glutinous and used as a tonic food and also used in making stews. The part used for this plant is its root that is similar in action to ginseng (*Panax* species), but it is milder and has a shorter-lasting effect. This herb grows to about 1m tall and has oval leaves and a yellowish brown, bell-shaped flower. Root of *C. pilosula* is in the form of long cylindrical, slightly curved, 10-35 cm long, 0.4-2 cm in diameter and the interior of the root is brittle and has light-colored central pith. It is soft and tenacious in texture, sweet in taste and is aromatic.



Figure 2.6 *Codonopsis pilosula* dried root

2.6.2.1 Phytochemical

Phytochemical in *C. pilosula* include fridelin, hexenyl, hexenyl glycosides, neoligan glycosides, tetraxeryl acetate (Zhu, 1998), alkenyl, alkenyl glycosides, Dspinaastrol, D₇-stigmastenol, sterin taraxerol, tanshinone I, II_A, II_B, hydroxytanshinionone, methyle tanshinquinone, przewatnshinquinone, isotashinone I

and II, isocryptotanshinine, tanshiquinine A, B and C (Chen and Chen, 2004). Its glucosides has also been identified (tangshenoside I, III, IV) (Liu *et al.*, 1990). The root also comprises alkaloids, saponins, proteins, starch, vitamin B1, B2 and glucose, inulin and sucrose (Tun-hai *et al.*, 2007).

2.6.2.2 Function and benefits

C. pilosula is one of the nine important herbs in Chinese medicine; it is a gentle tonic that increases energy levels and helps the body adapt to stress. It supports the spleen which can clean the blood and helps the production of red blood cells. It acts mainly on the lungs and stomach, raising secretion of body fluids and blood sugar levels, and stimulating the blood cell levels and can reduce adrenalin levels and decrease blood pressure (Chen and Chen, 2004).)

C. pilosula contracts the suppression of the immune system caused by radiation therapy, therefore it is a superior herb for cancer patients (Wang *et al.*, 1996). Studies in animals showed that it dilates peripheral blood vessels and inhibits adrenal cortex activity, causing a lowering of blood pressure (Reid, 1995). *C. pilosula* has an important function in cardiovascular system to prevent abnormal clotting, exert cardiotonic effects on the heart and is beneficial in the treatment of heart disease. It also helps increase immune system because it is rich in immune stimulating polysaccharides (Wang *et al.*, 1996) which are useful in helping the body's natural immunities in fighting and reducing inflammation. Wang *et al.*, (1996) showed that it increased haemoglobin and red endurance to stress and promotes alertness. *C. pilosula* intake is believed to be capable of stimulating the nervous system and enhance neural growth factor in PC12 cells by amplifying an up-stream step of the MAPK-dependent signaling pathway (Liu *et al.*, 2003) and increase body resistance to disease by promoting phagocytosis. It can also resist gastric mucosa injury, and reduce gastric acid secretion (Wang, 1997).

2.6.3 *Illicium verum* (Star anise)

Illicium Verum, also called Star Anise, is categorized in *Magnoliaceae* (Magnolia family). *I.verum* is also known as badian (Iran), Anis de la chin (France), bunga lawang (Malaysia) `mai dai hoi` (Vietnam) and ba jiao (China). It is one of the most important spices in Chinese cuisine, an ingredient of 'five spice powder' (Bown, 1995), and used widely in Chinese and Vietnamese cuisine. *I. verum* tree is a small to medium size and may grow up to 8 m in height. The leaves are lanceolate and the axillary flowers are yellow. The tree is propagated by seed and the leaves are 6-10 cm long, alternate, simple, leatherby, often clustered 3 to 4 together at the end of branches.

I. verum has large white-pink to red greenish-yellow flowers (1-1.5 cm in diameter), the star shaped fruits radiate between five and ten pointed boat-shaped sections, with each one contain a single seed and cruch fruit with a licorice aroma. The fruits are harvested before they ripen and dried by sunlight. It is available whole, or ground to a red-brown powder. The dried fruit are used as herbs or spices in the preparation of certain dishes.



A

Figure2.6 A:*Illicium verum* dried fruit



B

B. *Illicium verum* plant

2.6.3.1 Phytochemicals

I. verum seed contain 20% fatty oils with 45% oleic acid, 24% linoleic acid, 23% palmitic acid, and 2.5% stearic acid (Duke, 1985). Anethole or trans-anethole is the major (85-90%) chemical component in star anis, which gives the licorice aroma. There are a number of phytochemical compounds found in *I. verum* such as anethole, cinnamaldehyde, O-methoxycinnamaldehyde, cinnamic acid (Shan *et al.*, 2005). The other components such as phelanderen, safrole and terpineol have only small effect on the aroma.

The chemical constituents found in *I. verum* include 1, 4-cineole, B-bisabolene, B-farnesene, alpha-copaene, c-alpha-bergamotene, trans-alpha bergamotene, caryophyllene, nerolidol, methylanisoate, trans-methylisoeugenol, cadinene, fueniculine, 3-carene, d-alpha-pinene (Hand Book of Medicinal Herbs and Hager's Handbook) .

2.6.3.2 Health benefits

The fruit is used as a flavoring in curries, teas and pickles (Kunkel, 1984). *I.verum* is extensively cultivated in China for its fruit and medicinal essential oil (Huxley, 1992). *I.verum* is the source of oil, volatile, aromatic oil used for flavouring smoked meats, candies, liqueurs, and perfumes. The essential oil is used as a substitute in soft drinks and bakery products. It is a common flavouring for medicinal teas, cough mixtures and pastilles (Hedrick, 1972). *I.verum* has been used in a tea as a remedy for colic and rheumatism, and the seeds are sometimes chewed after meals to aid digestion and sweeten breath (Grieve, 1984). *I.verum* is used to control flatulence and nausea, and is considered an antispasmodic for the gastrointestinal system. It also has been used for its mild diuretic properties, and for its expectorant qualities in the treatment of bronchitis. *I. verum* is the industrial source of shikimic acid, a primary ingredient used

to create the anti-flu drug Tamiflu (oseltamivir). Tamiflu is regarded as the most promising drug to mitigate the severity of bird flu (H5N1).

Star anise has carminative, stomachic, stimulant and diuretic properties. It is a common flavouring for medicinal cough mixtures and pastilles. *I. verum* has carminative, stomachic, stimulant and diuretic properties. In addition crude extract of star anise showed potent antifungal activity against a range of plant pathogens. The fruit has antibacterial properties against some bacterial strains including *Salmonella E*, *Shigella D, F*, *E. coli* and *Enterobacter A* (Katzner, 1999; Yeung, 1985). It also boosts an antispasmodic effect on the intestine thus relieving cramps. However, caution is advised since it is said to be poisonous if it is consumed in excess quantity (Sary, 1983).

2.6.4 *Lycium barbarum*

Lycium barbarum or Chinese wolfberry is from *solanaceae* family. *L. barbarum* is a sweet herb that grows wild in the remote areas of central China near Inner Mongolia, but is also grown as a cultivated plant throughout Asia, Britain, the Middle East and North America. *L. barbarum* has been studied by Chinese physicians for thousands of years for use in treatments ranging from replenishing vital essences to strengthening and restoring major organs, and boosting the immune system.

L. barbarum (Figure 2.7) is a wild bush which grows to about 3 m tall. The leaves are 0.2-5.0 cm wide. The pinkish or violet flowers constrict of small cluster of 5-6 lobed 8-12 mm short funnels. The part of the plant used is its red fruits (15-20 mm diameters) containing flat seeds and sweet in taste.



Figure 2.7 Fresh and dried *Lycium barbarum*.

2.6.4.1 Phytochemical

L. barbarum contains more than 18 amino acids, 21 trace minerals, beta carotene and cryptoxantin (pro-vitamin A). It is also has high amounts of vitamin B1, B2, B6, vitamin C and vitamin E (Qi *et al.*, 1986), and is a powerful antioxidant with anti-aging properties. *L. barbarum* phytochemicals include; Scopoletin, vanillic acid,

dihydro-N-caffeoyltyramine, trans-N-feruloyloctopamine, and trans-N-caffeoyltyramine, lyoniresinol 3 α -O-beta-D-glucopyranoside; daucosterol, betaine hydrochloride, E-ferulic acid octacosyl ester, 3R,3R'-zeaxanthin dipalmitate, Kukoamine A, betaine, withanolides, cerebrosides, (S)-9-hydroxy-E-10,Z-12,Z-15-octadecatrienoic acids, cerebroside (1-O-(beta-D-glucopyranosyl)-(2S,3R,4E,8Z)-2-N-palmitoyl)-tadecasphinga-4,8-diene; LCC), glucose, 2-O-(beta-D-Glucopyranosyl) ascorbic acid, polysaccharide-protein complex (LBP3p). It also contains scopoletin (I), beta-sitosterol (II), p-coumaric acid (III), glucose (IV), daucosterol (V) and betaine (VI) (Xie *et al.*, 2001).

2.6.4.2 Function and benefits

Polysaccharides are a major constituent of *L. barbarum*, representing up to 31% of pulp weight. Cao *et al.*, (1994) verified that *L. barbarum* polysaccharides can be used as an adjuvant in cancer therapy. *L. barbarum* polysaccharides (LBP) were also shown to enhance the immune system for cancer patients (Lu *et al.*, 1991). LBP also enhances the antibody reaction to cell-dependent antigen (SRBC) which means that LBP selectively strengthens the T cell immune response (Huang *et al.*, 2003). Zhou (1991) points out that polysaccharides from wolfberries aim cell-mediated and humoral immune responses. Vital red blood cells were also shown to be protected by flavonoids found in goji berries.

The protective effects of the total flavonoids of *L. barbarum* goji berries on lipid peroxidation in liver mitochondria and red blood cells of rats induced by oxygen radicals was also documented by Huang *et al.* in 2003. The mitochondria lipid peroxidation was significantly inhibited by the total flavonoids of *L. barbarum* and the fluidity of mitochondria membrane was also protected effectively. *L. barbarum* fruits are used to help maintain overall health when suffering from chronic conditions, such as

tired muscles and joints, dizziness and ringing in the ear, visual degeneration, headaches, insomnia, chronic liver diseases, diabetes, tuberculosis, and hypertension (Yeung, 1985). Using this herb can also help improve overall liver health, and balance blood pressure and blood sugar levels. The berries of this herb work as a liver and kidney tonic and in Chinese medicine, the liver is associated with the eye health.

Preliminary studies have shown that *L. barbarum* , which is rich in is effective in treating or preventing visual degeneration (Chan *et al.*, 2007), and due to its β -carotene is best known for improving visual system. The berries also protect the liver from damage caused by exposure to toxins. Gu *et al.*, (2007) indicated that LBP can effectively prevent alcoholic fatty liver of rats. This may be due to its effects in inhibiting the hepatocyte CYP2E1 expression and prevention of lipid peroxidation. In addition LBP appeared to be highly effective in promoting immunity and increase production of interleukin (Cao *et al.*, 1994). In *vitro* study showed the acetone extract from wolfberry fruit inhibit the gene mutation in TA98 and TA100 *Salmonella* strain (Tao and Zhongliang, 1992). It was also demonstrated in LBP that 2-AF induced mutation in TA100 was inhibited.

2.6.5 *Momordica grosvenori*

Momordica grosvenori (Lo Han Kuo Chinese common name) is a flowering plant in the family of *Cucurbitaceae*. It originates from China and this plant is cultivated in the mountains of southern China. It has 10-20 cm long heart-shape leaves and the fruit is 5-7cm diameter, contains a sweet, fleshy, edible pulp and numerous seeds. The fruit is harvested in round green form (Figure. 2.8) and is dried before further use. The outer surface of it is round and smooth, green-brown, covered with fine, soft hair (Dai & Liu, 1986). American food and drug administration (FDA) has classified *M.grosvenori* as a GRAS (generally recognized as safe) products.

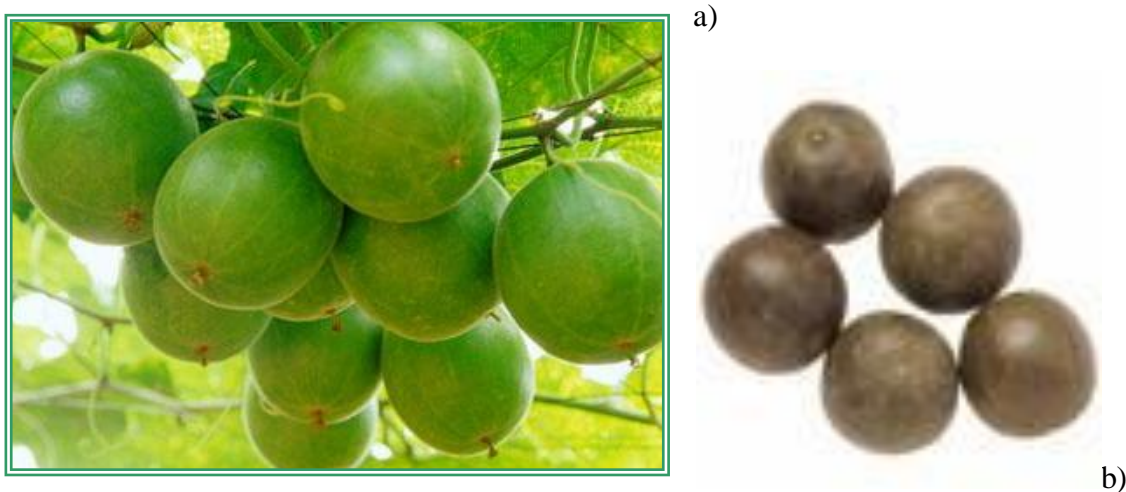


Figure 2.8 *Momordica grosvenori* a) plant b) dried fruit

The fruit water extract contains 80% mogrosides and offer a pleasant sweet taste with low calorie, without elevating blood sugar. The extract contains high amounts of amino acids, fructose, vitamins and minerals, as a nutritious food and a versatile natural sweetener that ideally suited to replace artificial sweeteners such as aspartame. *M. grosvenori* is an herb specializes in stimulating the organism of intestine and stomach, clearing away heat and stopping cough, preventing from being thirsty and reducing phlegm, keeping moist lung.

In comparison to other sweet additives *M. grosvenori*'s sweet taste is more than 250 times stronger than sucrose. *M. grosvenori* is currently being used in a wide range of lines such as foods, pharmaceutical, beverage and health care for the aged.

The fruits of *M. grosvenori* have been used for the treatment of pharyngitis or pharyngeus pain, and antitussive medicine in China and Japan as a folk medicine (Fangfang *et al.*, 2006). The sweet elements of this plant have been also assayed for oxidative modification of low density- lipoprotein. Two natural sweeteners, mogroside V and 11-oxo-mogroside V isolated from the fruits of *M. grosvenori* (Matsumoto *et al.*, 1990) exhibited strong inhibitory effect on the *in vivo* mouse skin carcinogenesis test (Takeo 2002). It also inhibits the promotion stages on two stage carcinogenesis induced by TPA (Konoshima and Takasaki, 2002). These compounds might be valuable as cancer chemo preventive agents for chemical carcinogenesis, and *M. grosvenori* might be valuable as a source of the chemo preventive agents. It was suggested that these natural sweetener are valuable as a substitute for sucrose in the food ingredient or the food additive (Takasaki *et al.*, 2003). Low calorie sweet elements may help to prevent cardiovascular disease, a complication of diabetes mellitus (DM). DM is associated with markedly increase risk of atherosclerosis cardiovascular disease, due to the modification of LDL by oxidation, glycosilation, or both.

2.6.6 *Psidium guajava*

Guava (*Psidium guajava*) is widely cultivated and consumed in Asia. The long history of guava's use has led modern-day researchers to study guava extracts. *P. guajava* has tranquilizing effect on intestinal smooth muscle, inhibit chemical processes found in diarrhoea and aid in the re-absorption of water in the intestines. An alcoholic leaf extract was reported to have a morphine-like effect, by inhibiting the gastrointestinal release of chemicals in acute diarrheal disease (Kamath *et al.*, 2008). This morphine-like effect was thought to be related to the chemical quercetin. In addition, lectin chemicals in guava have shown to bind to *E-coli* (a common diarrhea-causing organism), preventing its adhesion to the intestinal wall and thus preventing infection and resulting diarrhea (Watt and Branchwizk, 1969; Kamath *et al.*, 2008).



B



A

Figure 2.9 *Psidium guajava* a) plant b) fruit

2.6.6.1 Phytochemicals

The major components in *P.guajava* are: β -selinene, β -caryophyllene, caryophyllene oxide, squalene, selin-11-en-4 α -ol (Meckes *et al.*, 1996), guaijavarin, isoquercetin, hyperin, quercitrin and quercetin-3-O-gentobioside (Lozoya *et al.*, 1994), morin-3-O- α -L-lyxopyranoside and morin-3-O- α -L-arabopyranoside (Arima and Danno, 2002), β -sitosterol, uvaol, oleanolic acid and ursolic acid (Begum *et al.*, 2004). One new pentacyclic triterpenoid guajanoic acid, and four known compounds beta-sitosterol, uvaol, oleanolic acid, and ursolic acid have been isolated from the leaves of *P.guajava* by (Begum *et al.*, 2004).

2.6.6.2 Medicinal properties

Guava leaves are used for medicinal purposes, as a remedy for diarrhoea, and for their supposed antimicrobial properties (Lozoya *et al.*, 1990). According to Lutterodt and Maleque (1988) and Meckes *et al.* (1996), the leaf extract is used to treat diarrhoea, abdominal pain, convulsions, epilepsy, cholera, insomnia and has hypnotic effect. *P. guajava* leaf is a phytotherapeutic used in folk medicine to treat gastrointestinal and respiratory disturbances and is used as anti-inflammatory medicine (Abreu *et al.*, 2006). *P. guajava* leaf extracts may be beneficial in treating acne especially when they are known to have anti-inflammatory activities (Qadan *et al.*, 2005).

It was reported by Oh *et al.* (2005) that the extract from *P. guajava* leaves possesses antidiabetic effect in type 2 diabetic mice model and these effect is, at least in part, mediated via the inhibition of protein tyrosine phosphatase1B (PTP1B). A study revealed that guava leaf extracts comprise effective potential source of natural antioxidants (Qian & Nihorimbere, 2004). In addition *P. guajava* leaf extract was shown to have a wide spectrum of biological activities such as anticough, antibacterial, haemostasis (Jaiarj *et al.*, 1999; 2000). Some studies reported that the leaf extract and

its derivative identified as quercetin has effect on the intracellular calcium levels in gastrointestinal smooth muscle (Lutterodt, 1989; Lozoya *et al.*, 1990), in cardiac muscle cell (Morales *et al.*, 1994; Apisariyakul *et al.*, 1999) and in neuromuscular junction (Re *et al.*, 1999; Chaichana and Apisariyakul, 1996). More than twenty identified compounds from *P. guajava* leaf have been reported (Seshadri and Vasishta, 1965; Osman *et al.*, 1974; Lutterodt and Maleque, 1988).

Belemtougir *et al.*, (2006) reported that different leaf extracts of *P. guajava* and *Diospyros mespiliformis* inhibited caffeine induced calcium release from sarcoplasmic reticulum in a dose dependent manner. *P. guajava* popularly is used to treat hypertension in the central plateau of Burkina Faso (Nacoulma-Ouédraogo, 1996). In fact these results could explain their traditional use to treat many diseases and it can explain their use as hypertensive agent. Guava is rich in tannins have protein-binding properties and can interfere with many substances (Owen & Johns, 1999). It was suggested that tannins could reduce the intracellular calcium level by a decrease in the calcium inward current and by activation of the calcium pumping system (Chiesi and Schwaller, 1994). Zhu *et al.*, (1997) reported that tannins inhibited calcium channels and induced muscle relaxation.

CHAPTER 3: METHODOLOGY

3.1 MATERIALS

3.1.1 Milk

Pasteurized fresh cow's milk (Dutch lady's pasteurized and recombined full cream milk). All milk had balance of 7-10 days before expiry was purchased from supermarket.

3.1.2 Starter culture

Yoghurt pre-mix (Chr. Hansen, Denmark) was bought from local dealer. Every sachet provides 10 billion of selected acid resistant strains of friendly bacteria containing yoghurt powder and probiotic culture (4 billion *Lactobacillus acidophilus* LA-5, 4 billion *Bifidobacterium* Bb-12, 1 billion *Lactobacillus casei* LC- 01 and 1 billion *Streptococcus thermophilus* Th-4).

3.1.3 Herbs

Angelica sinensis, *Codonopsis pilosula*, *Illicium verum*, *lycium barbarum*, *Momordica grosvenori* were purchased from a Chinese medicinal shop whereas *Psidium guajava* leaves were collected from a tree located in the University of Malaya.

3.1.4 Chemicals

Chemical, unless stated otherwise were of analytical (A.R.) grade and these were purchased from Merck and Sigma Aldrich through local supplier.

3.2 METHODS

3.2.1 Preparation of herb powder

All herbs were first cleaned and washed thoroughly from visible contaminants and rinsed with distilled water prior to drying in an oven (Memmert; 45°C) until constant weight was achieved. The dried herbs, with the exception of *L. barbarum*, were ground (Kenwood BL335) to powder form. *L. barbarum* was purchased as semi dried and would be very sticky to grind. *L. barbarum* was kept in fridge, and was blended with distilled water on the day of experiment. Herbs powder was kept in airtight container in a cool and dry place and kept away from direct sunlight until use.

3.2.2 Preparation of herbal extract

The ground herbs were weighed and soaked in specified volume of sterilized distilled water (see Table 3.1). The mixture was mixed well and placed overnight in a warm water (70°C) bath (Julabo, Model Sw-21c or Haake Model SWD 20). The content was then centrifuged (2000 rpm for 10 minutes) and the supernatant was harvested and kept in a refrigerator (4°C). All herbal water extracts were used within 3 days. The percentage of each herbs used to make herbal-yoghurt were established in earlier study (Wai, 2006) to yield acceptable yoghurt taste and texture.

Table 3.1 Concentration of herbs in specified volume

	Herbs	Soaking %	Herb: water (g: ml)
1	<i>A.sinensis</i>	3.3%	5.0 g in 150ml distilled water
2	<i>C.pilosula</i>	6.3%	12.5 g per 200ml distilled water
3	<i>I.verum</i>	0.8%	2.3g per 150 ml distilled water
4	<i>L.barbarum</i>	7.5%	11.3 g per 150 ml distilled water
5	<i>M.grosvenori</i>	6.3%	9.4 g per 150ml distilled water
6	<i>P.guajava</i>	6.7%	16.6g per 250 ml distilled water

3.2.3 Yoghurt manufacturing

Yoghurt was prepared under a hygienic condition to minimize chances of contamination. Pasteurized fresh cow's milk (Dutch Lady pasteurized full cream, 1000ml) was placed in a sterilized glass beaker and the content was warmed up in a water bath (41°C). One sachet of yoghurt mix (lyophilized probiotics) was added followed by a uniform mixing using sterilized long spatula or glass rod. Aliquots (100ml) of milk containing probiotics were placed in 200ml disposable plastic containers. The containers were capped and incubated in shaking incubator (JElotech SI 900R) for up to 18 hours (see Sections 3.2.4a and 3.2.4b). The prepared yoghurts were stored in a refrigerator (4-6°C) for up to 3 weeks. A container of yoghurt was taken out for various analysis at 0, 3, 6, 7, 12, 14, and 21 days of refrigerated storage.

3.2.4 Preparation of herbal yoghurt

Herbal yoghurts (with 6 types of herbs) were prepared essentially as described for the plain yoghurt preparation with the exception that only 900 ml of fresh milk was used. Herbal extract (100ml) previously prepared (see Section 3.2.2) was added to the milk followed by the addition of Dutch-lady full cream milk powder (20g) to correct the

milk solid content. The mixture was mixed thoroughly with a spatula. Aliquots (100ml) of milk-herbal-probiotic mix were placed in disposable plastic containers (200ml) and the lids were put on tightly.

Two types of incubations were carried out:

- a) 18 hours incubation. Yoghurts were incubated in shaking incubator (41°C) and pH was monitored periodically for 18 hours. Yoghurts were cooled and stored at 4-6°C for up to 21 days for various analysis including physicochemical, microbiological, organoleptic evaluation, determination of peptides and amino acids concentration, ACE-inhibition activity and casein extraction for SDS-PAGE.
- b) pH 4.5 incubation. Yoghurts were incubated in shaking incubator (41°C) and pH was monitored periodically and incubation terminated when it reached 4.5. The yoghurt pH and TTA were measured every one hour. Yoghurts were immediately cooled after fermentation and stored at 4°C for up to 21 days for chemical and microbiological analysis subsequently at weekly intervals.

Evaluation of chemical, microbial, biochemical and therapeutical properties of yoghurt and herbal yoghurts in the present study was necessary to establish the effects of herbal water extract on yoghurt bacteria growth and metabolism during fermentation and refrigerated storage.

3.3 Physicochemical analysis

3.3.1 Sampling

Refrigerated set yoghurt was adequately stirred and samples were obtained using sterile spatula. The yoghurt was always kept on ice whenever the container was taken out of the refrigerator to ensure minimal yoghurt bacteria metabolic activity. Each assay was performed in triplicate.

3.3.2 pH evaluation

The pH of yoghurt is one of the important factors affecting growth, physiological activity, and bacteria viability. It also play main role in shelf life and assurance of acceptance of products post-fermentation. The pH changes were measured using digital pH meter (Mettler Toledo 320 USA) at room temperature (20°C). The pH meter was calibrated by using pH 7.0 and 4.0 standard buffer solutions.

Yoghurt sample was stirred evenly and samples (~3g) were weighed into a clean small beaker or test tube. Distilled water (9ml) was added and the mixture was mixed well. The pH electrode was immersed into the yoghurt slurry and the reading was recorded once it became stable (after ~10-15 seconds). The electrode was cleaned and rinsed with distilled water before and after each reading. Duplicate measurement was determined for each treatment.

3.3.2.1 pH changes during fermentation

The changes of pH during fermentation at 41°C with different concentration of starter culture were determined. Fresh milk (200ml) was pasteurized at 72°C for 15 second and was cooled immediately to 41°C. It was placed in a water-bath 41°C and one sachet of yoghurt mix was added and mixed evenly to dissolve probiotics in milk properly by using long glass rod (this concentration was considered as one time dilution). At the same time 40ml of this mixture was withdrawn and added to 160ml of pasteurized milk in same condition (five times dilutions). They were incubated at 41°C for 24 hours and the pH and TTA (total titratable acidity) were determined every 2 hours. After 24 hours 20 ml of fermented milk was added into 180ml pasteurized milk and the mixture was incubated at 41°C for another 24 hours (ten times dilution). pH and TTA were determined every 2 hours.

3.3.3 Total Titratable acidity (TTA)

TTA is a measure of total organic acids present in yoghurt. This was determined by titration using 0.1N NaOH to neutralize the acid. Yoghurts on day 0, 3, 6, 12 and 21 days storage were stirred thoroughly and each sample (5g) was diluted with 10 ml distilled water. Five drops of phenolphthalein 0.5% w/v (0.5g/100ml ethanol 95%) was added into the mixture. Titration was carried out using 0.1N NaOH under continuous gentle agitation of the flask until a definite pink colour developed. The volume of NaOH required to completely neutralize yoghurt acids was recorded. TTA was calculated by the following formula:

$$TTA = \frac{N(\text{NaOH}) \times V(\text{NaOH}) \times 0.0090}{W} \times 100$$

N= Normality of NaOH

V= Volume of used NaOH to neutralize acids

W= weight of yoghurt sample

3.3.4 Moisture content

Moisture content (MC) of yoghurt which measures the yield and quantity of solid content of yoghurts is much related to preserving characteristics. Moisture content of yoghurt was determined by deducting the total water evaporated from known yoghurt wet weight (Salwa *et al.*, 2004). Firstly glass plates were preheated in oven (Mettler) for one hour and cooled in a desiccator for 15 minutes. These plates were weighed electronically (OHAUS AdventureTM) correct to four decimal places. Two grams of every yoghurt samples was placed in dried plate and the weight of yoghurt and dried plate was recorded. The plate was placed in an oven (70°C) to constant weight. The dried samples were cooled in desiccators (to prevent of moisture absorption from the air) for 15 minutes and the dry weight was recorded.

Total yoghurt dry weight was calculated using the following formula:

$$\% \text{ Moisture content} = \frac{W1 - W2}{W_s} \times 100$$

W1= weight of plate + wet sample

W2= weight of plate + dry sample

Ws= weight of sample (2g)

%Total solid = 100 – %Moisture content

3.3.6 Sensory evaluation

Sensory evaluation of yoghurt was assessed by giving scores 1-10 points to the following characteristics: overall appearance, overall aroma, overall taste, sourness and bitterness. Assessment was carried out by 10 untrained individuals with mean age of 22 years. Every panellist was given the task to evaluate yoghurt samples (20g) that have been stored for 0, 7 and 21 days of storage.

3.4 Microbiology analysis

3.4.1. Preparation of dilutions

Yoghurt samples (100ml) were collected aseptically from the refrigerator on the day of analysis (day of 0, 3, 6, 12 and 21) and these were kept on ice. The surface of working area was cleaned with a suitable disinfectant (ethanol 70%). The yoghurt was serially diluted (1 part yoghurt in 9 part diluent) using sterile peptone water (2% w/v) as diluent. Diluted yoghurt was mixed thoroughly to ensure uniform distribution of the microorganisms present. Aliquots (1 ml or 0.1 ml) of the required dilutions were plated in duplicate on Petri dishes. These steps were carried out within 20 minutes of yoghurt sampling from the refrigerator. Each experiment was carried out three times and diluted yoghurt was prepared twice for microbial enumeration.

3.4.2 Enumeration of *Streptococcus thermophilus*

3.4.2.1 Preparation of culture media

M17 agar was used for the selective enumeration of *S. thermophilus*. It was prepared by re-hydrating M17 agar powder (48.2g; Oxoid) in 950ml distilled water followed by autoclaving (121°C; 15 minutes). The agar was cooled down to 50°C and membrane (0.22µm) filtered 10% Lactose monohydrate (w/v) solution (50 ml) was added. The mixture was mixed thoroughly immediately prior to pouring onto the Petri dishes. The agar was allowed to solidify for 15 minutes and the petri dish was sealed using parafilm. The plates were incubated in an inverted position over night at 37°C.

3.4.2.2 Inoculation and enumeration

Duplicate diluted yoghurt samples (0.1ml) were delivered by sterile pipette onto the surface of solidified agars. Sterile spreaders were used to spread inoculums evenly over the surface of the agar. This was done by placing the spreader on the surface of the inoculated agar, followed by a rotation of the spreader and wiping in a top-to-bottom and side-to-side motion. This operation must be carried out quickly to minimize the risk of contamination. After the inoculums have dried the plate was placed in inverted position under aerobic environment in an incubator (Memmert; 37°C, 24 hr). *S. thermophilus* was enumerated by counting visible colonies on the agar after 24 hours of incubation.

3.4.3 Enumeration of *Lactobacillus sp.*

3.4.3.1 Media preparation

MRS agar (62g/L; Oxoid) was used to enumerate *Lactobacillus spp.* (Bergamini *et al.*, 2005). It was autoclaved at 121°C for 15 minutes and cooled to 45-50°C. Bottles

containing sterile molten agar was placed in water bath (45°C) to maintain liquidity of agar while waiting to be plated.

3.4.3.2 Inoculation and enumeration of bacteria

A series of prepared decimal dilutions (normally 10^{-3} to 10^{-5}) of yoghurts were used. Since *Lactobacillus sp.* is anaerobic, the present study was carried out by pour plating followed by layering with molten MRS agar because anaerobic jar or 10% CO₂ there was not available in our laboratory. A pour plate is one in which a small amount of inoculum (1ml) from broth culture is mixed evenly with molten agar (45°C, 15ml) in a Petri dish. Pour plate allow micro-organisms to grow both on the surface and within the medium. The agar (first layer) was left undisturbed on a flat surface for about 10 minutes to allow the agar to completely gelled. A second layer of MRS agar was made by pouring 10 ml of molten MRS agar on top of the first layer. The lid was sealed with parafilm (Laboratory film) and the plate incubated in an inverted position at 37°C for 72 hours to allow the colonies to develop.

3.4.6 Viable cell count calculation

Population of bacteria in each gram of yoghurt samples was calculated by using the following formula:

$$\text{CFU/ml} = \frac{N \times (\text{D.F})}{\text{Yoghurt sample (ml)}}$$

N = number of colony forming units (CFU) on the agar plate

D.F = dilution factor

ml = the aliquot of yoghurt sample used to prepare dilution

3.5 Determination of peptides and free amino acids formation from milk protein proteolysis

3.5.1 Cd-ninhydrin method

Proteolysis in yoghurt was verified using a modified method of Folkertsma and Fox (1992) by Cd-ninhydrin to monitor free amino acids. Water soluble nitrogen extract (WSN) from yoghurt was prepared as follows: 5g of yoghurt samples on day 0, 7, 14 and 21 were weighed and homogenized (Polytron PT 2100) in 20ml of distilled water. The homogenate mixture was then incubated in water-bath (40°C; 1 hour), followed by centrifugation (6000rpm; 30 minutes; 4°C). The supernatant (WSN) was used to determine free amino acids in yoghurt.

Cd-ninhydrin reagent was prepared by mixing 80 ml of 99.5% ethanol and 10ml acetic acid. Ninhydrin (0.8g) was added to this mixture followed by mixing with 1 ml of CdCl₂ (Cadmium chloride) solution (1g/ml). WSN (200ml) was transferred into glass test tube containing 800 µl distilled water and the mixture was mixed thoroughly by vortexing. Cd-ninhydrin reagent (2 ml) was added to this mixture, briefly mixed, followed by heating (84°C; 5 minutes) and cooling to room temperature prior to being transferred into plastic cuvette for absorbance measurement (507nm, Thermo spectronic GENESYS four USA). Assays were performed in duplicate.

The free amino acids concentration was determined based on a standard curve using L-leucine (the most amino acid which present in milk products) as a reference. L-leucine standard solutions were prepared by making a serial dilution of 2 mM L-leucine stock solution to yield 0.01, 0.03, 0.05, 0.07, 0.1, 0.2, 0.3mM. These solutions were subject to free amino acid determination as described above.

3.5.2 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

3.5.2.1 SDS- PAGE solutions

Unpolymerized acrylamide is skin irritant and a neurotoxin. Thus it is important to carry out the preparation using gloves.

The following solutions were prepared according to Laemli (1970) system.

- Solution A: 30% (w/v) Acrylamide (60g) and 0.8% (w/v) N, N-methylen bisacrylamide (1.6g) were added to distilled water to a total volume of 200 ml. The mixture was stirred until completely dissolved. This solution can be stored at 4°C in refrigerator.
- Solution B: 1.5 M Tris-HCl, pH 8.8. Tris (18.17g) was initially dissolved in about 50 ml of distilled water. HCl was then added to adjust the pH to 8.8 in volumetric flask. The volume was made up to 100 ml using distilled water.
- Solution C: 10 % (w/v) SDS (Sodium Dodecyl Sulphate; minimum 98.5% GC, Sigma Aldrich). SDS (5.0g) was mixed with distilled water to a final volume of 50 ml. The mixture was stirred until completely dissolved.
- Solution D: Freshly prepared 10% (w/v) APS (Ammonium persulphate 98+%, Sigma). APS (0.1g) was added to 1ml of distilled water and stirred to dissolve. This solution was prepared on the day it was required.
- Solution E: TEMED (N,N,N',N'Tetramethyl-Ethylene Diamine 99%; Sigma)
- Solution F: 0.5 M Tris-HCl. Tris (6.06g) was dissolved in 50 ml distilled water and pH was adjusted to pH 6.8 with HCl, the volume was made up to 100 ml using distilled water
- Electrophoresis buffer (cathode buffer): 0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3. Tris, Glycine and SDS (3.8 g, 14.4g and 1.0 g respectively) were

mixed with distilled water, the pH was adjusted to 8.3 with 6N HCl and the final volume was made up to 1L with distilled water.

- Sample buffer: 0.2 SDS, 1ml glycerol, 1.25 solution F (Tris-HCl 0.5 M, pH 6.3), 5% (w/v) (0.5ml) reducing buffer β -mercaptoethanol, distilled water was added to 10 ml volume and Bromophenol blue (0.02%) to see blue colour in the buffer. It was stirred to dissolve properly.
- Fixing solution: 40% v/v methanol, 10% v/v acetic acid in water. Methanol (40ml) and acetic acid (10 ml) were mixed and the volume was made up to 100 ml with distilled water.
- Staining solution: 0.025% (v/v) Coomassie Blue in fixing solution. This solution was filtered before use and it was reused 8-10 times to stain the gel.
- Destaining solution: 5% v/v methanol, 7% v/v acetic acid in water. Methanol (5ml) and acetic acid (7ml) were mixed and the volume was made up to 100ml with distilled water.

3.5.2.2 Casein extraction

Caseins were obtained from yoghurts by using a method from Veloso *et al.*, (2002, 2004). Herbal and plain yoghurts on day 0, 7, 14 and 21 of storage were used. Samples (10g) were weighed and homogenized by the addition of 10 ml of 1M ammonium-acetate buffer, pH 4.3. The resulting suspension was kept at 8°C for 20 minutes followed by centrifugation (3000g; 15 minutes; 4°C) to recover the precipitated caseins. The caseins were dispersed in 1mM ammonium-acetate buffer pH 4.3, mixed and precipitated again by centrifugation (3000g; 10 minutes; 4°C). This procedure was repeated twice. Residual fat content was removed by washing the caseins with acetone and the casein was left to precipitate (15minute; room temperature). The upper layer, which contained fat dissolved in acetone, was poured off and the precipitated caseins were dried at room temperature. Dried casein was ground with pestle and mortar. The

powdered caseins were kept in airtight test tubes at 8°C until required for further analysis.

3.5.2.3 Preparation of SDS-polyacrylamide gels

a. Separating gel

The percentage of acrylamide in gel varies depending upon the size range of polypeptides present in the sample. Separating gel (15%; Table 3.2) was selected because it can show protein band which their molecular weights are between the range of 10000-100000 Dalton that is suitable for casein and other desired peptide in milk.

Table 3.2 Separating gel mix for total volume of 20ml

Content	volume(ml)
Solution A	10
Solution B	5
Solution C	0.2
Solution D	0.15
Solution E	0.014
Water	4.6
Total volume	20 ml

Procedure

1. SDS-polyacrylamide gels were cast in between two glass plates that were assembled according to the manufacture's instructions.
2. SDA-PAGE solutions (see section 3.5.2.1) were combined (Table 3.2) in a small glass beaker. Solutions A, B, C and water were initially mixed followed by the mixing with solution D and E. Extreme care was taken not to aggressively mix the mixture because excessive aeration will interfere with polymerization.

3. The assembled glass was kept in a vertical position and the separating gel mix was poured into the glass mould carefully using a pipette until the gel reached 1.5cm from the top of the glass.
4. The vacant space from meant for stacking gel and comb was filled with distilled water. Polymerization under a thin film of water helps to prevent evaporation of water from the gel.
5. A distinct interface was appeared between the gel and water when the gel was polymerized after 30-60 minutes. Water was poured off and the empty space was washed twice with distilled water.

b. Stacking gel

The following volume of stock solutions was mixed to prepare stacking gel 12.5%.

Table 3.3 Stacking gel mix

Content	volume(ml)
Solution A	0.65
Solution F	1.25
Solution C	0.05
Solution D	0.025
Solution E	0.005
Distilled Water	3.05
Total volume	5 ml

Procedure

Solution A, F, C and distilled water were initially mixed in a beaker followed by the addition of solution D and E. The contents were mixed by gentle swirling. The stacking gel mix was poured on top of the polymerized separating gel. A clean 10 well comb was carefully inserted into the gel sandwich until the bottom of the comb teeth reached to the top of the front plate. (Care was taken not to allow the inclusion of bubbles at the

ends of the teeth. Gel assembly was left (20-30 minutes) in a vertical position to allow the stacking gel to polymerize.

When the gel was polymerized, it was placed into gel electrophoresis tank after removing the bottom spacer. Electrophoresis buffer was added to the bottom and top reservoirs. All bubbles present at the bottom of gel between the glass plates were removed with a needle attached to a syringe to ensure buffer flows evenly. Comb was removed carefully. Distorted ears were repositioned using a Hamilton syringe. It is important to rinse the wells with electrophoresis buffer prior to loading in order to remove unpolymerized acrylamide and any contaminants.

c. Preparation of samples and markers

Preparation of sample for SDS-PAGE was carried out according to Ong *et al.*, (2005). An aliquot of herbal and plain yoghurt's caseins (40 mg) were suspended in a mixture of 1ml Tris (10mM)-EDTA (1mM) pH 8.0 buffer, 350 μ l of 10% SDS and 50 μ l of β -mercaptoethanol (Sigma). The suspensions were boiled in boiling water for 5 minutes to ensure a complete dissolve. Stock solution (25ml) was diluted with 200 μ l of sample buffer; and it was heated again for 3-5 minutes in boiling water.

For the present study six standard peptides from Sigma-Aldrich were used as markers for yoghurt peptides. α -, β - and κ -caseins, α -lactalbumin, β - lactoglobulin and Bovine serum albumin (BSA). Each marker (2mg) was dissolved in 1ml distilled water at neutral pH (concentration of 2mg/ml). From every diluted marker (20 μ l) was transferred into a micro tubes (eppendrof) and was mixed thoroughly. A 20 μ l aliquot of this mixture was diluted with 80 μ l sample buffer. All experiments were repeated two times with similar results. Protein bands were identified based on molecular weight and also by comparison with standards of casein (CN), α -CN, β -CN and κ -CN. α_{s1} CN and α_{s2} -CN could not be separated easily because the molecular weights of these two

fractions are very similar (Varnam & Sutherland, 1994). β - Lactoglobulin (MW 18, 300) and α -lactalbumin (MW 14, 000 Dalton) were identified based on their molecular weight. Degradative compound of caseins in yoghurts samples was determined by using Bio-Image densitometric analysis. The intensity and area of band were measured and subsequently used for the estimating of protein.

e. Loading the samples and markers

Depending upon the size of the wells, 15-40 μ l of the sample can be added into each well. A 15 μ l aliquot of each sample and 10 μ l of each marker were loaded by using Hamilton syringe. Sample buffer was loaded into unused wells, if there were any. The syringe was rinsed thoroughly with distilled water prior to loading different samples.

f. Running the SDS-page gel

The electrophoresis equipment was connected to a power supply. The polypeptides migrate towards the anode. SDS-gels were run at 120-150 V (5-10 V/cm of the gel). It was continued until the blue dye migrated to reach 1-5mm from the bottom of the gel, which normally took between 1 and 2 hours.

g. Staining the SDS-PAGE gels

The gel cassette was removed from electrophoresis tank and one of the plates was taken off by applying gentle pressure with the help of a spatula. The gels were first fixed with fixing solution for 30 minutes. Gloves were worn to prevent transfer of finger prints onto the gel. The gel was then transferred into the staining solution and was initially agitated to facilitate staining for 10-20 minutes followed by a further staining without agitation. The gel was then rinsed with de-staining solution and this was kept in that solution until protein bands became visible. This normally took 1-2 hours. The gels

were scanned (Bio-image software) and the gel images were analysed by using “Image MasterTM 2D Platinum 6.0”. Data from the analysis were expressed as the ratio of the area and the intensity of the bands.

3.5.3 Yoghurt water extraction for OPA and ACE experiments

An aliquot of 10 g of every herbal yoghurt from day 0, 7 and 14 was homogenized with 2.5 ml distilled water (1: 0.25 ratio). The pH of yoghurt was adjusted to 4.0 using 1M HCl followed by heating at 45°C in water bath for 10 minutes. Precipitated proteins were removed by centrifugation (5000 g; 10 minutes at 4°C; Centrifuge 5804R eppendorf). The pH of the supernatant was adjusted to 7.0 using 0.5 M NaOH. The supernatant was then centrifuged again (5000g, 10 min at 4°C) for further precipitation of salt and big proteins.

3.5.4 The OPA test

OPA method (Church *et al.*, 1983) was used to determine the peptide concentration in extract prepared from yoghurt. This method is a sensitive and rapid spectrophotometric assay for proteolysis of milk proteins.

3.5.4.1 Preparation of OPA Reagent

The OPA reagent was prepared as described by Church *et al.*, (1981). It was made by mixing 25ml of 100mM sodium tetraborat, 2.5 ml of 20% (w/w) sodium dodecyl sulphate (SDS), 40 mg of OPA (O-phthaldialdehyde; Sigma-25 g) dissolved in 1ml of methanol and 100 µl of β-mercaptoethanol. This mixture was diluted to a final volume of 50 ml with distilled water. OPA reagent was prepared on the day it was needed. The container was covered by aluminium foil to prevent reagent degradation by exposure to UV light.

3.5.4.2 Determination of peptide concentration

Determination of peptide concentration was carried out using diluted yoghurt water extract (2X dilution) and OPA reagent. Aliquots of 25 μ l (10-50 μ l) were added to test tubes containing 1ml of OPA reagent. The mixture was mixed thoroughly and was then incubated for 2 minutes at room temperature. Absorbance was measured at 340 nm using spectrophotometer (Thermo spectronic GENESYS four USA).

3.5.4.3 Preparation of Standard Curve

The peptide content was quantified using Tryptone as a standard peptide. A linear standard curve was prepared using serial dilution of 1.5 mg/ml standard (Tryptone dissolved properly in sterile distilled water). The Tryptone stock solution (1.5mg/ ml) was serially diluted to yield i.e. 1.25, 1.00, 0.75, 0.5, 0.25 and 0.125 mg/ml. Absorbance of these solutions were measured by adding 25 μ l of every dilution to 1ml OPA reagent followed by adequate mixing, Incubation was carried out for 2 minutes at room temperature and the absorbance was recorded at 340nm.

3.5.5 The ACE Inhibition Activity of herbal yoghurt

3.5.5.1 Substrates and chemicals

Furanacryloyl-Phe-Gly-Gly (FAPGG), angiotensin-converting enzyme from rabbit lung (Sigma), enalapril (Sigma), fresh rabbit lung, potassium phosphate buffer (50mM, pH 8.3), NaCl 400mM, Tris-HCl buffer (50 mM, pH 8.3), yoghurt water extract.

3.5.5.2 Preparation of Rabbit lung extract

The rabbit lung extract was prepared by homogenizing fresh rabbit lung with potassium phosphate buffer 50mM, pH 8.3. The homogenate was ultracentrifuged (60 min at 20,000 g; Biofuge Heraeus Kendolaboratory). The supernatant was then harvested and made to 500 µl aliquots.

3.5.5.3 ACE activity assay

Commercial angiotensin-converting enzyme from rabbit lung (0.1 U/ml; Sigma) was reconstituted with 1 ml demineralised water according to the manufacture recommendation. ACE reagent (500µl) and demineralised water (500 µl) or inhibitor solution were mixed and pre-incubated for 2 min at 37°C. After the addition of 100µl enzyme (commercial ACE) to this suspension, the reaction mixture was incubated for a total period of 20 min at 37°C. The absorbance of the reaction mixture was measured against demineralised water at 340 nm over a time interval of exactly 5 min. This assay was used as a standard to compare with the activities of different dilutions of fresh extract of rabbit lung (RLE). A 5 to 7 fold dilution of RLE that matches the standard rabbit lung extract was routinely obtained. The activity of the angiotensin-converting enzyme is expressed in U/l, whereby 1 unit is defined as the amount of enzyme that catalyzed the formation of 1 mMol FAP per minute under the assay conditions of the Sigma-Aldrich ACE assay.

3.5.5.4 ACE inhibition assay

ACE activity was measured spectrophotometrically with Furanacryloyl Phe Gly Gly (FAPGG, Sigma) as substrate and fresh extract from rabbit lung as ACE source according to Vermirssen *et al.*, (2002). The ACE activity is determined upon hydrolysis of a Furanacryloyl tripeptide (FA-Phe-Gly-Gly, FAPGG) leading to the production of

the corresponding amino acid (FA-Phe, FAP) and dipeptide (Gly-Gly, GG). The release of these peptides decreased the absorbance at 340nm wavelength. ACE reagent containing 1mM FAPGG was prepared by dissolving 25 mg FAPGG in 62.5 ml of diluent (equal volume of 400mM and 50mM Tris-HCl buffer pH 8.3).

Aliquots of 500 µl were placed in microtubes (Eppendorf) and these were kept at -20°C until required. The reaction mixture contained 500 µl ACE reagent, and 300µl of appropriately diluted yoghurt extract (between 1-2x dilutions). This mixture was pre-incubated for 2 min at 37°C followed by the addition of 300µl of diluted (5x) rabbit lung extract and further incubation in water-bath (37°C). The decrease in absorbance (340nm) was monitored every 5 minutes from 0 minute until 20 minutes. Distilled water was used as a blank. Reagent blank contains 500 µl ACE reagent, 300µl of distilled water (instead of yoghurt sample or any other inhibitor) and 300µl of rabbit lung extract. This blank was treated in the same manner as for samples. Percent of inhibition was calculated based on standard rabbit lung enzyme activities in the absence of inhibitor. The ACE inhibitor, Enalapril maleate salt (10^{-9} M, Sigma) was used as a positive control.

3.5.5.5 Inhibition of ACE by herb extract

The herbs extract used for ACE inhibition was the same as the extract used in herbal yoghurt preparation. The extract was diluted using distilled water to achieve the same concentration as in herbal-yoghurt. The ACE inhibition assay was carried out as described in section 3.5.5.4. ACE reagent (500 µl) and 300µl of appropriately diluted herb extract was mixed and was pre-incubated for 2 min at 37°C followed by the addition of 300µl of diluted (5x) rabbit lung extract and further incubation in water-bath (37°C). The decrease in absorbance (340nm) was monitored every 5 minutes from 0 minute to 20 minutes of total incubation period. Distilled water was used as a blank.

Reagents for control consisted 500 µl ACE reagent, 300µl of distilled water (instead of herb extract or any other inhibitor) and 300µl of rabbit lung extract. This blank was treated in the same manner as herb samples.

3.6. Statistical analysis

Statistical analyses were carried out using SPSS 14.0 (Chicago, IL, USA) for Windows. All experiments were performed in at least duplicates. A total of 3 batches of yoghurts were used to run the assays. Means, standard errors and standard deviations were calculated and the results were analyzed using One-way ANOVA procedures and Duncan test for means comparison were used for the statistical analysis of data. The level of significance was preset at $p < 0.05$.

CHAPTER 4: RESULTS

4.1 Chemical and Microbial Analysis of yoghurts with different stages of fermentation

The aims of the present study were to examine the effect of the presence of herbs on fermentation and post-acidification of yoghurt, viability and metabolic activity of probiotic bacteria and the anti-angiotensin-1 converting enzyme activities.

4.1.1 Changes of pH in fermentation of milk in the presence of herbs' water extract at 41°C

Figure 4.1 (and Table 4.1 in Appendix) show the changes in pH of milk with time during fermentation at 41°C. There was a lag phase for all treatments during the first 4 hours of fermentation. Changes in pH for plain-yoghurt (control) started to occur after 5 hours of incubation, whereby the pH decreased from 6.73 to 4.49 at the 12 hour of fermentation. Other yoghurts treated with different herbs also started to decrease sharply from the 5th hour of fermentation to reach pH 4.5 over a period ranging from 11-13 hours. *A. sinensis*-, *C. pilosula*-, *I. verum*-, *L. barbarum*- and *M. grosvenori*-yoghurts reached pH 4.5 after 11 hours of incubation which was faster than control (12 hour). *P. gujava*-yoghurt however took 13 hours of fermentation to reach pH 4.5.

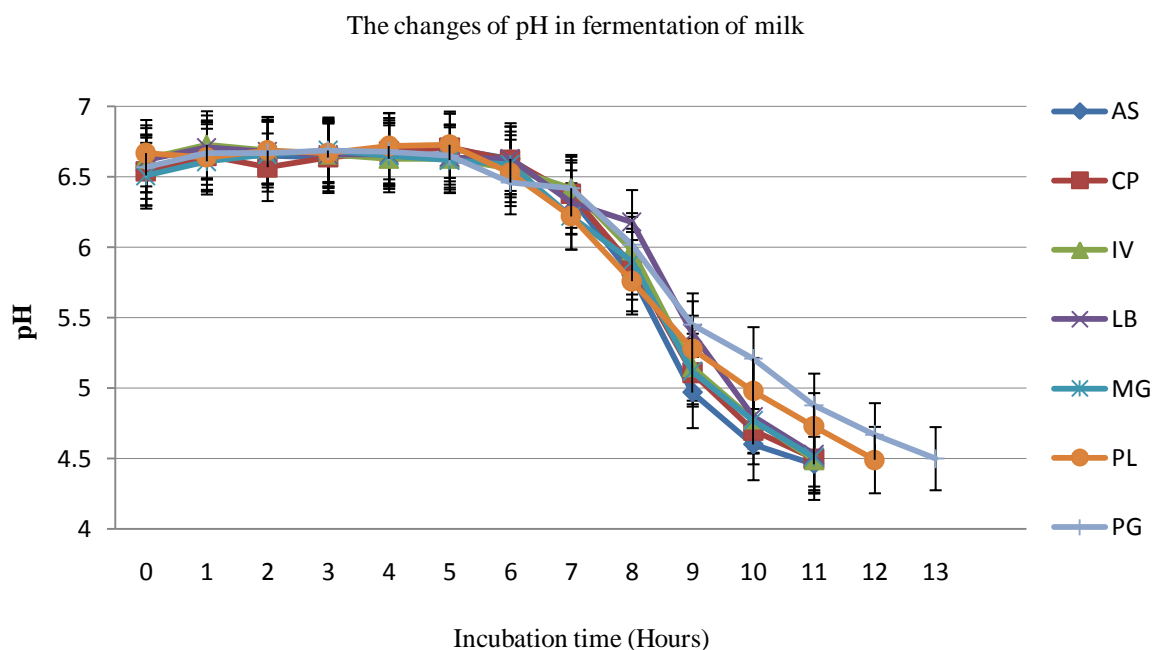


Figure 4.1: Changes of pH during the fermentation of milk¹ in the presence of different herbs² at 41°C

¹ Milk was incubated at 41°C, ² AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

4.1.2 Changes of TTA during yoghurt fermentation in the presence of herbal water extract.

Changes in the percentage of lactic acid production during the fermentation of milk in the presence of herbal water extract are as shown in Figure 4.2 (and Table 4.2 in Appendix). There were undetectable changes in TTA concentration during the first 3 hours of fermentation. TTA percentage started to increase after the 3rd hour of incubation and differences in TTA concentration were evident after the 6th hour of incubation. The TTA of yoghurt at pH 4.5 (when incubation was terminated) was 0.75% for the control and *P. guajava*-yoghurt whereas the TTA for *A. sinensis*-, *L. barbarum*-, *C. pilosula*-, *I. verum*-, and *M. grosvenori*-yoghurts were higher (0.81%, 0.85% ($p > 0.05$); 0.9%, 0.8% and 0.9% ($p < 0.05$) respectively).

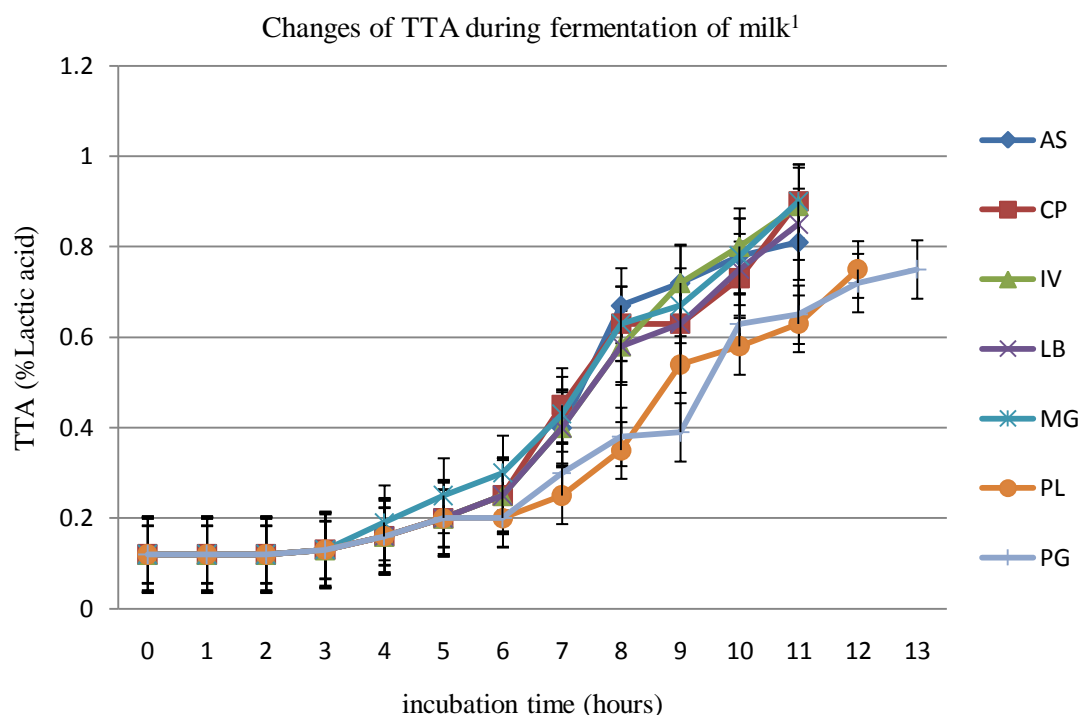


Figure 4.2: Changes of TTA¹ during fermentation of milk in the presence of different herbs² at 41°C

¹ TTA, Total titratable acidity; AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

4.1.3 Changes in pH and TTA of yoghurts with initial pH 4.5 during storage at 4°C

Figure 4.3 (and Table 4.3 in Appendix) shows the changes in yoghurt pH from day 0 to day 21 of storage at 4°C. Plain- and herbal-yoghurts showed a linear decrease in pH during the 3 weeks. On average, all yoghurts showed a 0.2 unit of pH decrease throughout the 21 days storage. No significant differences between treatments were noted.

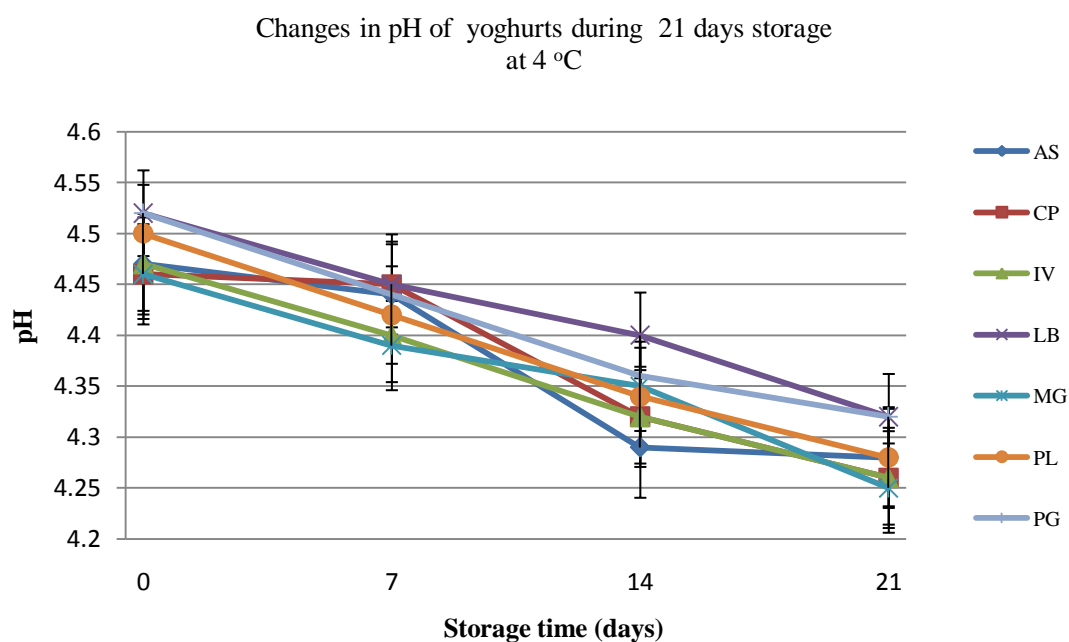


Figure 4.3 Changes in pH of yoghurts² during 21 days storage at 4°C

¹ yoghurt with initial pH 4.5 after fermentation, ² AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

Means in percentage of TTA of herbal yoghurts during storage is as shown in Figure 4.4 and Table 4.4. Plain- and herbal-yoghurts showed a linear increase in TTA during the first two weeks of storage ($P < 0.05$). This linear increase in TTA continued in *A. sinensis*- and *M. grosvenori*-yoghurt for the next one week whereas no further increase in TTA was noted for *C. pilosula*-, *I. verum*-, *L. barbarum*- and *P. guajava*-yoghurt. Differences in TTA were significantly ($P < 0.05$) different from control (0.93) on day 21 of storage for *A. sinensis*-, *I. verum*-, *L. barbarum*- and *M. grosvenori*-yoghurts (1.1, 1.05, 1.05, and 1.1 respectively).

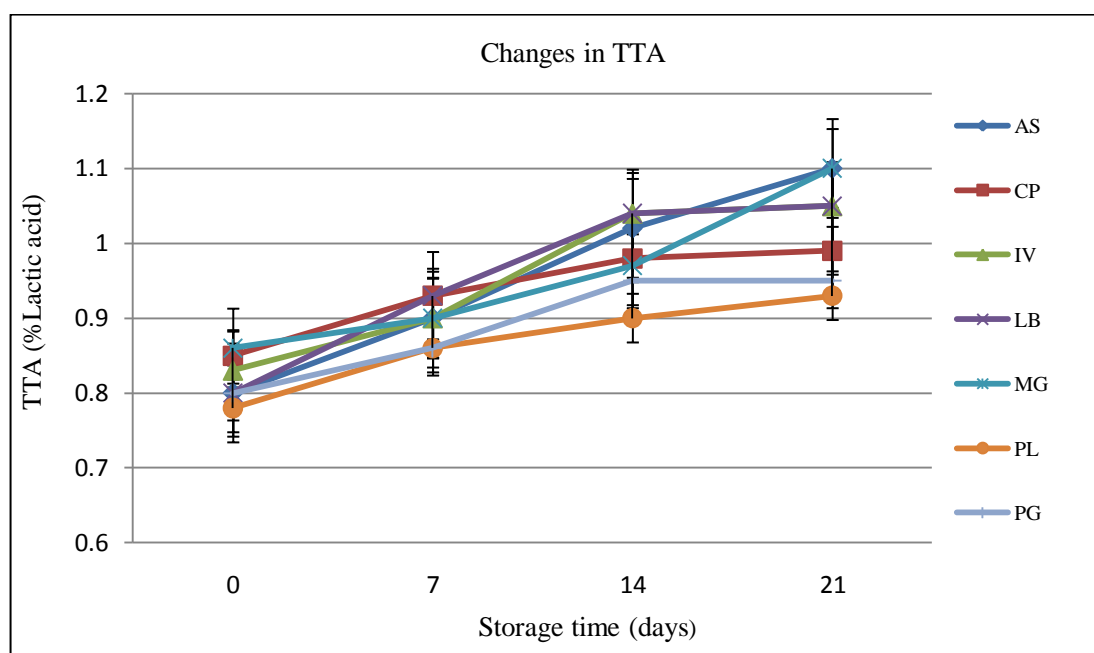


Figure 4.4 Changes in TTA of yoghurts¹ during storage at 4°C

¹ yoghurt with initial pH 4.5 after fermentation, AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

4.1.4. Enumeration of *Lactobacillus* spp. during storage in MRS Agar

Viable count of *Lactobacillus* spp. and *Lactobacillus acidophilus* in plain and herbal yoghurts during storage are presented in Figure 4.5 and Table 4.5. The *Lactobacillus* spp. counts in plain-yoghurt (control) declined sharply from 9.3×10^5 CFU/ml (day 0) to 2.2×10^5 CFU/ml (day 7) after which the viable cell counts reduce slowly to 0.85×10^5 CFU/ml (day 21). *P. guajava*-yoghurt showed higher bacterial counts on day 0 (15.0×10^5 CFU/ml; $P < 0.05$), than those in plain- and other herbal-yoghurts. The bacterial counts reduced to 6.3×10^5 CFU/ml which was significantly higher ($p < 0.05$) than all other treatments on day 0 and day 7. *A. sinensis*-yoghurt had similar bacterial counts (10.1×10^5 CFU/ml) to the control (9.3×10^5 CFU/ml). *M. grosvenori*-, *C. pilosula*-, *L. barbarum*- and *I. verum*-yoghurts ($5.3 - 6.3 \times 10^5$ CFU/ml)

were lower ($P<0.05$) than control yoghurt but the viable count for these yoghurts were indistinguishable from plain yoghurt on day 7 of storage.

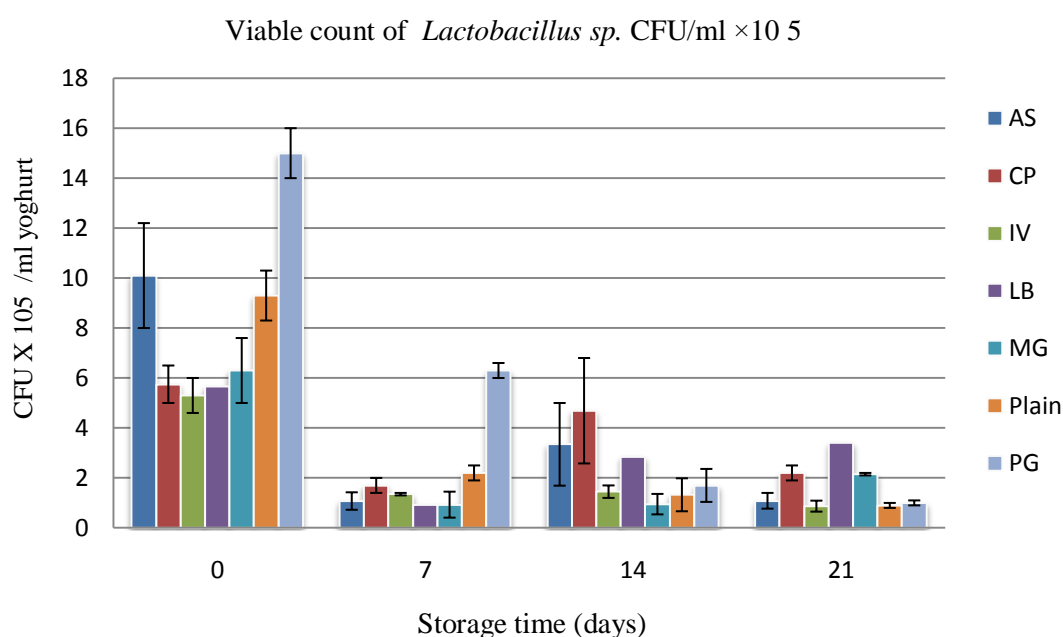


Figure 4.5: Viable count of *Lactobacillus* spp. in yoghurts¹ during storage at 4°C

¹ yoghurt with initial pH 4.5 after fermentation, AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

Table 4.5 Enumeration of *Lactobacillus* spp. CFU $\times 10^5$ /mL in yoghurts^{1,2} during storage at 4°C

Day (s)	AS	CP	IV	LB	MG	PL	PG
0	10.1±2.1 ^{ax}	5.8±0.8 ^{bx}	5.3 ± 0.7 ^{bx}	5.65±1.7 ^{bx}	6.3±1.3 ^{bx}	9.3 ±0.7 ^{ax}	15.0±1.0 ^{bx}
7	0.85±0.4 ^{ay}	1.7± 0.3 ^{by}	1.4±0.1 ^{by}	0.75±0.4 ^{ay}	0.7±0.5 ^{ay}	2.2 ±1.5 ^{ay}	6.3± 0.3 ^{by}
14	3.4±1.7 ^{ay}	4.7±2.1 ^{ax}	1.4±0.3 ^{by}	2.85±1.2 ^{ax}	0.95±0.4 ^{ay}	1.3±0.5 ^{ay}	1.7 ± 0.1 ^{by}
21	1.1 ± 0.3 ^{ay}	2.2 ± 0.3 ^{by}	0.8±0.2 ^{ay}	3.41±0.1 ^{bx}	2.1±0.1 ^{by}	0.85±0.2 ^{ay}	1.0 ±0.1 ^{ay}

¹ Yoghurt at an initial storage pH = 4.5

AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

^{ab} different superscripts in the same row differ significantly ($p<0.05$)

^{xy} different superscripts in the same column differ significantly ($p<0.05$)

4.1.5. Viability of *Streptococcus thermophilus* in yoghurts in M17 Agar

Effect of herbs on *S. thermophilus* viable counts are as shown in Figure 4.6 and Table 4.6. Plain-yoghurt (control) had a viable cell count of $7.8 \pm 0.1 \times 10^8$ CFU/ml on 0 day which increased to about $13.8 \pm 0.7 \times 10^8$ CFU/ml and $13.3 \pm 0.3 \times 10^8$ CFU /ml on the 2nd and 3rd week respectively prior to a reduction to $8.2 \pm 0.2 \times 10^8$ CFU /ml on day 21 of storage. In comparison, *P.guajava*-yoghurt which had similar viable counts ($7.4 \pm 0.1^a \times 10^8$ CFU /ml) with plain yoghurt, maintained its viable cell counts during the first week, but reduced steadily thereafter to $4.1 \pm 0.1^b \times 10^8$ CFU /ml.

M. grosvenori– and *I. verum*-yoghurts had similar viable cell count on day 0, which were higher than plain-yoghurt. The former however showed continue reduction in number during 21 days storage whereas the latter had increased viable count to $20.3 \pm 0.5 \times 10^5$ CFU /ml on day 7 of storage prior to a reduction in viable counts on day 14 and 21.

P. guajava- and *L. barbarum*-yoghurts had similar bacterial counts (7.4 ± 0.1 and $8.3 \pm 0.3 \times 10^5$ CFU /ml respectively) on day 0 which remain relatively unchanged on day 7 of storage. The bacterial counts in the former however began to reduce over the next two weeks of storage to $4.1 \pm 0.1 \times 10^8$ CFU /ml whereas the *S. thermophilus* counts in *L. barbarum*-yoghurt increased to $14.9 \pm 0.9 \times 10^8$ CFU /ml on day 14 prior to a decrease ($7.3 \pm 0.6 \times 10^8$ CFU /ml) on day 21 of storage. *A. sinensis*- and *C. pilosula*-yoghurts also had higher viable counts (12.6 ± 0.6 and $13.3 \pm 0.1 \times 10^8$ CFU /ml respectively) on day 0 which increased in the first week to 16.7 ± 0.2 and $16.9 \pm 0.9 \times 10^8$ CFU /ml respectively and then decreased on the second ($11.7 \pm 0.7 \times 10^8$ CFU /ml and $9.3 \pm 0.3 \times 10^8$ CFU /ml respectively) and third week ($6.9 \pm 0.4 \times 10^8$ CFU /ml and $9.1 \pm 0.3 \times 10^8$ CFU /ml respectively).

Viable count of *S. thermophilus* in yoghurt

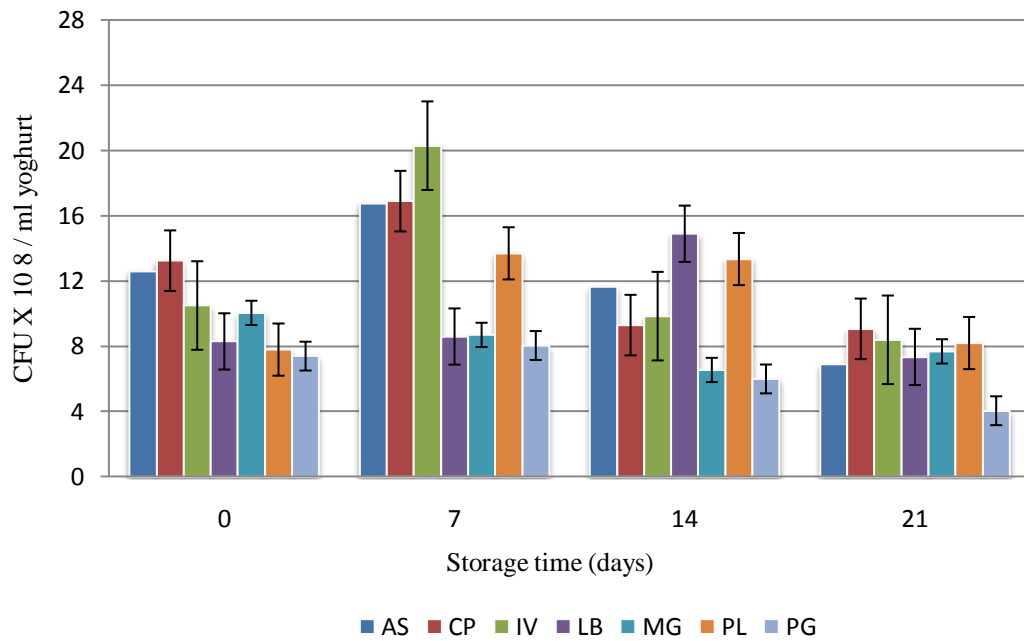


Figure 4.6: Viable count of *Streptococcus. thermophilus* (CFU×10⁸/mL) in yoghurt^{1,2} during storage

¹ yoghurt with initial pH 4.5 after fermentation, ² AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

Table 4.6 Enumeration of *S. thermophilus* (CFU×10⁸/mL) in yoghurts^{1,2} stored (4°C) up to 21 days

days	AS	CP	IV	LB	MG	Plain	PG
0	12.6±0.6 ^{bx}	13.25±0.1 ^{bx}	10.5±0.5 ^{bx}	8.3±0.3 ^{ax}	10.05±0.2 ^{bx}	7.8±0.1 ^{ax}	7.40±0.1 ^{ax}
7	16.75±0.3 ^{by}	16.9±0.9 ^{bx}	20.3±0.5 ^{by}	8.6±0.1 ^{bx}	8.7±0.9 ^{bx}	13.7±0.7 ^{ay}	8.05±0.4 ^{bx}
14	11.65±0.7 ^{bx}	9.30±0.3 ^{bx}	9.85±0.4 ^{bx}	14.9±0.9 ^{ay}	6.55±0.5 ^{bx}	13.35±0.4 ^{ax}	6.0±1.0 ^{bx}
21	6.92±0.4 ^{by}	9.07±0.3 ^{ax}	8.40±0.1 ^{ax}	7.35±0.6 ^{bx}	7.69±0.3 ^{ax}	8.2±0.2 ^{ax}	4.05±0.2 ^{bx}

¹ yoghurt with initial pH 4.5 after fermentation, ² AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

^{ab} different superscripts in the same row differ significantly (p<0.05)

^{xy} different superscripts in the same column differ significantly (p<0.05)

4.2. Acidity of yoghurts during refrigerated storage (4°C)

Fermentation of yoghurts was also carried out for 18 hours to evaluate the differences in microbial population in the presence of herbs. The extent of post-acidification and enumeration of viable probiotic bacteria in these yoghurts during refrigerated storage were also investigated.

4.2.1 Changes in pH of yoghurts

Changes in pH during cold storage at 4°C of yoghurts are as shown in Figure 4.7 (and Table 4.7 in Appendix). The initial pH of milk (6.55-6.66) at the beginning of the fermentation decreased to within a range of pH 4.0 - 4.3 after 18 hours fermentation for all yoghurts. The reduction or increase of pH during storage period in herbal yoghurts was minimal and not significant ($p>0.05$) and all yoghurts had pH changes within 0.15 units during that period.

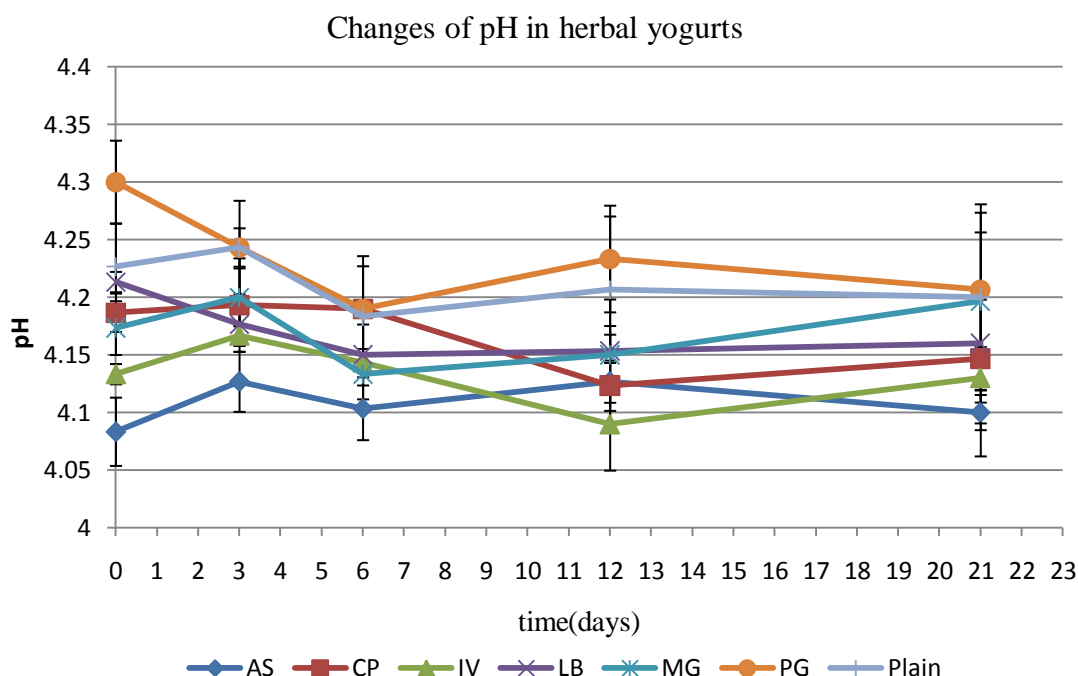


Figure 4.7: Changes of pH in yoghurts^{1,2} during storage

¹ yoghurt with 18 hours incubation, ² AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

4.2.2 Changes of Total Titratable Acidity of yoghurts.

The changes in TTA during refrigerated storage are presented in Figure 4.8 (and Table 4.7 in Appendix). *A. sinensis*-, *C. pilosula*-, *I. verum*-, *L. barbarum*- and *M. grosvenori*-yoghurts had marginally higher ($p < 0.05$) initial TTA values than that for plain-yoghurt. The mean TTA values for each yoghurt remained essentially the same throughout the storage period. *A. sinensis*-yoghurt has the highest TTA% value (1.02 ± 0.01) on day 0 but not on day 3, in agreement with the pH value that was the lowest in this yoghurt compared with others. *P. guajava*-yoghurt's TTA was consistently the same as plain-yoghurt throughout the storage period.

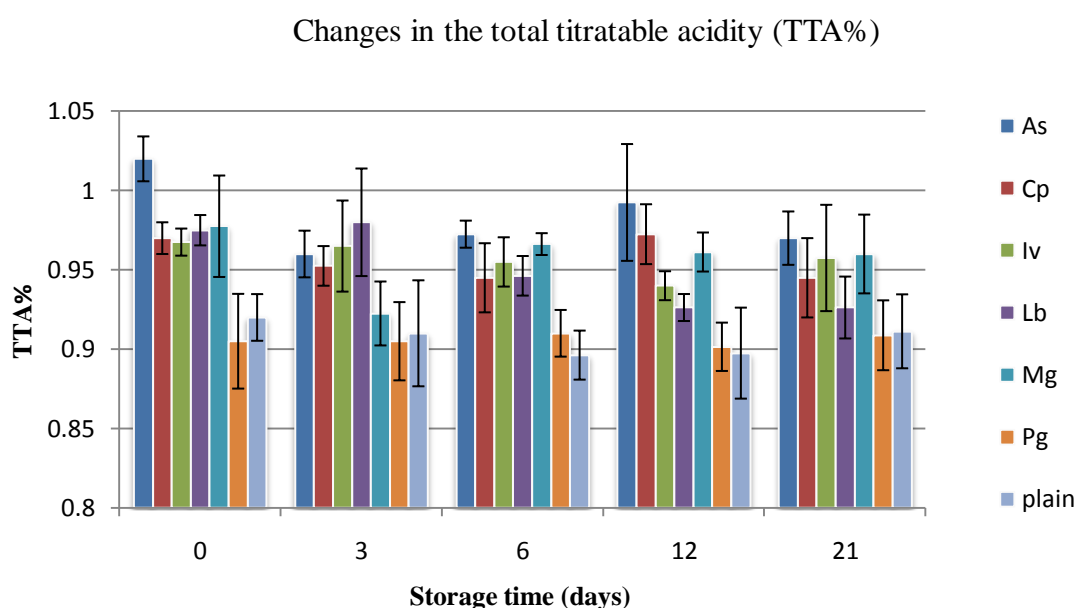


Figure 4.8: Changes in total titratable acidity (TTA) of yoghurts^{1,2} during storage

¹ yoghurts after 18 hours incubation, ² AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

4.3 Viable cells count in yoghurts during refrigerated storage (4°C)

4.3.1 Changes in viable cells count of *Lactobacillus spp.*

Results of enumeration of *Lactobacillus spp.* in yoghurts are as shown in Figure 4.9 and Table 4.8. The population of *Lactobacillus spp.* in plain yoghurt on day 0 after fermentation was $59 \pm 17.8 \times 10^6$ CFU ml⁻¹ and this was maintained during the first week of storage. A 75% decrease in cells count occurred by day 12 followed by a further 74% decline (from day 12) by day 21 of storage. *L. barbarum*-yoghurt and *P. guajava*-yoghurt had higher *Lactobacillus spp.* counts ($109 \pm 21.4 \times 10^6$ CFU ml⁻¹ and $126 \pm 26.3 \times 10^6$ CFU ml⁻¹ respectively; $p < 0.05$) on day 0. However, only *L. barbarum*-yoghurt continued to have *Lactobacillus spp.* ($112 \pm 28.8 \times 10^6$ CFU ml⁻¹) higher than control ($52 \pm 14.1 \times 10^6$ CFU ml⁻¹) on day 3 of storage. *L. verum*-yoghurt's cells count which was not different from that of plain-yoghurt had lower *Lactobacillus spp.* counts than that in plain-yoghurt on day 3 and day 6 of storage. *C. pilosula*-yoghurt was not different compared to control with respect to *Lactobacillus spp.* counts at all storage days determined. Although all yoghurts showed a decrease in microbial counts during storage, the *Lactobacillus sp.* counts were still within acceptable range of 6-8 log CFU ml⁻¹ (Donkor *et al.*, 2005).

Table 4.8 Enumeration ¹of *Lactobacillus spp.* (CFU×10⁶/mL) in yoghurts^{2,3} during storage.

Day	AS	CP	IV	LB	MG	PG	Plain
0	55.7±12.8 ^{ax}	70.0±20 ^{bx}	51.7±15.8 ^{ay}	109.3±21.5 ^{by}	49.3±11 ^{ax}	126.3±26.3 ^{by}	59.7±17.8 ^{ax}
3	40.0±8.0 ^{ax}	67.3±8.1 ^{ax}	26.5±7.2 ^{by}	112.7±28.8 ^{by}	43.7±11.5 ^{ax}	54.7±5.0 ^{ay}	52.8±14.1 ^{ax}
6	26.7±5.0 ^{bx}	51±15.5 ^{ax}	12±5.01 ^{by}	38.3±14.8 ^{ay}	18.0±3.8 ^{by}	18.8±4.5 ^{ay}	47.0±13 ^{ax}
12	13.8±5.3 ^{ax}	17.8±6.6 ^{ay}	11.7±9.1 ^{ax}	13.8±5.0 ^{ax}	7.30±0.7 ^{ax}	13.1±7.9 ^{ax}	11.7±4.1 ^{ax}
21	5.3±1.0 ^{ax}	4.9±0.9 ^{ay}	2.95±1.8 ^{ax}	3.0±0.87 ^{ax}	2.55±0.6 ^{ax}	6.7±1.3 ^{ax}	2.97±0.4 ^{ax}

¹ Enumeration was carried out on MRS agar (see section 3.4.3)

² yoghurt with 18 hours incubation, ³ AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

ab different superscripts in the same row differ significantly (p<0.05)

^{xy} different superscripts in the same column differ significantly (p<0.05)

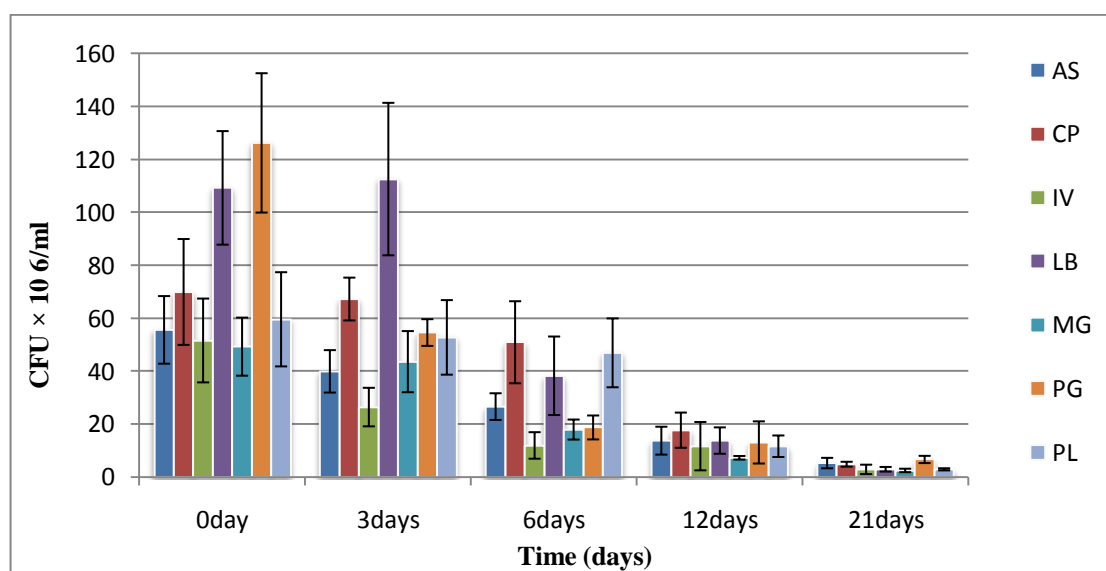


Figure 4.9 Changes of *Lactobacillus spp.* count (CFU ×10⁶/ml) of yoghurts¹ during storage

¹ yoghurt with 18 hours incubation, ² AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

4.3.2 Changes in viable cells count of *Streptococcus thermophilus*

The changes in *S. thermophilus* bacteria in yoghurts after fermentation and during storage at 4°C are presented in Table 4.9 and Figure 4.10. Viable *S. thermophilus* in

plain yoghurt was relatively unchanged during the first 6 days of storage. The cell counts on day 12 ($7.9 \pm 2.2 \times 10^8$ cfu /ml) was lower ($p < 0.05$) when compared to those on day 0 ($13.1 \pm 1.0 \times 10^8$ cfu /ml). *S. thermophilus* counts on day 0 and day 3 were higher for *A. sinensis*- and *L. barbarum*-yoghurts than that in plain-yoghurt. The presence of *L. barbarum* in yoghurt also resulted in higher ($p < 0.05$) *S. thermophilus* counts on day 6 ($42.9 \pm 4.8 \times 10^8$ cfu /ml) than that in plain-yoghurt ($16.8 \pm 11.1 \times 10^8$ cfu /ml). The viable cell counts for *S. thermophilus* in *C. pilosula*-yoghurt were consistently higher at all storage periods, but the difference was only significant on day 21. Only *A. sinensis*- and *C. pilosula*-yoghurts showed higher ($p < 0.05$) *S. thermophilus* counts than plain-yoghurt on day 21 of storage.

Table 4.9 Enumeration¹ of *S. thermophilus* of yoghurts² during storage

Storage Day	Type of yoghurts ³ (CFU $\times 10^8$ /mL)						
	As	Cp	Iv	Lb	Mg	Pg	Plain
0	53.0 \pm 9.0 ^{bx}	16.3 \pm 3.3 ^{ax}	28.7 \pm 11.9 ^{bx}	97.0 \pm 19 ^{bx}	13.2 \pm 2.1 ^{ax}	15 \pm 5.3 ^{ax}	13.1 \pm 0.97 ^{ax}
3	44.3 \pm 13 ^{bx}	28.5 \pm 15.1 ^{ay}	12.9 \pm 4.5 ^{bx}	37.7 \pm 5.8 ^{ay}	28.6 \pm 23.5 ^{ay}	20 \pm 8.7 ^{ay}	23.6 \pm 20.7 ^{ay}
6	15.5 \pm 1.3 ^{ax}	37.8 \pm 22.9 ^{by}	8.4 \pm 3.2 ^{bx}	42.9 \pm 4.8 ^{bx}	5.6 \pm 1.4 ^{ay}	5.2 \pm 1.03 ^{by}	16.8 \pm 11.1 ^{ax}
12	10.3 \pm 2.5 ^{ax}	10.4 \pm 4.1 ^{ax}	3.9 \pm 2.7 ^{ax}	6.3 \pm 0.8 ^{ay}	5.03 \pm 2.3 ^{ax}	5.6 \pm 1.4 ^{ay}	7.9 \pm 2.2 ^{ax}
21	11.6 \pm 3.4 ^{ay}	23.9 \pm 7.7 ^{by}	5.12 \pm 0.8 ^{ay}	5.6 \pm 0.9 ^{ax}	9.47 \pm 1.6 ^{ay}	5.9 \pm 1.9 ^{ay}	6.6 \pm 0.98 ^{ax}

¹Enumeration was carried out on M17 agar (see section 3.4.2.)

²Yoghurt was incubated for 18 hours prior to refrigerated storage at 4 °C.

³s, *A. sinensis*-yoghurt; Cp, *C. pilosula*-yoghurt; Iv, *I. verum*-yoghurt; Lb, *L. barbarum*-yoghurt; Mg, *M. grosvenori*-yoghurts; Pg, *P. guajava*-yoghurt; Plain, Plain-yoghurt

^{ab} different superscripts in the same column differ significantly ($p < 0.05$) from day 0 refrigerated storage ^{xy} different superscripts in the same row differ significantly ($p < 0.05$) from plain-yoghurt

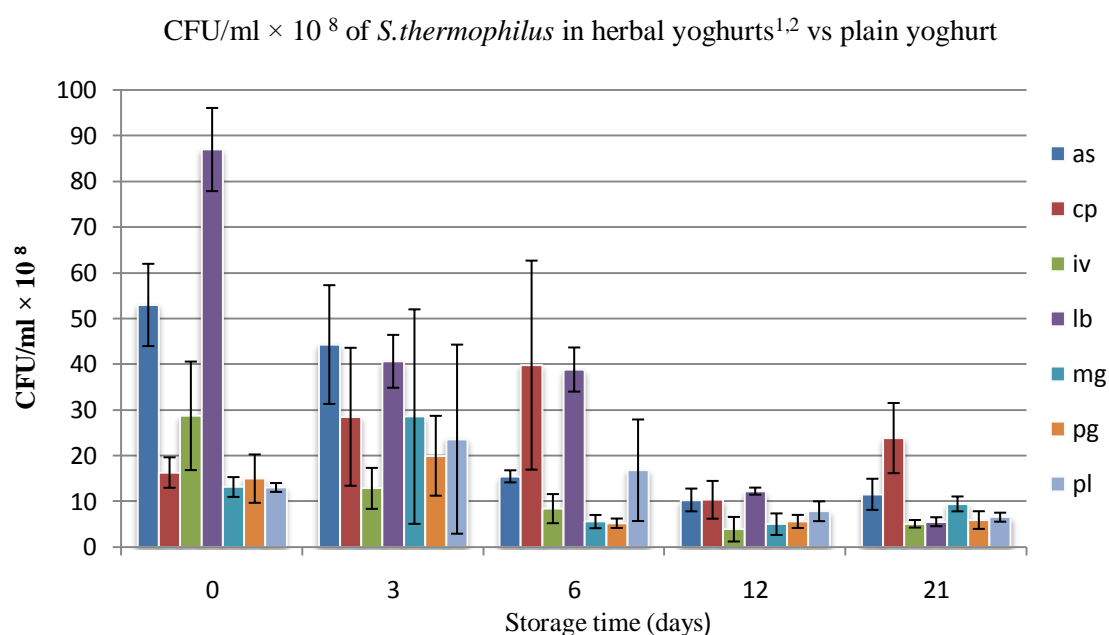


Figure 4.10: Changes of *Streptococcus thermophilus* counts (CFU $\times 10^8$ /ml) in yoghurts during storage at 4°C

¹ yoghurt with 18 hours incubation, ² AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

4.4 Proteolysis of yoghurts during refrigerated storage

4.3.1 Effects on Peptide Concentration

OPA assay was carried out on yoghurts at three different storage days (i.e. 0, 7 and 14 days) and the changes in peptide concentrations are shown in Table 4.10 and Figure 4.11.

Plain-yoghurt peptide concentration on day 0 (19.75 ± 2.1 mg/ml) was increased to 25.4 ± 1.09 mg/ml on day 7, but decreased to 20.75 ± 1.3 mg/ml on the day 14 of storage. All herbal-yoghurts followed similar changes in peptide concentration during the 14 day period as that shown by plain-yoghurt. *L. barbarum*-, *A. sinensis*- and *I. verum*-yoghurts had higher ($p < 0.05$) whereas *C. pilosula*-yoghurt had lower ($p > 0.05$) peptide concentration than plain-yoghurt on day 7 of storage. Peptide concentration in *A. sinensis*- and *I. verum*-yoghurts remained higher ($p < 0.05$) than that in plain-yoghurt on

day 14 of storage. *C. pilosula*-yoghurt which had lower peptide concentration than that in plain-yoghurt showed significant difference ($p<0.05$) on day 14.

Table 4.10 Changes of peptide concentration (mg/ml) of yoghurts¹ during storage

Day	AS	CP	IV	LB	MG	PG	Plain
0	23.7±2.1 ^{ax}	16.8±0.9 ^{ax}	20.3±1.7 ^{ax}	21.4±2.1 ^{ax}	17.0±2.0 ^{ax}	17.4±1.8 ^{ax}	19.8±2.1 ^{ax}
7	28.6±0.4 ^{by}	22.8±1.9 ^{ay}	30.6±2.2 ^{by}	32.0±2.0 ^{by}	25.5±4.2 ^{ay}	25.9±3.8 ^{ay}	25.4±1.1 ^{ay}
14	24.8±1.8 ^{ax}	16.6±1.6 ^{ax}	24.0±0.4 ^{ax}	22.0±1.3 ^{ax}	22.9±3.9 ^{ax}	17.3±2.4 ^{ax}	20.8±1.3 ^{ax}

¹ AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

^{ab} different superscripts in the same row differ significantly ($p<0.05$)

^{xy} different superscripts in the same column differ significantly ($p<0.05$)

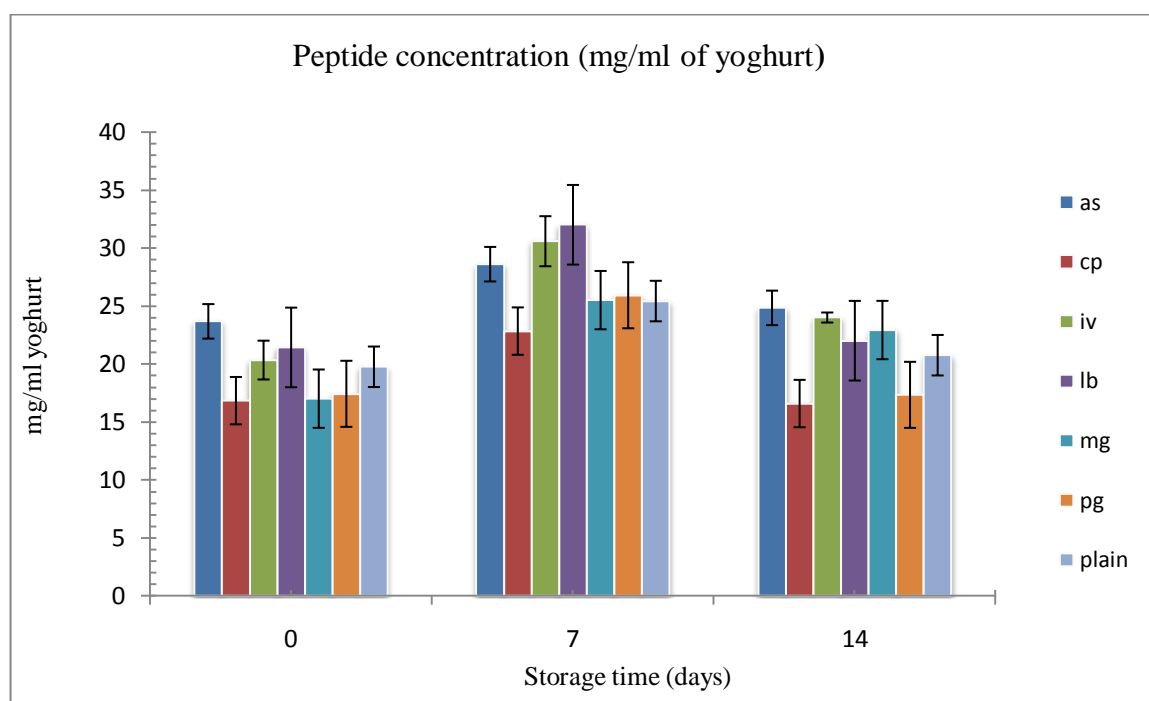


Figure 4.11: Changes of peptide concentration (mg/ml) in yoghurts^{1,2}

¹ yoghurt with 18 hours incubation, ²AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

4.3.2 Effects on Total Free Amino Acids

Free amino groups (expressed as mM leucine equivalent/ g yoghurt) on day 0 up to 21 days are presented in Table 4.11. There was a tendency of an increase in total free amino acids in yoghurts during refrigerated storage for all with significance ($p<0.05$) obtained by day 21 of storage compared to day 0 for *A. sinensis*-, *L. barbarum*- and *P. guajava*-yoghurts. Only *A. sinensis*-yoghurt showed higher ($p<0.05$) total free amino acids compared to control for all storage period determined.

Table 4.11 Changes of total free amino acids (mM of leucine eq/ml yoghurt) in yoghurts¹ during storage

Day of storage	Type of yoghurts						
	As	Cp	Iv	Lb	Mg	Pg	Plain
0	0.31±0.03 ^{ay}	0.17±0.02 ^{ax}	0.14±0.02 ^{ax}	0.20±0.02 ^{ax}	0.12±0.00 ^{ax}	0.15±0.01 ^{ax}	0.16±0.05 ^{ax}
7	0.33±0.03 ^{ay}	0.19±0.03 ^{ax}	0.19±0.02 ^{ax}	0.21±0.03 ^{ax}	0.16±0.01 ^{ax}	0.18±0.03 ^{ax}	0.19±0.07 ^{ax}
14	0.30±0.02 ^{ay}	0.17±0.01 ^{ax}	0.20±0.07 ^{ax}	0.21±0.07 ^{ax}	0.14±0.01 ^{ax}	0.16±0.00 ^{ax}	0.18±0.08 ^{ax}
21	0.43±0.12 ^{by}	0.20±0.02 ^{ax}	0.17±0.03 ^{ax}	0.27±0.11 ^{ax}	0.15±0.00 ^{bx}	0.17±0.00 ^{bx}	0.20±0.08 ^{ax}

¹As, *A. sinensis*-yoghurt; Cp, *C. pilosula*-yoghurt; Iv, *I. verum*-yoghurt; Lb, *L. barbarum*-yoghurt; Mg, *M. grosvenori*-yoghurts; Pg, *P. guajava*-yoghurt; Plain, Plain-yoghurt

^{ab} different superscripts in the same column differ significantly ($p<0.05$) from day 0 refrigerated storage

^{xy} different superscripts in the same row differ significantly ($p<0.05$) from plain-yoghurt

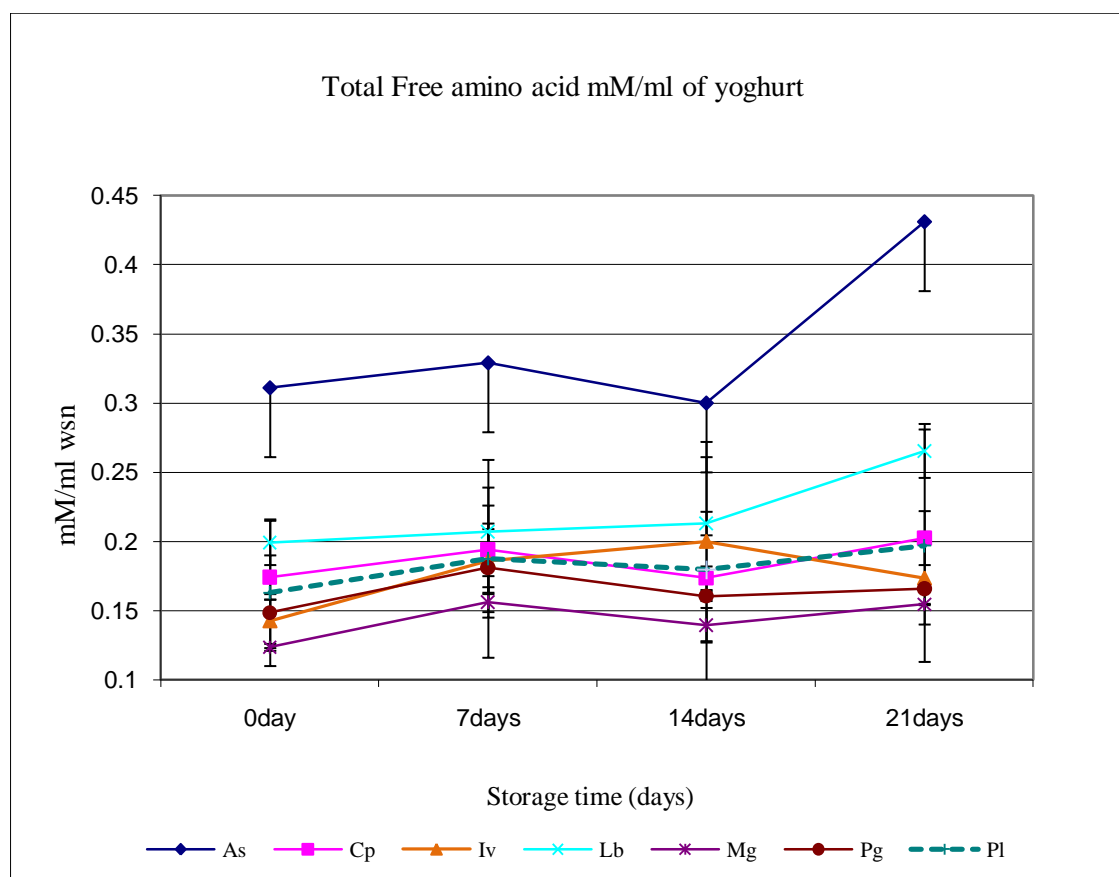


Figure 4.12: Total free amino acid (mM of leucine eq/ml yoghurt) of yoghurts¹ during storage at 4°C

¹ AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

4.3.3 Effects on Angiotensin-1 Converting Enzyme (ACE) activity

A. sativum-, *C. pilosula*-, *I. verum*-, *L. barbarum*- and *P. guajava*-water extracts were assayed for their inhibitory effects on ACE activity, both immediately after extraction and after pre-incubation at 41°C for 4 hours. As shown in Figure 4.13 and 4.14 (and Table 4.12 in Appendix), all herbal-water extracts caused lower ACE activity in comparison to control treatment. *P. guajava*-water extract showed the highest reduction in ACE activity in cold and incubated extract (0.0067 and 0.0040 unit/min/mg respectively) followed by *L. barbarum*- and *C. pilosula*-water extracts. Exposure to

mild heat (41°C) which mimicked changes occurring to herbal-water extracts during yoghurt incubation resulted in higher ($p<0.05$) inhibition on ACE activity for *L. barbarum*- and *P. guajava*-water extracts (an increase unit 21.3% and 18% respectively) than their respective unheated treatments.

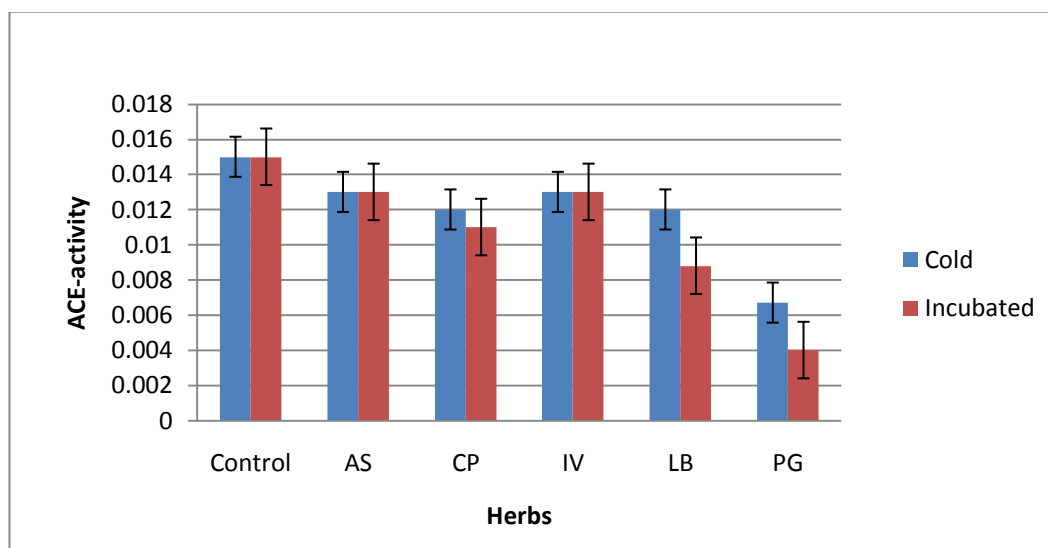


Figure 4.13. ACE activity of herb extracts

¹ AS, *A. sinensis*; CP, *C. pilosula*; IV, *I. verum*; LB, *L. barbarum*; PG, *P. guajava*; Control, Water. Herb-water extract was prepared and used in the measurement of ACE-inhibition (see Section 3.5.5.5).

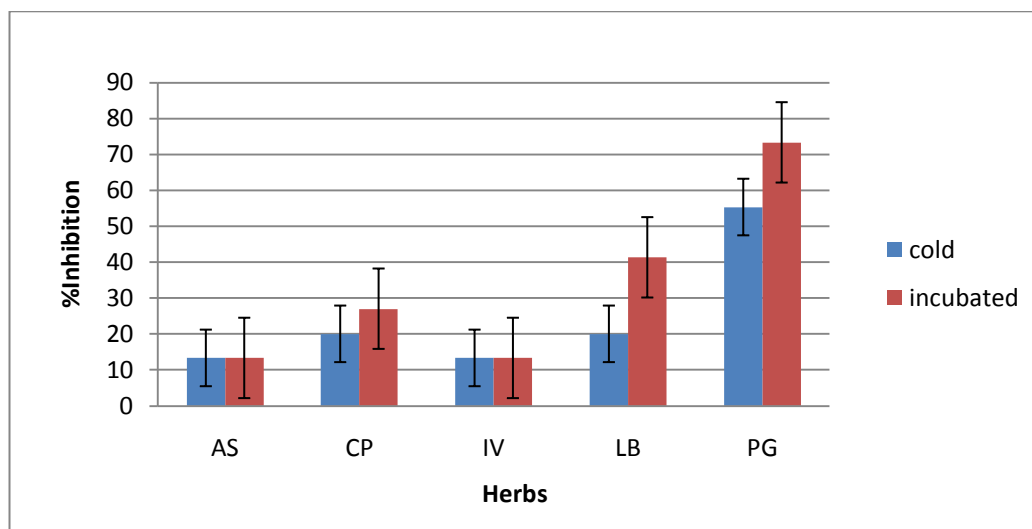


Figure 4.14 Percentage of ACE inhibition of herb extract

¹ AS, *A. sinensis*; CP, *C. pilosula*; IV, *I. verum*; LB, *L. barbarum*; PG, *P. guajava*; Control, Water. Herb-water extract was prepared and used in the measurement of ACE-inhibition (see Section 3.5.5.5).

4.3.4 ACE-Inhibitory effects of yoghurts

ACE activity in the presence of yoghurt water extract was expressed as % activity of untreated rabbit lung extract (100%). The inhibitory effect of plain yoghurt increased from 38.6 ± 5.4 % to 52.9 ± 3.1 % over 7 days of storage and increased to 57.0 ± 8.1 % after 14 days refrigerated storage period. *A. sinensis*-, *L. barbarum*- and *I. verum*-yoghurts had higher ($p < 0.05$) inhibition of ACE activity (60.7 ± 7.5 , 64.4 ± 9.9 % and 72.5 ± 0.6 %) on day 7 compared to plain yoghurt (52.9 ± 3.1 %).

Table 4.13: Percentage of ACE- Inhibition in herbal and plain yoghurts¹ within two weeks storage

Day	AS	CP	IV	LB	MG	PG	Plain
0	57.3±5.7 ^{ax}	31.4±6.3 ^{ax}	53.4±5.2 ^{bx}	53.3±3.9 ^{bx}	58.4±8.6 ^{bx}	36.9±2.5 ^{ax}	38.6±5.4 ^{ax}
7	60.7±5.6 ^{ax}	52.1±5.5 ^{ay}	72.5±0.6 ^{by}	64.4±9.9 ^{by}	57.±10.6 ^{ax}	61.3±10.1 ^{by}	52.9±3.1 ^{ay}
14	57.4±7.5 ^{ax}	55.0±5.3 ^{ay}	58.9±3.6 ^{ax}	55.3±5.2 ^{ax}	63.8±6.7 ^{ax}	47.0±17.8 ^{ax}	57.0±8.1 ^{ay}

¹ AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

^{ab} different superscripts in the same row differ significantly (p<0.05)

^{xy} different superscripts in the same column differ significantly (p<0.05)

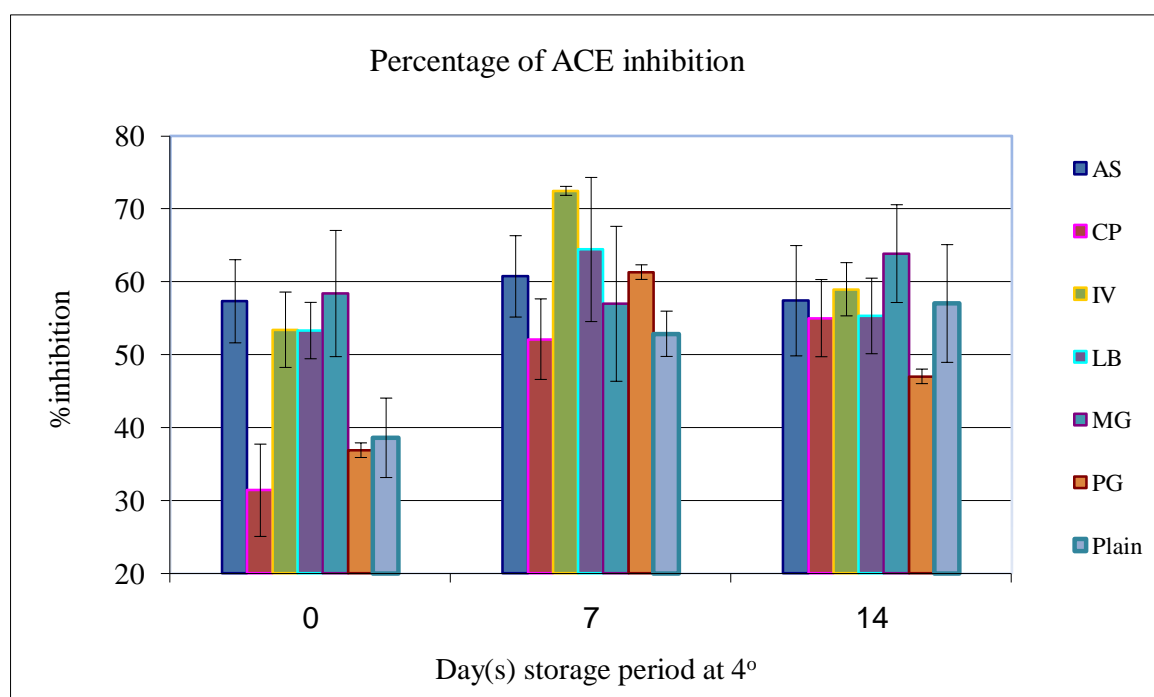


Figure 4.15: percentage of inhibition in yoghurts during storage at 4°C

¹ AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt. Herb-water extract was prepared and used in the measurement of ACE-inhibition (see Section 3.5.4).

The inhibitory effects of yoghurts were expressed over the peptide contents to obtain the specific activity of ACE-inhibition. Specific activity values take into consideration the

peptide content (which was assumed to contribute directly to ACE inhibition) when describing the inhibitory effects of yoghurt water extract Plain-yoghurt showed the lowest ACE-inhibition specific activities (0.54 ± 0.05 unit/minute/mg peptide) on day 7 compared to day 0 and 14 of storage (0.65 ± 0.22 and 0.64 ± 0.07 unit/minute/mg peptide respectively). All herbal yoghurts also showed similar trend. A lower ACE specific activity indicates stronger inhibitory effect of yoghurt extract on ACE activity. *C. pilosula*- and *P. guajava*- yoghurts (0.97 ± 0.13 and 0.94 ± 0.05 unit/minute/mg peptide respectively) showed lower ($p < 0.05$) ACE-inhibition activity compared to control-yoghurt (0.65 ± 0.22 unit/minute/mg peptide) on day 0 whereas *A. sinensis*-yoghurt caused a higher (0.49 ± 0.06 unit/minute/mg peptide; $p < 0.05$) ACE-inhibition.

All herbal-yoghurts, except *C. pilosula*-yoghurt caused a higher ACE-inhibition activities (range 0.32-0.43 unit/min/mg peptides) on day 7 of storage than that in control-yoghurt (0.54 ± 0.22) with significant ($p < 0.05$) effects showed by *A. sinensis*- (0.35 ± 0.04), *I. verum*- (0.33 ± 0.06) and *L. barbarum*-yoghurts (0.32 ± 0.08). *A. sinensis*-, *I. verum*-, *L. barbarum*- and *M. genosvenori*-yoghurts continued to have higher ($p < 0.05$) ACE-inhibition activities (0.46 ± 0.12 , 0.40 ± 0.04 , 0.49 ± 0.07 and 0.40 ± 0.05 unit/min/mg peptides respectively) compared to control yoghurt (0.64 ± 0.07 unit/min/mg peptides) on day 14 of storage.

Table 4.14 Changes of specific activity (unit/min/mg peptides) in yoghurt¹ during storage

Day	<i>A.sinensis</i>	<i>C.pilosula</i>	<i>I.verum</i>	<i>L.barbarum</i>	<i>M.grosvenori</i>	<i>P.guajava</i>	Plain
0	0.49±0.06 ^{bx}	0.97±0.13 ^{bx}	0.61±0.10 ^{ax}	0.66±0.09 ^{ax}	0.57±0.05 ^{ax}	0.94 ±0.05 ^{bx}	0.65±0.22 ^{ax}
7	0.35±0.04 ^{by}	0.55±0.03 ^{ay}	0.33±0.06 ^{by}	0.32±0.08 ^{by}	0.42±0.10 ^{ay}	0.43±0.12 ^{ay}	0.54±0.05 ^{ay}
14	0.46±0.12 ^{ax}	0.64±0.07 ^{ay}	0.40±0.04 ^{by}	0.49±0.07 ^{by}	0.40±0.05 ^{by}	0.72 ± 0.16 ^{ay}	0.64±0.07 ^{ax}

¹ AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

^{ab} different superscripts in the same row differ significantly (p<0.05)

^{xy} different superscripts in the same column differ significantly (p<0.05)

Figure 4.15 and Table 4.13 present the effect of refrigerated storage of plain and herbal-yoghurts on *in vitro* ACE inhibitory activity. There was a decrease in IC₅₀ values during the first week of refrigerated storage followed by gradual increase in IC₅₀ values during the remaining period of storage for all yoghurts. *A. sinensis*-yoghurt showed lower (p<0.05) IC₅₀ values compared to plain-yoghurt at all time points except on day 7 of storage. All herbal-yoghurts except *C. pilosula*-yoghurt showed lower IC₅₀ values than plain-yoghurt on day 21 of storage. *C. pilosula*-yoghurt IC₅₀ values were higher (p<0.05) than plain-yoghurt at all storage periods.

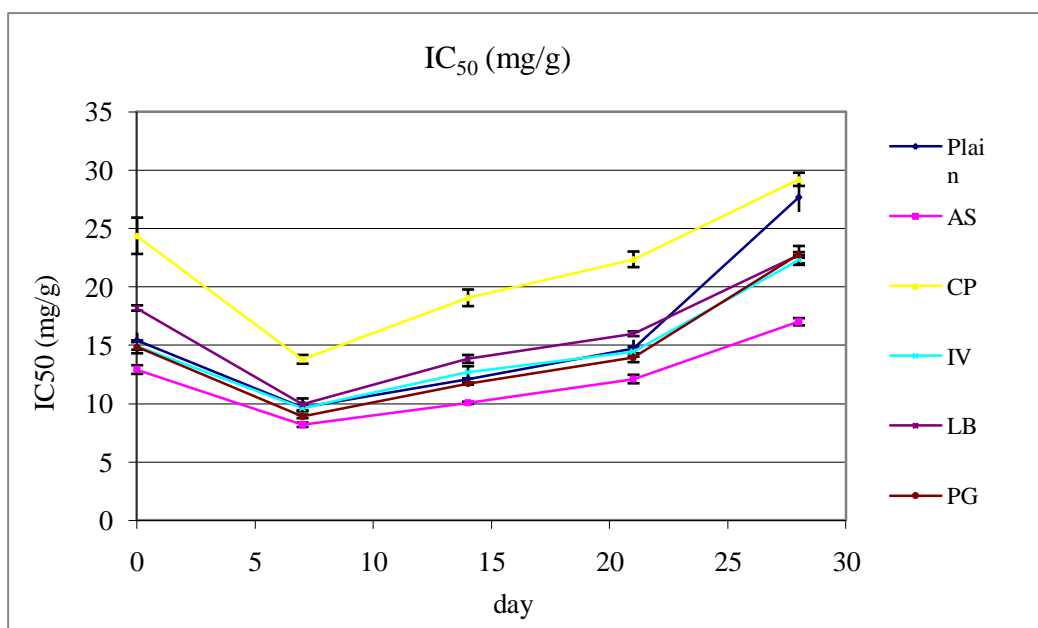


Figure 4.16: Changes of IC₅₀ (mg/g) of yoghurts¹ against storage days

Herb-water extract was prepared and used in the measurement of ACE-inhibition (see Section 3.5.4).¹ AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt, There is no result for *M. grosvenori*-yoghurts in this experiment.

Table 4.15 IC₅₀ % of yoghurts¹ during 4 weeks storage at 4°C

Day	0	7	14	21	28
Plain	15.42±0.6 ^{ax}	9.69±0.1 ^{ay}	12.07±0.6 ^{ax}	14.73±0.7 ^{ax}	27.69±1.2 ^{ay}
As	12.92±0.4 ^{ax}	8.19±0.2 ^{ay}	10.07±0.1 ^{ax}	12.11±0.4 ^{ax}	17.02±0.3 ^{by}
Cp	24.39±1.6 ^{bx}	13.8±0.4 ^{by}	19.07±0.7 ^{by}	22.37±0.6 ^{by}	29.23±0.6 ^{ay}
Iv	14.95±0.3 ^{ax}	9.61±0.3 ^{ay}	12.69±0.5 ^{ax}	14.46±0.4 ^{ax}	22.31±0.4 ^{ay}
Lb	18.2 ± 0.2 ^{ax}	9.94±0.5 ^{ay}	13.83±0.3 ^{bx}	15.99±0.2 ^{ax}	22.75±0.2 ^{ay}
Pg	14.87±0.5 ^{ax}	8.91±0.1 ^{ay}	11.72±0.1 ^{ax}	13.94±0.4 ^{ax}	22.70±0.7 ^{ay}

¹ AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

There is no result for *M. grosvenori*-yoghurts in this experiment.

^{ab} different superscripts in the same column differ significantly (p<0.05) compared to plain yoghurt (plain)

^{xy} different superscripts in the same row differ significantly (p<0.05) from day 0 refrigerated yoghurt

4.4. SDS-PAGE, Gel Electrophoretic patterns of yoghurts during fermentation and storage

The extent of proteolysis of yoghurts during storage was also monitored by SDS-PAGE. The changes in milk protein break down on day 0, 7, 14 and 21 of storage at 4 °C are as shown in Figure 4.17 to Figure 4.22. Very little detectable visual differences in intensity were observed between control (plain-yoghurt) and herbal-yoghurts. The ratio of intensity of herbal yoghurts vs their respective controls (relative proteolysis) at each storage period was calculated. Values of more or less than 1.0 indicate inhibitory or stimulatory effect of herbs on proteolysis respectively. The κ -casein in different treatment had different intensity and did not observed a similar trend for all samples.

The intensity of bands as described by Park *et al.* (1998), expressed by integrated intensity of band \times area of the band for all herbal-yoghurts and their respective control. As shown in table 4.16 to Figure 4.21 calculating of density of bands by multiply intensity and area, indicated significant differences between each herbal-yoghurt and its control in *A.sinensis*-, *C.pilosula*- and *M.grosvenori*-yoghurts. The relative proteolysis (RP) of α -, β - and κ -CNs in *A. sinensis*-, *M. grosvenori*- and *C. pilosula*-yoghurts in day 0 and 7 of storage were inhibited (RP > 1.0) whereas those in others were not (i.e. close to 1.0). RP values for all CNs in day 14 and 21 of storage tended to be lower than their respective day 1 or day 7 refrigerated storage. Proteolysis of β -LG was inhibited in *A. sinensis*-yoghurt only (RP values ranged 1.3 – 1.8; Table 4.16) throughout the study period whereas for the others (*C. pilosula*-, *I. verum*-, *M. grosvenori* and *L. barbarum*-yoghurts), the RP values were close to 1.0. An exception in the proteolysis of milk protein was seen in *P. guajava*-yoghurt whereby the RP values for α -, β - and κ -CNs and β -LG changed in cyclical manner (alternate inhibition-stimulation of proteolysis week-to-week) during the 4 weeks refrigerated storage. There were different patterns in protein degradation of α -Lac in herbal-yoghurts throughout the refrigerated storage periods. The inhibition of proteolysis of α -Lac in *A. sinensis*-

yoghurt (Table 4.16) was reduced with storage time whereas that in *C. pilosula*-yoghurt (Table 4.17) was prominently strong in day 7 and 14 of refrigerated storage. Proteolysis of α -Lac was constantly stimulated in *I. verum*-yoghurt (Table 4.18) but was increasingly stimulated in *M. grosvenori*-yoghurt (Figure 4.20). In contrast to other herbal-yoghurts, proteolysis of α -Lac in *L. barbarum*- (Table 4.19) and *P. guajava*- (Table 4.21) yoghurts showed a cyclical inhibition-stimulation pattern during refrigerated storage.

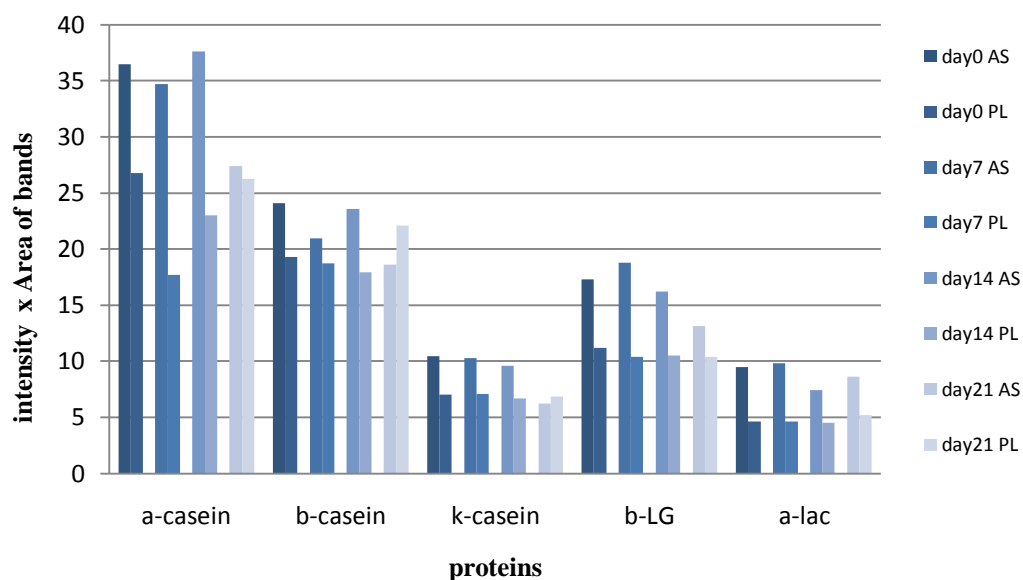


Figure 4.17. Changes of protein from SDS-PAGE analysis *A.sinensis*-yoghurts (AS) and control (PL) during storage at 4 °C

Tabel 4.16. Comparison of densitometric values of SDS-PAGE protein (intensity of band \times area) in *A.sinensis*-yoghurt with respective control (plain-yoghurt) at different refrigerated (4 °C) storage periods

Storage	yoghurts	I.i*. \times area				
		α -CN*	β -CN	k-CN	β -LG	α -Lac
day0	AS	36.5	24.07	10.46	17.3	9.5
	PL	26.77	19.29	7.003	11.2	4.6
	AS/PL	1.31	1.24	1.49	1.54	2.06
day7	AS	34.68	20.93	10.3	18.8	9.8
	PL	17.7	18.7	7.1	10.4	4.6
	AS/PL	1.95	1.11	1.45	1.81	2.13
day14	AS	37.63	23.6	9.58	16.2	7.4
	PL	23.03	17.9	6.69	10.5	4.5
	AS/PL	1.63	1.32	1.43	1.54	1.64
day21	AS	27.38	18.6	6.2	13.11	8.6
	PL	26.24	22.1	6.86	10.4	5.2
	AS/PL	1.04	0.84	1.1	1.26	1.65

* I.i: Intensity of bands; * CN: casein

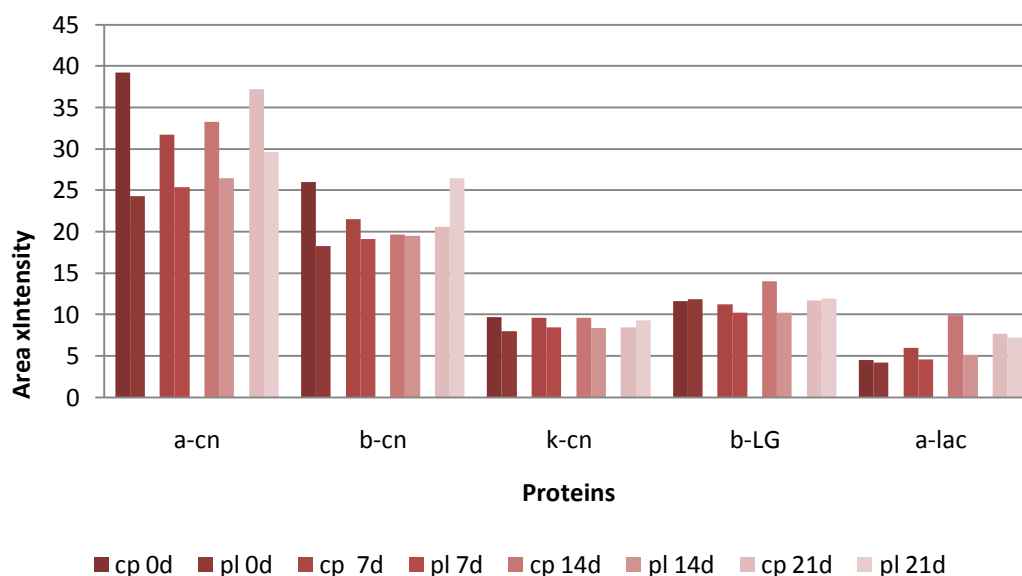


Figure 4.18. Changes of protein from SDS-PAGE analysis *C.piloula*-yoghurts (CP) and control (PL) during storage at 4 °C

Table 4.17: Comparison of densitometric values of SDS-PAGE protein (intensity of band \times area) in *C.pilosula*-yoghurt with respective control (plain-yoghurt) at different refrigerated (4°C) storage periods

storage	yoghurt	I.i \times area				
		α -CN	β -CN	k-CN	β -LG	α -Lac
Day0	CP	24.3	18.25	8.02	11.87	4.2
	PL	39.2	26	9.7	11.65	4.5
	CP/PL	1.61	1.42	1.21	0.98	1.07
Day7	CP	25.4	19.1	8.42	10.24	4.56
	PL	31.75	21.5	9.63	11.2	5.96
	CP/PL	1.25	1.13	1.14	1.1	1.3
Day14	CP	26.5	19.48	8.4	10.21	5.1
	PL	33.3	19.69	9.6	14	9.9
	CP/PL	1.26	1.01	1.14	1.17	1.94
Day21	CP	29.64	26.5	9.3	11.9	7.2
	PL	37.2	20.6	8.43	11.69	7.7
	CP/PL	1.25	0.78	0.9	0.98	1.07

* I.i: Intensity of bands

* CN: casein

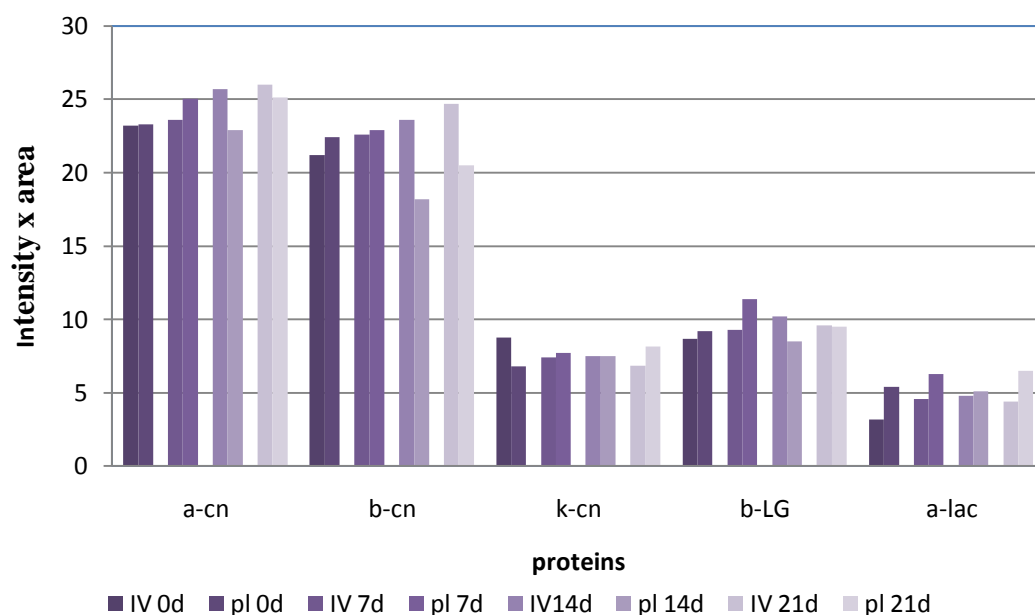


Figure 4.19. Changes of protein from SDS-PAGE analysis *I.verum*-yoghurts (IV) and control (PL) during storage at 4 °C

Table 4.18. Comparison of densitometric values of SDS-PAGE protein (intensity of band \times area) in *I. verum*-yoghurt with respective control (plain-yoghurt) at different refrigerated (4°C) storage periods

storage	yoghurts	I.i \times area				
		a-cn	b-cn	k-cn	b-LG	a-lac
Day0	IV	23.2	21.2	8.78	8.7	3.2
	PL	23.3	22.4	6.8	9.2	5.41
	IV/PL	0.99	0.95	1.28	0.94	0.59
Day7	IV	23.6	22.6	7.4	9.3	4.6
	PL	25.05	22.88	7.7	11.4	6.3
	IV/PL	0.94	0.99	0.96	0.81	0.73
Day14	IV	25.7	23.6	7.5	10.2	4.8
	PL	22.9	18.2	7.5	8.5	5.1
	IV/PL	1.12	1.3	1	1.2	0.94
Day21	IV	26	24.7	6.85	9.6	4.4
	PL	25.12	20.5	8.14	9.5	6.5
	IV/PL	1.03	1.2	0.84	1.01	0.68

* I.i: Intensity of bands

* CN: casein

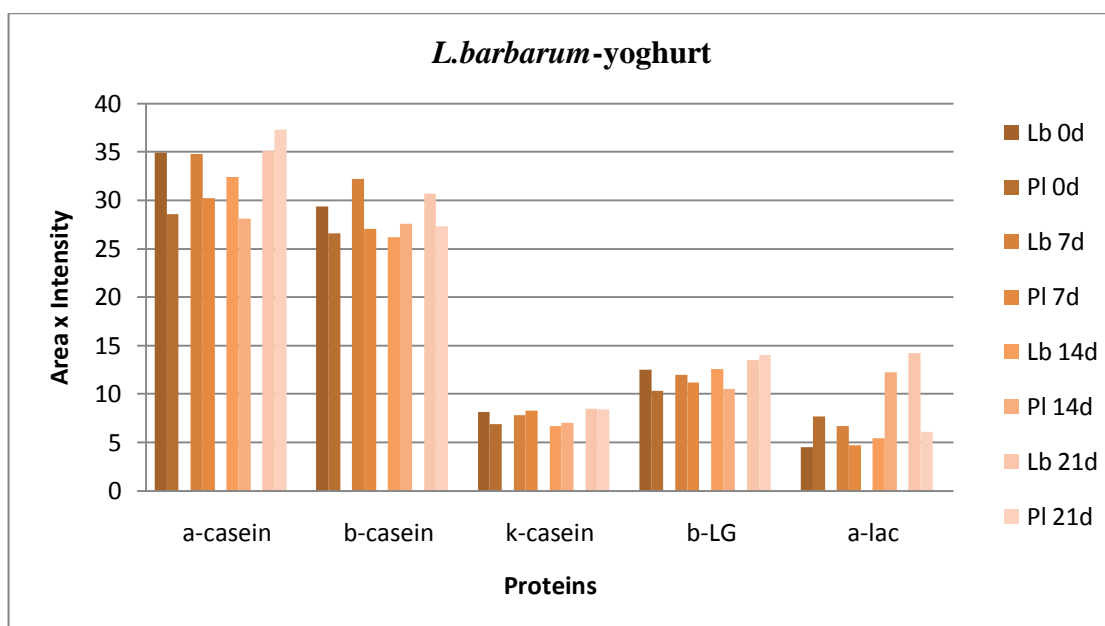


Figure 4.20. Changes of protein from SDS-PAGE analysis *L.barbarum*-yoghurts (LB) and control (PL) during storage at 4 °C

Table 4.19. Comparison of densitometric values of SDS-PAGE protein (intensity of band \times area) in *L.barbarum*-yoghurt with respective control (plain-yoghurt) at different refrigerated (4°C) storage periods

storage	Yoghurt	I.i*.×area				
		α -CN*	β -CN	k-CN	β -LG	α -Lac
day0	LB	34.8	29.4	8.14	12.4	4.5
	PL	28.7	26.6	6.7	10.3	7.8
	LB/PL	1.21	1.11	1.21	1.2	0.58
day7	LB	34.8	32.2	7.8	12.2	7.1
	LPL	30.2	27.1	8.3	11.3	4.8
	LB/PL	1.15	1.19	0.94	1.08	1.48
day14	LB	29.5	25.9	6.7	12.5	5.4
	PL	28.2	27.6	6.9	10.5	12.3
	LB/PL	1.05	0.93	0.97	1.19	0.43
day21	LB	35.2	30.7	8.5	13.5	14.1
	PL	37.3	31.2	8.5	14.1	6.2
	LB/PL	0.94	0.98	1	0.96	2.27

* I.i: Intensity of bands * CN: casein

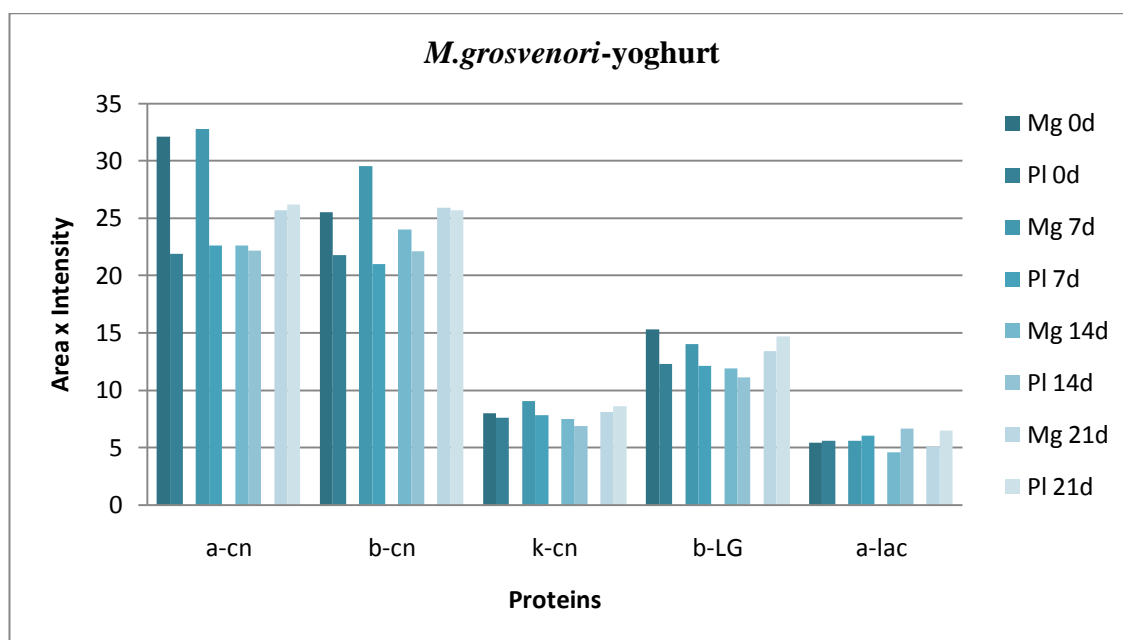


Figure 4.21: Changes of protein from SDS-PAGE analysis *M.grosvenori*-yoghurts (MG) and control (PL) during storage at 4 °C

Table 4.20. Comparison of densitometric values of SDS-PAGE protein (intensity of band \times area) in *M.grosvenori*-yoghurt with respective control (plain-yoghurt) at different refrigerated (4°C) storage periods

Storage	Yoghurt	I.i*. \times area				
		α -CN*	β -CN	k-CN	β -LG	α -Lac
day0	MG	32.1	25.5	8	15.4	7.9
	PL	21.2	21.9	6.9	12.3	5.4
	MG/PL	1.51	1.16	1.16	1.25	1.46
day7	MG	32.2	29.5	9	14.1	6
	PL	22.5	21	7.8	12.2	6
	MG/PL	1.43	1.4	1.15	1.16	1
day14	MG	22.6	24	7.5	11.9	4.7
	PL	22.3	22.1	6.9	11.2	6.7
	MG/PL	1.01	1.09	1.09	1.06	0.69
day21	MG	25.8	26	8.2	13.5	5.1
	PL	26.2	25.7	8.6	14.9	6.5
	MG/PL	0.98	1.01	0.95	0.91	0.78

* Li: Intensity of bands

* CN: casein

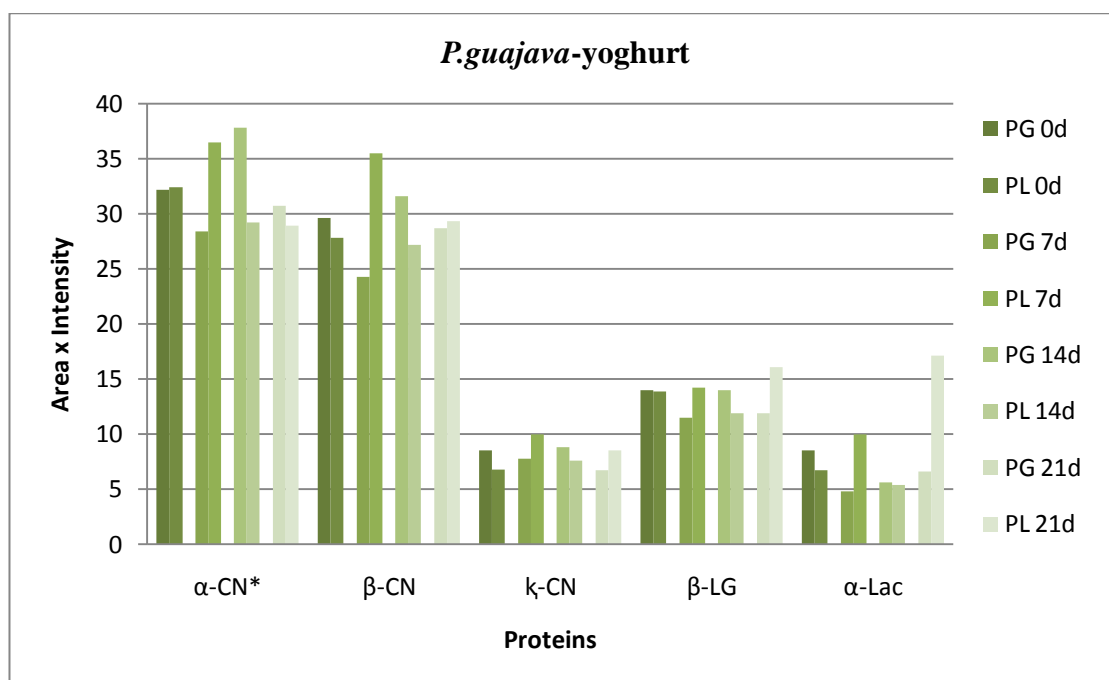


Figure 4.22. Changes of protein from SDS-PAGE analysis *P.guajava*-yoghurts (PG) and control (PL) during storage at 4 °C

Table 4.21. Comparison of densitometric values of SDS-PAGE protein (intensity of band × area) in *P.guajava*-yoghurt with respective control (plain-yoghurt) at different refrigerated (4°C) storage periods

storage	Yoghurt	I.i*. ×area				
		α-CN*	β-CN	k-CN	β-LG	α-Lac
day0	PG	32.2	29.6	8.5	13.97	8.5
	PL	32.4	27.8	6.8	13.88	6.7
	PG/PL	0.99	1.06	1.25	1.01	1.27
day7	PG	28.4	24.3	7.8	11.5	4.8
	PL	36.5	35.5	10	14.2	10
	PG/PL	0.97	0.68	0.78	0.79	0.48
Day14	PG	37.8	31.6	8.84	13.99	5.6
	PL	29.2	27.2	7.6	11.89	5.4
	PG/PL	1.29	1.16	1.12	1.18	1.04
day21	PG	30.7	28.7	6.7	11.9	6.6
	PL	28.9	29.3	8.5	16.1	17.1
	PG/PL	1.06	0.98	0.79	0.74	0.39

* I.i: Intensity of bands

* CN: casein

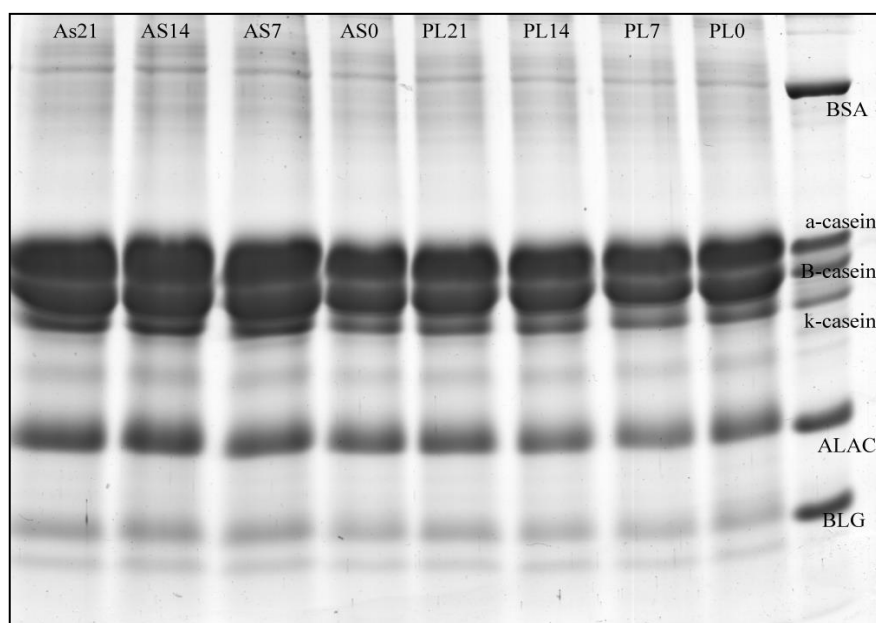


Figure 4.23. SDS-PAGE patterns of proteins in *A.sinensis*-yoghurt and plain-yoghurt

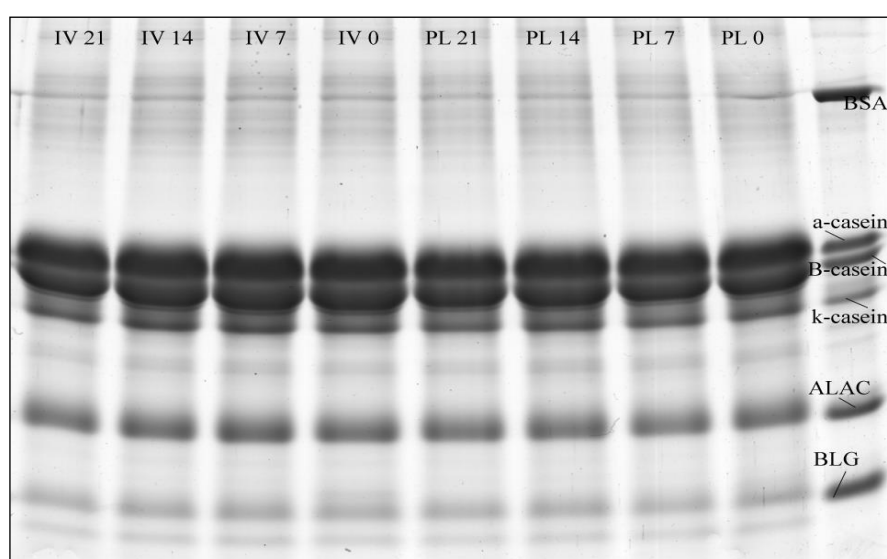


Figure 4.24. SDS-PAGE patterns of proteins in *I.verums*-yoghurt and plain-yoghurt

4.5 Average of moisture content changes in yoghurts during storage

No significant differences in moisture content between herbal-yoghurts and plain-yoghurt was found. Changes in the moisture content of yoghurts during storage was shown in Appendix 10.

4.6 Sensory changes of herbal-yoghurts and plain-yoghurt

Changes in the organoleptic properties of yoghurt in the presence of herbs are as shown in Figures 4.25 to 4.29. The taste of herbal-yoghurts in comparison to plain samples (Figure 4.27) were not different ($p>0.05$) except for *I. verum*-yoghurt (day 0, 7 and 14 of storage) and *M. grosvenori*-yoghurt (day 7 and 21 of storage) . *M. grosvenori*-yoghurt enhanced the overall aroma and overall taste during the storage (Figure 4.25 and 4.27 respectively). *P.guajava*- and *C.pilosula*- yoghurts had higher score for sourness in comparison with plain yoghurt in day 7 and 14 of refrigerated storage (Figure 4.28). *A.sinensis*-yoghurt showed the highest score for bitterness (Figure 4.29) at all stages of storage whereas *C. pilosula*-yoghurt was considered bitter compared to plain-yoghurt on day 0 but not on day 7 and 14 of refrigerated storage.

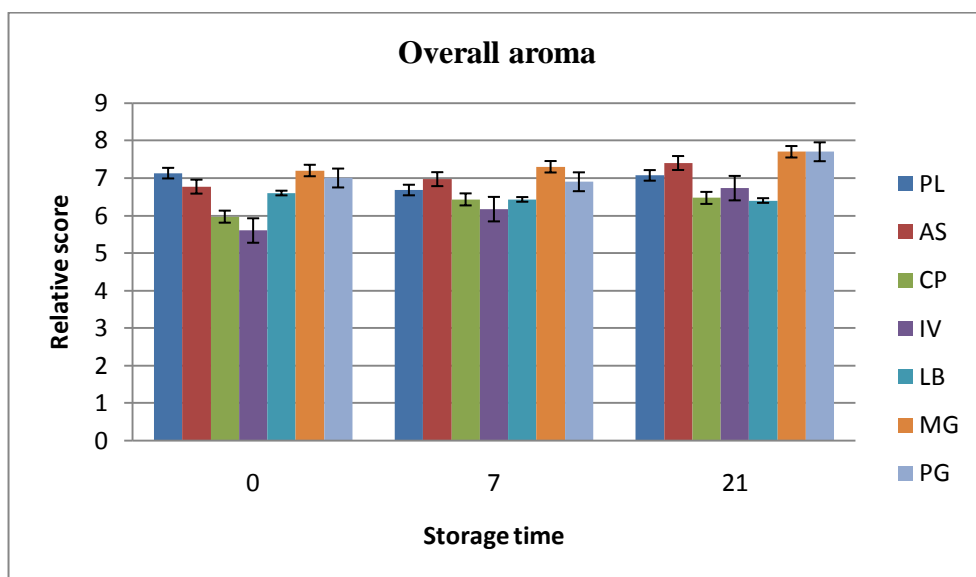


Figure 4.25 Changes of overall aroma in herbal-yoghurts and control during storage at 4 °C
AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

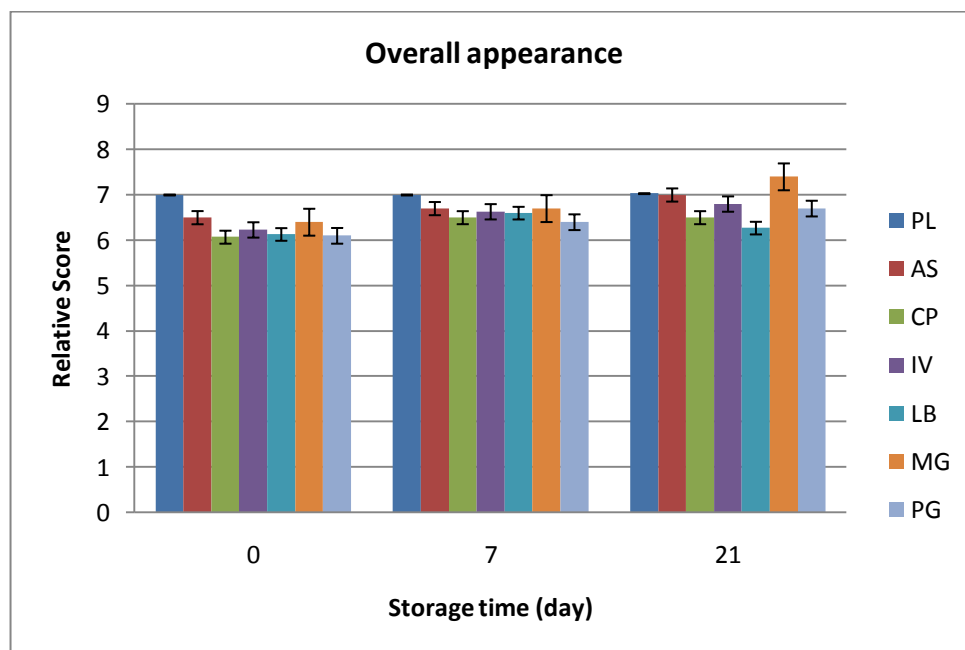


Figure 4.26 Changes of overall appearance in herbal-yoghurts and control during storage at 4 °C
AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

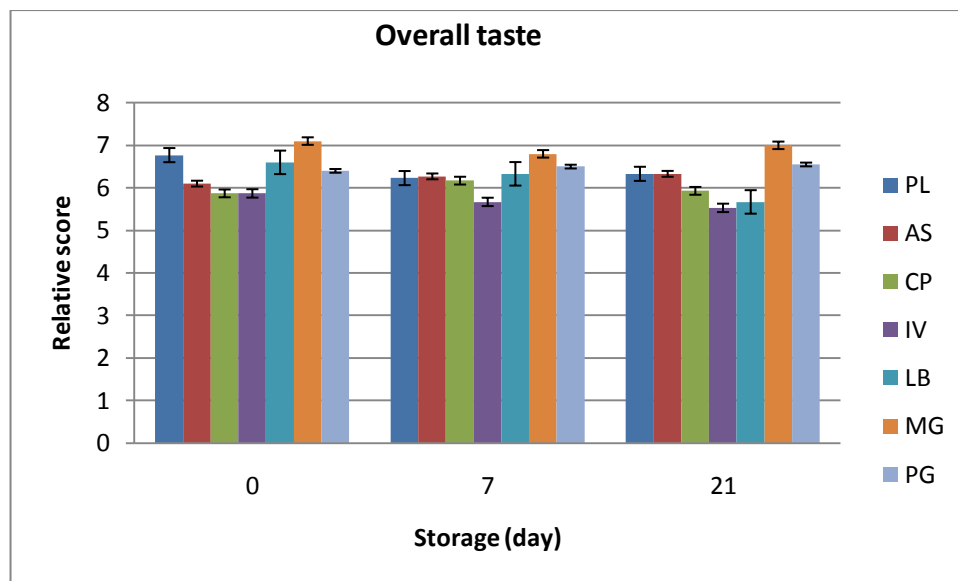


Figure 4.27 Changes of overall taste in herbal-yoghurts and control during storage at 4 °C

AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

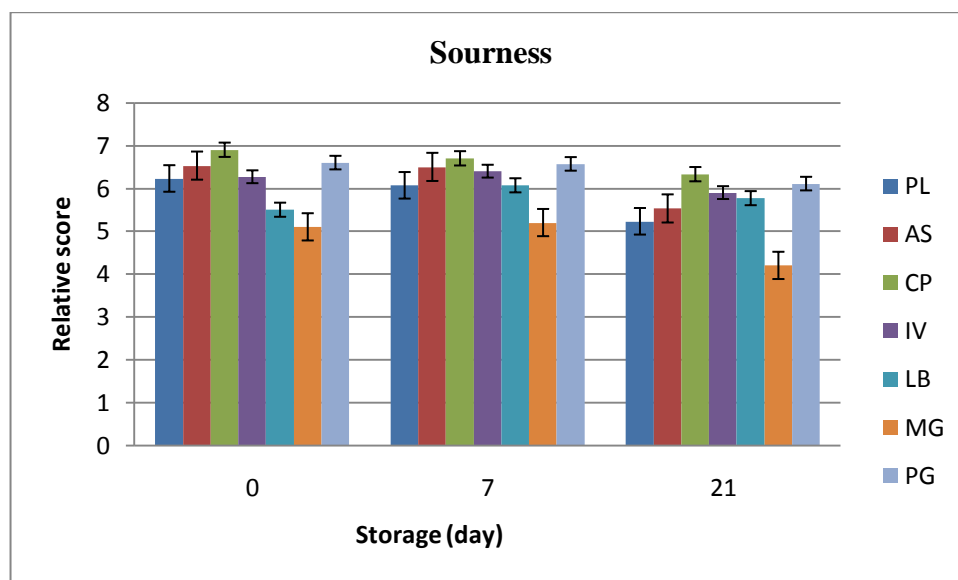


Figure 4.28 Changes of sourness in herbal-yoghurts and control during storage at 4 °C

AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

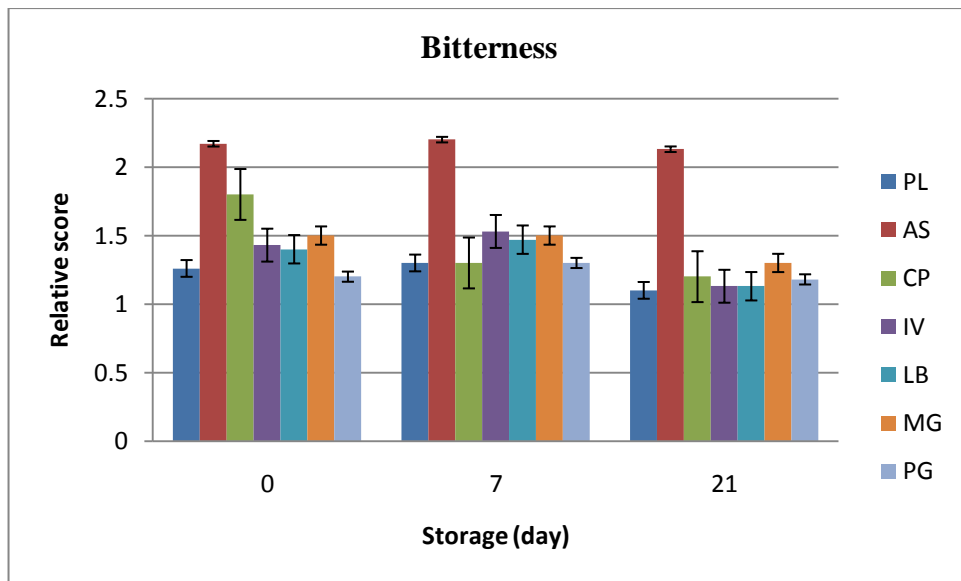


Figure 4.29 Changes of bitterness in herbal-yoghurts and control during storage at 4 °C

AS, *A. sinensis*-yoghurt; CP, *C. pilosula*-yoghurt; IV, *I. verum*-yoghurt; LB, *L. barbarum*-yoghurt; MG, *M. grosvenori*-yoghurts; PG, *P. guajava*-yoghurt; PL, Plain-yoghurt

CHAPTER 5: DISCUSSION

Yoghurt should fulfill several basic requirements in order to meet current demands for marketable probiotic products. These requirements include the probiotics' survival and activity in the product, and stability of the product during storage. Probiotic should not adversely affect the taste, aroma or acidification during the shelf life of the yoghurt (Heller 2001). These considerations should also apply to herbal-yoghurts, a relatively new dairy product which aims to enhance the nutritional and therapeutical values of yoghurt. In the present studies the effects of six common medicinal herbs on yoghurt formation was investigated in relation to microbial counts, physicochemical properties and anti-angiotensin-1 converting enzyme activities.

5.1 Acidification of herbal yoghurt

5.1.1 Acidification of yoghurt during fermentation and storage

The shelf life of yoghurt is determined by the extent of physical, chemical or sensory characteristics changes, and these may affect consumption. Thus the measurements of these parameters during fermentation and storage are important. The acidification during milk fermentation and thus the coagulation of milk protein is the key mechanism for producing yoghurt. Caseinate particles destabilize at pH=5.3–5.2 whereas complete precipitation occurs at pH=4.7–4.6 (Rasic and & Kurmann 1978). Therefore in the present study the pH and total titratable acidity (TTA) of all yoghurts were analyzed to monitor the changes in yoghurts during fermentation and during 21 days of storage. The pH measures free hydrogen ions concentration whereas the TTA is a measurement of total organic acids present in yoghurt that can be neutralized (titrated) by NaOH. Organic acids are weak acids and each type has their own dissociation constant. Since the qualitative organic acids are different in different yoghurts, i.e. poorly reflected by yoghurt pH, the acids produced by fermentation is better described by TTA.

The present study evaluated the acidification at the end of fermentation (described as either after an 18 hr incubation [Method 18hr] or once yoghurt pH reaches 4.5 [Method pH4.5]), and the post-acidification of yoghurt thereafter during storage at 4°C. Fermentation of milk during yoghurt formation involves successive growth of *S. thermophilus* and *Lactobacillus spp.* The microbial population of LAB at the end of fermentation in Method 18hr was in general more than that in yoghurt fermented using Method pH4.5. The post-acidification that occurred during storage however is due to β -galactosidase which is still active at 0–5 °C. In this case, pH may decrease to less than 4.2, resulting in whey separation and affecting also the LAB viability, due more to hydrogen ions than ions of lactate (Rasic & Kurman, 1978).

Extensive post-acidification in yoghurts during storage can become a concern in commercial production of yoghurt. Thus, starter cultures that are devoid of *L. delbureckii ssp. Bulgaricus* (which may cause extensive post-acidification) such as ABT (*L. acidophilus*, *Bifidiobacterium* and *S. thermophilus*) are commonly used. This means *S. thermophilus* acts as the sole fermenting organism since other bacteria in ABT starter cultures are less proteolytic than *L. delbrueckii ssp. bulgaricus* (Shihata and Shah 2000). The presence of *A. sinensis* and *C. pilosula* in yoghurts resulted in higher viable counts on day 0 which subsequently increased after 7 days. Both types of yoghurts also had higher *S. thermophilus* counts for incubation Method 18hr than for incubation Method pH 4.5 (See section 4.1.3). Extraordinarily higher microbial counts may contribute to significant amount of amino acids (Figure 4.12) for LAB growth and metabolisms, an unwanted condition indeed since this may have contributed to the pronounced post-acidification (Figure 4.8)

The pH changes in the control and herbal-yoghurts during fermentation (Figure 4.1) and storage at 4 °C for a period of 3 weeks (Table 4.3 (See Appendix 2) showed that the duration of fermentation (either reaching pH 4.5 or reaching constant pH) for all herbal-

yoghurts, except *P. guajava*-yoghurt, were shorter than that for control-yoghurt. These are indications that the presence of these plants stimulated LAB growth. The pH at which the fermentation was terminated can affect the viability of bacteria either due to one or several of the following factors i.e. initial pH, the rate of pH reduction prior to pH 4.5 (sigmoidal pH decrease) or the different levels of organic acids produced at the end of fermentation. The reduction in pH was less during the storage period if fermentation was terminated at pH 4.5 than at 18 hrs. This may be explained by the extent of microbial growth at different pH (Donkor *et al.*, 2006) and in the present studies as a result of the addition of *A.sinensis* (Figure 4.7). A requirement of minimal extent of post-acidification reflects the importance to terminate yoghurt fermentation at pH 4.5, a step which is commonly practiced in commercial yoghurt production (Dave and Shah, 1997^a).

The final pH (at end of 3 weeks storage) were lower (Table 4.7; see Appendix 6) for yoghurts in Method 18hr than those for yoghurts fermented using Method pH4.5. The presence of *C. pilosula* and *M. grosvenori* enhanced further the acidification and post-acidification (Table 4.1 and Table 4.3 respectively; see Appendix 2 and 4) of yoghurts. The effects of these herbs may be unique to the plants as the inclusion of other herbs in the present studies and others (How, 2008) did not affect the acidification of yoghurts. The differences in the extent of post-acidification of yoghurt by the two fermentation methods may be explained by the viable LAB counts at the end of the fermentation period, as discussed in section 5.2.

5.2 Microbial population in herbal-yoghurts

Probiotic bacteria are slow acid producers (Marshall & Tamime, 1997), which necessitate the inclusion of *S. thermophilus* as starter culture because of its proteolytic

activity. *S. thermophilus* is active even at refrigerated temperature and these microbes can still produce small amounts of lactic acid by fermentation of lactose which results in noticeable pH decrease (Shah *et al.*, 1995). All yoghurts showed higher viability of starter culture bacteria but lower count of probiotic (*L. acidophilus*) which is in agreement with other studies (Dave and Shah, 1997a). The presence of *P.guajava* may however enhance the growth of *L. acidophilus* (Figure 4.5) whereas *L.barbarum* stimulated the growth of *S. thermophilus* (Figure 4.11). In either case the counts of *L. acidophilus* were above 10^5 CFU/ml, in agreement with the suggested minimum level (10^5 - 10^6 CFU /ml; Kurmann & Rasic, 1991; Salwa, 2004; Samona & Robinson, 1994) for the consumption of probiotic in fermented milk to produce therapeutic benefits. The population of *S. thermophilus* in the herbal-yoghurts in the present studies was 10^8 CFU/ml, i.e. close to the higher range (10^7 - 10^8 CFU/ml or g) suggested by Lourens-Hattingh & Viljoen, (2001).

Poor viability of probiotics in the market as reported by Lina *et al.* (2006) and Shah *et al.*, (1995) has drawn the attention of researchers to increase viability of these probiotics. In general the growth of lactobacilli was seen to decrease from day 0 until day 21 (Figure 4.5) for both control and herbal yoghurts but the population of this microorganism was higher ($p \leq 0.05$) for each treatment at week 1 in the absence or presence of herbs. However, the counts of *Lactobacillus spp.* and *S.thermophilus* in all treatments declined with storage time. This is not surprising since Hood and Zootola, (1998) and Shah and Ravula (2000) have reported that the loss of cell viability are associated with decreasing pH during product storage (post-acidification) and the accumulation of organic acids as a result of growth and fermentation.

A competition between the lactobacilli or the dwindling storage of residual lactose (Kailsapathy and Iyer, 2005) could slow the growth of probiotic lactobacilli. However, it appeared that the presence of herbs slowed down the rate of microbial mortality in *P.*

guajava-yoghurt or enhanced those in *M. grosvenori*-, *C. pilosula*-, *L. barbarum*- and *I. verum*-yoghurts (Figure 4.5). In addition, no significant interaction effect was observed between the storage period and yoghurt type, and that the number of colony forming units in each yoghurt type was affected by storage period in a similar way. Both *S. thermophilus* and *Lactobacillus spp.* are known not to have symbiotic relationship and in fact whenever the population of the latter were higher, the former was lower (Figure 4.5 & Figure 4.6). For instance, the counts for *S. thermophilus* remained well above the lactobacilli throughout the storage of all yoghurts which is in agreement with previous studies in this laboratory (Choong, 2006) and that of others (Kneifel *et al*, 1993; 80% of their yoghurts had higher counts of cocci than rods). Such changes inevitably point to the fact that the viability of yoghurt bacteria is very much dependant on factors such as the availability of nutrients, growth promoters and inhibitors, concentration of solutes (osmotic pressure), inoculation level, incubation temperature, fermentation time and storage temperature (Dave and Shah, 1997; Shah, 2000b). The findings from the present studies thus offered options of identifying which herbal plant that may be used to modify LAB counts that can eventually have a considerable effect on acidification of yoghurts during storage.

The present studies have further explored the relationship between i) the decrease in the pH of the medium and accumulation of organic acids as a result of growth and fermentation (Shah, 2000b; Shah and Jelen, 1990) and ii) the ultimate pH at the end of yoghurt fermentation and the growth and viability of *L.acidophilus* (Laroia and Martin, 1991; Shah and Ravula, 2000). When fermentation was stopped at pH 4.5, *Lactobacillus spp.* population in *P.guajava*-yoghurt was more than those in control-yoghurt and other herbal-yoghurts. This suggests the phytochemicals in *P.guajava* enhanced the viability of *Lactobacillus spp.* during fermentation up until when pH 4.5 was achieved. However, extended acidification during storage will impede the growth

of this microorganism. In contrast, *S. thermophilus* growth was suppressed by *P. guajava*. *P. guajava*-yoghurt had the lowest CFU/ml, both after fermentation and throughout the storage (Figure 4.6 and Figure 4.10). Other herbal yoghurts did not enhance or affect the viability of microbes except *C. pilosula*-yoghurt which may have increased *S. thermophilus* on day 14 (Figure 4.6). *C. pilosula* contains mainly steroidal compounds. In the presence of this herb the viability of *S. thermophilus* was higher than those in control-yoghurt up to day 7 of storage but was decreased lower than control by day 14 of storage. This is possibly attributed to acid inhibition as evident from the increased lactic acid concentration (Figure 4.4). On the other hand, the counts for *S. thermophilus* in *A. sinensis*-yoghurt and *I. verum*-yoghurt were higher than that in control at the end of first week of storage but the microbial count decreased lower than control values on day 14. This may be related to decrease of pH and increment of TTA on day 14 ($p>0.05$) or may be due to competition with probiotics for essential growth factors (Donkor *et al.*, 2005).

The presence of *M. grosvenori* in yoghurt resulted in lower pH and higher titratable acidity. Such an acidic condition had a suppressive effect (Figure 4.9 & Figure 4.10) on viable cell counts and on the metabolism of both probiotics and *S. thermophilus*. One plausible explanation is that the momorgrosvin (a sweetener present in *M. grosvenori*) known to act as ribosome inactivating protein (Wang and Ng, 2001) may exert inhibitory effect on enzymatic activities. Hence, momosgrosvin could have affected bacterial growth in the present studies in a manner seen in other studies which reported suppressed growth of *S. thermophilus* in the presence of sweeteners such as sucrose and fructose (Tramer, 1973). The low number of *Lactobacillus* after storage for more than 7 days (Figure 4.5) may indicate a condition of either limited nutrient or they are not able to survive in yoghurt made with full cream milk. This is despite the fact that *L.*

acidophilus has high tolerance to acidic medium, in the pH range from 3.5 to 4.5 in the yoghurt (Vinderola *et al.*, 2000).

In contrast to “Method pH 4.5”, the pH and titratable acidity of yoghurts in “Method 18hr” did not change significantly during storage. This might be due to the lowest acidity values in these yoghurts were already reached by 18 hrs of incubation. It is interesting however to note that *P. guajava*-yoghurt which had the highest total count of *Lactobacillus* on day 0 after fermentation decreased its count rapidly during the first 3 days of storage. The pH of *P. guajava*-yoghurt was higher than control and other herbal-yoghurts and the TTA was lower. This result is in agreement with the microbial profile in the first experiment i.e. the survival of *Lactobacillus* was stimulated with the higher pH and lower TTA. The counts of *S. thermophilus* in *P. guajava*-yoghurt were lower than control and other treatments (Figure 4.6). These microbes are known to be sensitive to low pH and the presence of *P. guajava*’s phytochemicals (e.g phenolic compounds) may have increased the microbial sensitivity to the changes in pH brought about by *Lactobacillus* as shown by Davidson and Naidu (2000). The compounds responsible for antimicrobial activity of most spices are primarily phenolic components of essential oil fraction (Beuchat, 1999) which are different according to geographic origin and crop to crop variations. This could partially explain the differences in the antimicrobial activity of whole spices or essential oils (Moyler, 1994). For instance, the presence of herbal-water extracts (thus anti-microbial activities) may partially explain the low viability of both probiotic and *S. thermophilus* during the storage of *I. verum*-yoghurt.

It is interesting to note that the viability of *S. thermophilus* and *lactobacillus spp.* in *A. sinensis*-yoghurt decreased steadily during storage (Figure 4.10). Such a low cell count of *lactobacillus spp.* in *A. sinensis*-yoghurt compared to other herbal yoghurts may be ascribed to water-soluble polysaccharides isolated from *A. sinensis*. The neutral

polysaccharide fraction of *A. sinensis* which is a mixture of glucan and arabinogalactan, is rich in glucose (65.00%), galactose (3.66%), and arabinose (9.15%) (Sun *et al.*, 2005).

It was also shown in the present studies that the extent of yoghurt fermentation affected the relative *S. thermophilus* and *Lactobacillus spp.* population at the termination of incubation and during storage at 4°C. Higher *Lactobacillus spp.* population (at least 1 log; despite similar *S. thermophilus* population) in yoghurt prepared using Method 18h than that when Method pH 4.5 was used suggest that with the longer incubation *Lactobacillus spp.* had more time to reproduce with subsequent reduction in pH and acidity enhancement. When data relating the viability of *S. thermophilus* and TTA% were analysed it was found that *I. verum*-, *L. barbarum*-, *A. sinensis*- and *P. guajava*-yoghurts had positive correlations ($r = 0.908, 0.773, 0.414$ and 0.391 respectively), as opposed to weak correlation for plain yoghurt ($r = 0.089$), or negative correlations for *C. pilosula*- and *M. grosvenori*-yoghurts (-0.852 and -0.796 respectively; Figure 5.1, Appendix 8). Positive correlation between pH and *S. thermophilus* ($r = 0.848, 0.711, 0.636, 0.627, 0.399$ and 0.288 for *L. barbarum*, *M. grosvenori*, *P. guajava*, *C. pilosula*, plain and *I. verum* respectively) were also noted. Thus, despite lower reproduction of yoghurt bacteria at 4°C (Foster *et al.*, 1958; Tayeb *et al.*, 1984) a higher population of lactobacillus at the end of fermentation enable more post-acidification to occur during refrigerated storage. The generally higher yoghurt bacteria counts in herbal-yoghurts at the time of consumption can ensure sufficient delivery of intestinal-friendly bacteria to the large intestine. However it is important to establish the survival of the bacteria upon exposure to harsh acidic environment in the stomach and alkaline secretion in pancreatic juices. *In vitro* approach such as those of Pinto *et al.* (2006) may provide crucial initial information on the potential stability of yoghurt bacteria in the gastrointestinal tract.

5.3. Changes in proteolytic activity

Proteolysis is a key physical and chemical changes that occur during the production of yoghurt (Tamin& Deeth, 1980). This process involves the progressive hydrolysis of the milk casein proteins to polypeptides, peptides and amino acids which is critical to fulfill the microbial needs for essential and growth stimulating amino acids and peptides (Kunji *et al.*, 1996; Christensen *et al.*, 1999; Donkor *et al.*, 2007).

5.3.1. Herbs and its Effect on Peptide Concentration of Yoghurt

The OPA spectrophotometric assay used in the present study is a rapid, convenient and sensitive method for the determination of the products of proteolysis in milk and milk protein. Peptides concentration of some herbal yoghurt was generally higher than plain yoghurt on day 0, day 7 and day 14. Peptides concentration within treatment were always highest on day 7 with lower concentration recorded on day 0 and 14. The increase of peptide and free amino acid in yoghurt is attributed to the proteolytic activity of *L. bulgaricus* and *S. thermophilus* (Rajagopal and Sandine, 1990). In fact there was 2-3 log CFU/ml higher *S. thermophilus* counts than probiotic bacteria in both yoghurts (herbal-yoghurt and plain-yoghurt) which was in line with other reports (Shihata and Shah, 2000). The counts of *S. thermophilus* remained 2 logs above the counts of lactobacilli throughout the storage of all yoghurts (Figure 4.6 & 4.10). Akalin *et al.*, (2004) and Rohm *et al.*, (1990) also reported a high ($P<0.05$) growth and survival of *S. thermophilus* in all batches of yoghurt during production and storage in various studies. The viability of *S. thermophilus* increased on day 7 compared to 14 days (Figure 4.6). The changes in pH and TTA brought about by one microbial species as described earlier (section 5.2) is the most crucial factor for the survival of other microbes. The addition of different herbal water extract yielded different responses in increases in peptide concentration on day 7 with highest values recorded in *L. barbarum*-yoghurt followed

by *I. verum*-, *A. sinensis*-, *P. guajava*-, plain-, *M. grosvenori*- and *C. pilosula*-yoghurts.

It is highly possible that the presence of phytochemicals in these herbs influenced the activity of *S. thermophilus* in different manner and hence the accumulation of metabolic product such as titratable acids which subsequently determine the activity of *Lactobacillus spp.* The little effect of the presence of flavouring materials added to yoghurt on the survival of yoghurt culture perhaps illustrate the lack of activities these compounds had compared to phytochemicals (Venizelou *et al.*, 2000). Although yoghurt culture exhibited a substantial level of proteolytic activity in the plain-yoghurt, the amount of liberated peptides was further enhanced by the presence some herbal extracts (Figure 4.11). Consequently, the increased proteolytic activity could have resulted in better survival of probiotics in certain yoghurts during storage (Donkor *et al.*, 2006) and in the present studies.

5.3.2. Effects of herbs on total free amino acids during storage in yoghurts

The free amino acid content in yoghurts increased due to proteolytic activity of microorganisms, as assessed by the release of free NH₃ groups by the CD-Ninhydrin method. The ability of LAB to grow to high cell densities in milk is dependent on a proteolytic system that can liberate essential amino acids from casein-derived peptides (Christensen *et al.*, 1999). Significant ($p < 0.05$) increase in proteolysis in yoghurt was recorded in the presence of *A. sinensis* and *L. barbarum*, whereas those in *C. pilosula*-, *I. verum*- and *M. grosvenori*-yoghurts were not different ($p > 0.05$) from plain-yoghurt. In contrast to OPA peptides (section 5.3.1), the amount of liberated amino acids was significantly higher ($p < 0.05$) on day 21 of storage instead of on day 7. Amino acids at this stage may be regarded as accumulated proteolytic products of polypeptides taken place during the storage. Despite the importance of free amino acids for microbial

growth, improved proteolytic activity in *A. sinensis*-yoghurt appeared not to play prominent role in sustaining the viability of LAB during storage (Figure. 4.5 and Figure. 4.6). Similar observation was also reported (Hayaloglu *et al.*, 2005) and the relative increase in pH and reduced viable microbes in *A. sinensis*-yoghurt suggest extensive autolysis of LAB, which is not a desirable fermentation condition. Future studies need to be carried out to elaborate the benefits of apparent advantages of increased proteolysis as opposed to reduced viable lactic acid bacteria caused by *A. sinensis*.

5.3.3. ACE inhibitory activities of herbal-yoghurts

The presence of peptides that inhibit angiotensin-I converting enzyme (ACE) have been reported in food (Ariyoshi, 1993). Milk proteins are rich source of biologically active peptides, which can be released via enzymatic activities in *vitro* and in *vivo*. Gobbetti *et al.* (2000) reported that proteolytic activity results in the release of bioactive peptides from specific amino acid sequences within the proteins and can provide physiological benefits. Milk fermentation with lactic acid bacteria was also reported (Yamamoto & Takano, 1999) to generate a large number of peptides including some with potentially bioactive properties against ACE (Nakamura *et al.*, 1995). This could explain the negative correlation between the OPA peptides and ACE activity (Table 5.2).

However, the production of antihypertensive peptides by milk fermentation is not routinely reported. In particular, milk fermentation with commercial starter strains cannot generate peptides with ACE-I activity although these fermented products can act as precursors of ACE-I peptides after hydrolysis with gastrointestinal enzymes (Herna'ndez-Ledesma & Recio, 2004; Pihlanto-Leppa *et al.*, 1998). It is generally agreed that a strong proteolytic starter is required to produce antihypertensive peptides (Fuglsang *et al.*, 2004; Leclerc *et al.*, 2002; Nakamura *et al.*, 1995a; Sipola *et al.*, 2002; Yamamoto *et al.*, 1999). Two tri-peptides, IPP and VPP are recognised as two

of the most potent ACE-inhibitors from these fermented products. Thus, the present studies considered it more accurate to describe the relative ACE inhibition to peptide contents (specific activity) in yoghurt. This measurement can be considered more representative in determining the inhibition potential of each mg of peptide in various samples. Different storage days will produce different amount of peptides depending on the rate of proteolysis produced by LAB in the presence of herbs. ACE specific activity (Table 4.14) in the plain yoghurt (control) was generally higher than *A. sinensis*-, *I. verum*-, *L. barbarum*-yoghurt on day 0 and *M. grosvenori*-yoghurt on day 14.

The low IC_{50} for *A. sinensis*-yoghurt (Figure 4.16) reaffirms the higher ACE-inhibitory than control and other treatments except *M. grosvenori*-yoghurt. Pripp *et al.* (2006) also found that a high ACE-inhibitory potential was accompanied by a high content of OPA-peptides and a high degree of proteolysis. This allow reliable correlation between OPA-peptides and anti-ACE activity be made. However, the various sizes of protein generated during proteolysis must be taken into account when considering the enzyme-inhibitory properties of these peptides. Results from day 14 of refrigerated storage indicated low specific activity, less IC_{50} and high ACE-inhibitory due to high peptides concentration and free amino acids. This result shows that high peptides concentration illustrate extensive proteolysis which results in higher bioactivity i.e. less specific activity with high ACE inhibition. The lower the rate of specific activity of ACE, the better the potency of the peptides is going to act as ACE inhibitors. Heng, (2008) reported similar IC_{50} (mg/ml) for herbal-yoghurt and plain-yoghurt. Similar trend was observed for all yoghurt with a significant reduction in IC_{50} on day 7 and increased until 28 days of storage.

Specific activity of *I. verum*-yoghurt on 0 day was less than control and *C. pilosula*-, *L. barbarum*- and *P. guajava*-yoghurts. Incidentally, the peptide concentration in *I. verum*-yoghurt was higher than control, *C. pilosula*-, *M. grosvenori*- and *P. guajava*-yoghurts.

IC₅₀ of *I. verum*-yoghurt at this time point was less than *C. pilosula*-, *L. barbarum*-, and control-yoghurts. Further study using HPLC is needed to establish correlation between specific protein fragments formation and ACE inhibitory activity. This should be followed by *in vivo* study to establish which specific peptides in herbal yoghurts can have a blood pressure-lowering effect.

The ACE-inhibition activity seen in *I. verum*-yoghurt, or for other herbal-yoghurts, may be attributed more to the alteration of milk fermentation products produced in the presence of added herbs than due to the intrinsic effects of the herbs. This can be demonstrated by the diminishing inhibitory effects on ACE by herbal-yoghurts as the storage of yoghurts proceeded longer than 14 days (Figure 4.15). In addition, the altered fermented milks due to the addition of herbs may be considered as suitable functional foods, since the IC₅₀ values of most of the ACE-inhibitory peptides found are compatible with the amounts (10 to 60 mg) of bioactive peptides potentially produced during proteolysis of 1 g of CN (Maruyama, 1987).

5.3.4 SDS-PAGE, Gel electrophoresis of yoghurts during fermentation and storage

In the present studies the concentrations of caseins decrease and the concentration of lower molecular weight breakdown products of the caseins increases as the storage time of yoghurt increases. The SDS-PAGE analysis clearly displayed that the α -casein bands of yoghurt proteins were at the top of all vertical PAGE bands in consistent with previous reports (Basch *et al.*, 1989; Jaubert and Martin, 1992), which showed α_{s2} -casein band migration in PAGE and reverse phase HPLC to be the slowest of all casein fractions. The slower migration of α -casein compared to other caseins is due to the fact that it has the highest molecular weight (α_{s1} -CN and α_{s2} -CN; 23,600 and 25,150 respectively).

The electrophoretic patterns of fermented milk (especially cheese during several months ripening at 4 °C) showed that as the ripening time increases, the concentrations of α -casein and β -casein decreased and the concentration of lower molecular weight breakdown products of the caseins increased. These products, which appeared in SDS-PAGE in the area between β -casein and β -lactoglobulin were suggested (Basch *et al.*, 1989; Brandsma *et al.*, 1994) to serve as substrates for microbial proteinases and peptidases, which lead to the formation of smaller peptides and amino acids.

Ong *et al.* (2005) reported the α -casein was hydrolysed faster in almost all probiotic cheeses by the disappearance of the α -casein band at the early stage of ripening period but in the present study this band did not disappear, probably due to the short storage time (3 weeks) as opposed to studies in cheese (greater than 3 months). Lawrence *et al.*, (1987) noted that the most important contributor to the continuous casein matrix of a Cheddar cheese is α ₁-casein and hydrolysis of this casein is believed to be responsible for the softening of Cheddar cheese texture (Grappin, & Olson, 1985). During ripening, β -casein does not undergo as much degradation as α s-casein (Brandsma *et al.*, 1994; Mistry & Kasperson, 1998). This shows that the bacterial peptidases were able to hydrolyse α s-casein faster than β -casine.

Coisson *et al.* (2005) found no significant differences between the electrophoresis patterns in the protein composition of the yoghurt after the addition of the herbs. Visual comparison of SDS-PAGE patterns is not able to readily differentiate difference between plain and herbal yoghurt in each gel. However by measuring intensity and area of band a high density of casein was detected. Using this method the ratio of α -casein in *A.sinensis* tend to decrease upon refrigerated storage which indicate a stimulatory effect of this herb on bacteria and proteolysis in yoghurt (Table 4.16). β -casein also revealed similar results although with k-casein showing minimal reduction during refrigerated storage.

Fox (1993) investigated several important variables which influence the rate of hydrolysis of this casein fraction, including milk quality, storage temperature, bacterial count and species involved and the effects of different manufacturing procedures. Most of these variables were kept constant in the present studies and thus increases in the rate of proteolysis may be attributed mainly due to the enhanced peptidase activity by the bacteria. This is further supported by the amount of protein in WSN and yoghurt extract which contained more product of proteolysis, especially the free amino acids.

5.4 Organoleptic properties of herbal-yoghurts

Sensory characteristics of yoghurts evaluated to preserve yoghurt natural quality during storage because it play important role in acceptance by consumers. Typical yoghurt aroma is mainly due to the presence of acetaldehyde (Pette and Lolkema, 1950). Other flavour compounds that have isolated from yoghurt are found in considerable amount such as ethanol, acetone, diacetyl and 2-butanone (Kneifel *et al.*, 1992). Acetaldehyde and acetone both are produced by single culture of *S.thermophilus* (Accolas *et al.*, 1980).

In the present study yoghurts had gained good scores in overall aroma. The mean appearance scores and aroma for all samples were 6.0 or higher (Appendix 14 and Appendix 15). The score of 6-7 corresponds to moderate liking of the samples, and the score of 5 corresponds to indigent liking of the samples. The score for overall appearance showed insignificant differences between herbal-yoghurts and plain yoghurt. Small differences seen may be explained by multitude of factors; the enhanced *M. grosvenori*-yoghurt taste may be attributed to the sweet mogrosides, the high sourness in *C.pilosula*-, *A.sinensis*- and *P.guajava*-yoghurts may be correlated to pH or TTA (Figure 4.7 & 4.8), whereas *P.guajava*-yoghurt sourness may be contributed to its viability of *Lactobacillus spp.* that was higher than other samples after fermentation. In

general herbal yoghurts were more bitter than plain yoghurt (Figure 4.29) and *A.sinensis*-yoghurt in particular has the highest score of bitterness. The bitter phytochemicals in *A.sinensis* are the most likely reasons that impart the bitter taste to the yogurt. Despite the relatively high sourness and bitterness score that may reduce the organoleptic properties of herbal-yoghurts, the potentially high therapeutical values (i.e. ACE-I inhibition) of these yoghurts make herbal-yoghurts worthy of further research in the future.

5.5 CONCLUSION

The present studies have demonstrated that the probiotic organisms (*L. acidophilus*, *Bifidobacterium spp.* and *L. casei*) survived in presence of medicinal herbs extract in milk fermentation. Yoghurt bacteria are also able to maintain their viability and that *S.thermophilus* had higher viability than *Lactobacillus spp.* during refrigerated storage. The incorporation of herbs extract did not alter the physicochemical properties (pH, TTA, moisture content, organoleptics and shelf life) of yoghurts during 21 days refrigerated storage. Most of the changes in microbial proteolysis of milk protein during storage resulted in the formation of bioactive peptides with anti-ACE activities. Seven days-old yoghurts contain the highest concentration of peptides with anti-ACE activity. Fitting wonderfully into herbal-yoghurt concept is the stable yoghurt bacteria population during refrigerated storage with unique taste and flavour with potentially enhanced phytochemical contents and anti-hypertensive properties.

REFERENCES

- Abbas, Z. and Jafri, W. (1992). Yoghurt (dahi): a probiotic and therapeutic view. *J Pak Med Assoc* **42**, 221-4.
- Abreu, P., Almeida MC, Bernardo RM, Bernardo LC, Brito LC, Garcia EA, Fonsec AS, and M, B.F. (2006). Guava extract (*Psidium guajava*) alters the labelling of blood constituents with technetium-99m. *J Zhejiang Univ. Sci. B.* **7**, 429-35.
- Abu-Tarboush, H. M. (1996). Comparison of associative growth and proteolytic activity of yoghurt starters in whole milk from camels and cows. *Journal of Dairy Science* **79**, 366-371.
- Accolas, J. P., Hemme, D., Desmazeaud, M. J., Vassal, L., Bouillanne, C. and Veaux, M. (1980). Thermophilic lactic starters: properties and behaviour in dairy technology: a review. *Lait*, **60**: 487-524.
- Afonso, I. M. and Joao, M. M. (1999). Rheological monitoring of structure evolution and development in stirred yoghurt. *Journal of Food Engineering* **42**, 183-190.
- Akalin, A. S., Fenderya, S. and Akbulut, N. (2004) Viability and activity of *bifidobacteria* in yoghurt containing fructooligosaccharide during refrigerated storage. *International Journal of Food Science & Technology*. **6 (39)** 613 - 621
- Amatayakul, T., Halmos .A.L., F., S. and N.P. Shah (2006). Physical characteristics of yoghurts made using exopolysaccharide-producing starter cultures and varying casein to whey protein ratios. *International Dairy Journal* **16**, 40-51.
- Ana C. Simões, E. Silva, A.C., José S.S. Diniz, J.S., Filho, A. and Santos, R. A. (2004). The renin angiotensin system in childhood hypertension: Selective increase of angiotensin-(1-7) in essential hypertension. *Journal of pediatrics* **145**, 93-98.
- Ana, C. A., Veloso, T. N. E., Peres, A. M., Mendonça, A. and Isabel, M.P.L.V.O., Ferreira. (2004). Evaluation of cheese authenticity and proteolysis by HPLC and urea-polyacrylamide gel electrophoresis. *Food Chemistry* **87**, 289-295.
- Analie, L.-H. and Bennie, C. V. (2001). Yogurt as probiotic carrier food. *International Dairy Journal* **11**, 1-17.
- Anonymous (1981). Sensory evaluation guide for testing food and beverage products. *Food Technology* **11**, 50 - 65.
- Ariyoshi J. (1993) Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends Food Sci Technol*, **(4)** 139- 44.
- Apisariyakul, A., Chaichana, N. and Takemura, H. (1999). Dual effects of quercetin on contraction in cardiac and skeletal muscle preparations. *Research Communications in Molecular Pathology and Pharmacology*, **105**, 129-138.
- Arima, H., and Danno, G. I. (2002). Isolation of antimicrobial compounds from guava (*Psidium guajava* L.) and their structural elucidation. *Biosci. Biotechnol. Biochem* **66**, 1727-1730.

- Augustin, H., Braun K, Telmenakis I. and Modlich U, W., K. (1995). Ovarian angiogenesis, phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. *American Journal of Pathology* **147**, 339-51.
- Basch, J.J., Farrell, H.M., Walsh, R.A., Konstance, R.P. and Kumosinski, T.F., (1989). Development of a quantitative model for enzymecatalyzed, time-dependent changes in protein composition of Cheddar during storage. *J. Dairy Sci.* **72**, 591–603.
- Begum, S., S.I., H., Ali, S. N. and Siddiqui, B. S. (2004). Chemical constituents from the leaves of *Psidium guajava*. *Nat Prod. Res*, **18**, 135-140.
- Belemtougir, R. G., Constantin, B., Cognard, C., Raymond, G. and L., S. (2006). Effects of two medicinal plants *Psidium guajava* L. (Myrtaceae) and *Diospyros mespiliformis* L. (Ebenaceae) leaf extracts on rat skeletal muscle cells in primary culture. *J Zhejiang Univ SCIENCE B* **7**, 56-63.
- Bensky, D. and Gamble, A. (1986). "Chinese Herbal Medicine:" Materia Medica, Seattle WA.
- Bensky, D. and Gamble, A. (1993). "Chinese Herbal Medicine," Materia Medica Eastland Press, Washington.
- Bensky, D. and R., B. (1990). "Chinese Herbal Medicine, Formulas and Strategies." Eastland Press, Washington.
- Bergamini, C. V., Hynes, E. R., Quiberoni, A. and Suarez, c. a. (2005). Probiotic bacteria as adjunct starters: influence of the addition methodology on their survival in a semi-hard argentinean cheese. *Food Research International* **38**, 597-604.
- Bertrand-Harb, C., Ivanova, I. V., Dalgalarondo, M. and Haertll, T. (2003). Evolution of b-lactoglobulin and a-lactalbumin content during yoghurt fermentation. . *International Dairy Journal* **13**, 39-45.
- Beuchat, L. R. (1999). Survival of *Escherichia coli* O157:H7 in bovine feces applied to lettuce and ineffectiveness of chlorinated water as a disinfectant. *J. Food Prot* **62**, 845-849.
- Bown, D. (1995). "Encyclopaedia of Herbs and their Uses." Dorling Kindersley, London.
- Brandsma, J. B., E. Floris, A. R. D. Dijkstra, L. Rijnen, J. A. Wouters, and W. C. Meijer. (2008). Natural diversity of aminotransferases and dehydrogenase activity in a large collection of *Lactococcus lactis* strains. *Int. Dairy J.* **18**, 1103–1108.
- Brown, A. C., Shovic, A., Ibrahim, S. A., Holck, P. and Huang, A. (2005). A non-dairy probiotic's (poi) influence on changing the gastrointestinal tract's microflora environment. *Altern Ther Health Med* **11**, 58-64.

- Brunner, J. R. (1977). Milk proteins, In Food Proteins.Inc., Connecticut, AVI Publishing Company, Pp 291-314.
- Campbell-Platt, G. (1994). Fermented foods- a world perspective. *Food Research International* **27**, 253.
- Cao, G. W., Yang, W. G. and Du, P. (1994). "Observation of the effects of LAK/IL-2 therapy combining with *Lycium barbarum* polysaccharides in the treatment of 75 cancer patients."
- Cao. W., Li X.Q., Liu. L., Y., T-H, L. C., H.T Fan, M., Jia, Z. G. L. and Mei, Q. B. (2006). Structure of an anti-tumor polysaccharide from *Angelica sinensis* (Oliv.) Diels. *Carbohydrate Polymers* **66**, 149-159.
- Caplice, E. and Fitzgerald, G. F. (1999). Food fermentations: role of micro-organisms in food production and preservation. *International Journal of Food Microbiology* **50**, 131-149.
- Cerning, J. and Marshall, V. (1999). Exopolysaccharides produced by the dairy lactic acid bacteria. *Recent Results and Developments in Microbiol.* **3**, 195-209.
- Chaichana, N. and Apisariyakul, A. (1996). Cholinergic blocking effect of quercetin. *Thai J. Pharmacol* **18**, 1-15.
- Chan, H. C., Chang R.C.C., AKC, I.p., Chiu K, Yuen W.H., Zee S.Y. and KF, S. (2007). Neuroprotective effects of *Lycium barbarum* Lynn on protecting retinal ganglion cells in an ocular hypertension model of glaucoma. *Exp Neurol* **203**, 269-273.
- Chen Guan-Wen, Tsai Jenn-Shou , and Sun, P. B. (2007). Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation. *International Dairy Journal* **17**, 641-647.
- Chen, J. K. and Chen, T. T. (2004). "Chinese Medicinal Herbology and Pharmacology," Art of medicine Press Inc.
- Chiesi, M. and Schwaller, R. (1994). Reversal of phospholamban- induced inhibition of cardiac sarcoplasmic reticulum Ca²⁺-ATPase by tannin. *Biochemical and Biophysical Research Communications* **202**, 1668-1673.
- Choong, W.S. (2006). The effects of *Codonopsis pilosula* and fish collagen on the proteolysis of milk proteins in yogurt. 3rd year biochemistry project report. Institute of Biological Science, Faculty of Science, University of Malaya.
- Choo, P. W., Rand, C. S. and Inui, T. S. (1999). Validation of patient reports, automated pharmacy records and pill counts with electronic monitoring of adherence to antihypertensive therapy. *Med Care* **37**, 846-57.

- Christensen J.E., Dudley E.G., Pederson J.A. and Steele J.L., (1999) Peptidases and amino acid catabolism in lactic acid bacteria, *Antonie van Leeuwenhoek* **(76)** 217–246.
- Chu, D. (1986). "A contribution to my Knowledge of Dong Quai," Calgary, Alberta.
- Church, F. C., H. E. Swaisgood, D. H. Porter, and Catignani., G. L. (1983). Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins *J. Dairy Sci.* **66**, 1219-1227.
- Cor`sson, J. D., Travaglia .F, Piana. G.a, Capasso. M. and Arlorio. M. (2005). *Euterpe oleracea* juice as a functional pigment for yogurt. *Food Research International* **38**, 893-897.
- Collins, M., Glenn, D. and Gibson, R. (1999). Probiotics, prebiotics and symbiotics: Approches for modulating the microbial ecology of the gut. *American J Clin Nutri* **69**, 1052s-57s.
- Commane, D., Roisin Hughes , Colette Shortt , and Ian Rowland (2005). Review The potential mechanisms involved in the anti-carcinogenicaction of probiotics. *Mutation Research* **591**, 276-289.
- Conway, P. L., Gorbach, S. L. and Goldin, B. R. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *Journal of Dairy Science* **70**, 1-12.
- Craig, W. J. (1997). Phytochemicals: Guardian of our health. *J.American dietetic association* **97**, 199-204.
- Crittenden, R., Bird, A. R., Gopal, P., Henriksson, A., Y.K., L. and Playne, M. J. (2005). Probiotic research in Australia, New Zealand and the Asia-Pacific Region. *Current Pharmaceutical Design* **11**, 37-53.
- Dai, Y. and Liu, C. (1986). "Fruit as Medicine," The Ram's Skull Press, Kuranda, Australia.
- Dalglish, D. G. (1982). "Milk proteins-chemistry and physics, In Food Proteins," Applied Science Publishers Ltd., Essex.
- Daniel Commane, Roisin Hughesa, Colette Shorttb, and Rowland, I. (2005). The potential mechanisms involved in the anti-carcinogenic action of probiotics. *Mutation Research* **591**, 276-289.
- Dave, R. I. and Shah, N. P. (1997a). Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. *Int Dairy J* **7**, 31-41.
- Dave, R. I., and Shah, N. P. (1998). Ingredient supplementation effects on viability of probiotic bacteria in yoghurt. *J Dairy Sci* **81**, 2804-16.
- Davidson, P. M. and Naidu, A. S. (2000). "Natural food antimicrobial system," CRC press, LLC.

- Davidson, R. H., Duncan, S. E., Hackney, C. R., Eigel, W. N. and Boling, J. W. (2000). Probiotic culture survival and implications in fermented frozen yogurt characteristics. *J Dairy Sci* **83**, 666-73.
- De Vuyst, L. and Degeest, B. (1999). Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiology Reviews* **15**, 153- 177.
- Deeth, H. C. and Tamime, A. Y. (1981). Yoghurt: nutritive and therapeutic aspects. *J. Food Prot* **44**, 78-86.
- Donkor, O. N., Henriksson, A., Singh, T. K., Vasiljevic, T. and Shah, N. P. (2007). ACE-inhibitory activity of probiotic yoghurt. *International dairy journal* **17**, 1321-1331.
- Donkor, O. N., Henriksson, A., Vasiljevic, T. and N.P. Shah (2006). Effect of acidification on the activity of probiotics in yoghurt during storage. *Int. Dairy Journal* **Vol 16**, 1181-1189.
- Donkor, O. N., Henriksson, A., Vasiljevic, T. and Shah, N. P. (2005). Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yoghurt. *Journal of Food Science* **70**, M375-M381.
- Donkor, O. N., Henriksson, A., Vasiljevic, T. and Shah, N. P. (2007). Rheological properties and sensory characteristics of set-type soy yoghurt. *J Agric Food Chem* **55**, 9868-76.
- Duke, J. A. (1985). "CRC Handbook of Medicinal Herbs," CRC Press, Boca Raton.
- Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O' Sullivan, G. C., Shanahan, F. and Collins, K. (2001). *In-vitro* selection criteria for probiotic bacteria of human origin: Correlation with *in vivo* findings. *American Journal of Clinical Nutrition* **73**, 386-392.
- Dutta, S. M., Kuila, R. K., Ranganathan, B. and Laxminarayan, H. (1971). Biochemical changes produced in milk by selected cultures of lactic acid bacteria. *Ind. J. Dairy Sci.* **24**, 107.
- Elisa, B. M., Anna Beninia, Marta Marzottob, Andrea Sbarbatic, Orazio Ruzzenented, Rossano Ferrarioe, Henno Hendriksf, and Franco Dellaglio (2004). Assessment of novel probiotic *Lactobacillus casei* strains for the production of functional dairy foods. *International Dairy Journal* **14**, 723-736.
- Elmer, G. W. (2001). Probiotics: "living drugs". *Am J Health Syst Pharm* **58**, 1101-9.
- Falk, P. G., Hooper, L. V., Midtvedt, T., and Gordon, J. I. (1998). Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from gnotobiology. *Microbiology and Molecular Biology Reviews* **62**, 1157-1170.

- Fangfang Song, Weijun Chen, Wenbo Jia , Ping Yao, Andreas K. Nussler, Xiufa Sun , and Liegang Liu (2006). A natural sweetener, *Momordica grosvenori*, attenuates the imbalance of cellular immune functions in alloxan induced diabetic mice. *Phytotherapy Res.* **20**, 552-560.
- Farnworth, E. R. (2003). "Handbook of fermented functional foods," CRS PRESS.
- Fernandes, C. F., K. M. Shahani, and M.A. Amer (1987). Therapeutic role of dietary *lactobacilli* and lactobacillic fermented dairy products. *FEMS Microbiology Reviews* **46**, 343-356.
- Fernandes, C. F., Shahani. K.M., Staudinger, W. L. and Amer, M. A. (1991). Effect of *Lactobacillus acidophilus* on growth of Ehrlich ascites tumor in Swiss mice. *J. Nutr. Med.* **2**, 25 -34.
- Fernandes, C. F. and Shahani, K. M. (1989)^a. Lactose intolerance and its modulation with *lactobacilli* and other microbial supplements. *Journal of Applied Nutrition* **42**, 50-64.
- Fernandes, C. F. and Shahani, K. M. (1989)^b. "Anticarcinogenic and Immunological Properties of Dietary Lactobacilli," University of Nebraska, Lincoln.
- Fiat, A. M. and Jolles, P. (1989). Caseins of various origins and biologically active casein peptides and oligosaccharides. *Mol. Cell. Biochem* **87**, 5-30.
- Fiszman, S. M., and Salvador, A. (1999). Effect of gelatin on the texture of yoghurt and acid heat induced milk gels. *Food Research and Technology* **208**, 100-105.
- FitzGerald, R. J., and Meisel, H. (2003). "Milk protein hydrolysates and bioactive peptides " 3rd/Ed. Kluwer Academic/Plenum Publishers, NewYork/Boston/Dordrecht/London/Moscow.
- Fuglsang, A., Nilsson, D. and Nyborg, N. (2002) Cardiovascular effects of fermented milk containing angiotensin-converting enzyme inhibitors evaluated in permanently catheterized spontaneously hypertensive rats. *Appl. Envir. Microbiol.* **68**: 3566–3569.
- Folkertsma, B. and Fox., P. F. (1992). Use of the cadmium-ninhydrin reagent to assess proteolysis in cheese during ripening. *J. Dairy Res.* **59**, 217-224.
- Foltz, M., Meynen, E. E., Bianco, V., van Platerink, C., Koning, T. M. and Kloek, J. (2007). Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *J Nutr* **137**, 953-8.
- Fostere, M., Nelson, F. E., Speck, M. L., DOE , R. N. and OLSON, J. C. (1958). "Dairy Microbiology " Macmillan, London.
- Fox, P. F. (1993). "Cheese: Chemistry, physics and microbiology," 2nd /Ed. Chapman & Hall, London, UK.

- Fox, P. F. and McSweeney, P. L. H. (1998). "Dairy Chemistry and Biochemistry," Blackie Academic & Professional London.
- Fox, P. F., McSweeney, P. L. H., and Lynch, C. M. (1998). Significance of non-starter lactic acid bacteria in Cheddar cheese. *Aust. J. Dairy Technol* **53**, 83-89.
- Fredrickson, A. G. (1977). Behavior of mixed cultures of microorganisms. *Annual Review of Microbiology* **31**, 63-87.
- Friend, B. A. and Shahani, K. M. (1984). Antitumor properties of *lactobacilli* and dairy products fermented by *lactobacilli*. *Food Prot.* **47**, 717-723.
- Fuglsang, A., Nilsson, D. and Niels. Nyborg , C. B. (2004). Using spectrophotometry to determine in vitro turnover rates of peptides in plasma. *Journal of Biochemical and Biophysical Methods*, 2(**58**), 139-151.
- Fuller, R. (1989). Probiotics in man and animals. A review. *J. Appl. Bacteriol* **66**, 365-378.
- Fuller, R. (1992). "History and development of probiotics. In: Probiotics," Chapman & Hall, New York.
- Fuller, R. and Gibson, G. R. (1997). Modification of the intestinal microflora using probiotics and prebiotics. *Scand J Gastroenterol Suppl* **222**, 28-31.
- Gardiner, G. E., Heinemann, C., Bruce, A. W., Beuerman, D. and Reid, G. (2002). "Persistence of *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 but not *L. rhamnosus* GG in the human vagina as demonstrated by randomly amplified polymorphic," DN. *Clin Diagn Lab Immunol* **9**(1): 92-96
- Gavras, H. and Brunner, H. R. (2001). Role of angiotensin and its inhibition in hypertension, ischemic heart disease, and heart failure. *Hypertension* **37**, 342-345.
- Ghafar, A. M. (1992). Volatile flavour compounds of yoghurt, *International Food Science and Technology*, (**27**) 87 – 91.
- Gilliland, S. E. (1989). Acidophilus milk products: a review of potential benefits to consumers. *Journal of Dairy Science* **72**, 2483-2494.
- Gobbetti, M., P. Ferranti, E. Smacchi, F. Goffredi, and Addeo., F. (2000). Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii* subsp. bulgaricus SS1 and *Lactococcus lactis* subsp. cremoris FT4. *Appl. Environ. Microbiol.* **66**, 3898-3904.
- Gobetti, M., Minervini, F. and G., R. C. (2004). Angiotensin I-converting-enzyme-inhibitory and antimicrobial bioactive peptides. *International Journal of Dairy Technology* **57**, 173-188.
- Grappin, R., Rank, T.C. and Olson, N.F., (1985). Primary proteolysis of cheese proteins during ripening. *J. Dairy Sci.* **68**, 531-540, A review.

- Greenberg, N. A., and Mahoney, R. R. (1982). Production and characterization of P-galactosidase from *Streptococcus thermophilus*. *Journal of Food Science* **41**, 1824-1829.
- Grieve (1984). "A Modern Herbal," Penguin. ISBN 0-14-046-440-9.
- Gu, S., Wang, P.L. and Jiang, R. (2007). "A study on the preventive effect of *Lycium barbarum* polysaccharide on the development of alcoholic fatty liver in rats and its possible mechanisms." *Zhonghua Gan Zang Bing Za Zhi* 2007;15:204-8
- Gu, Z., Eleswarapu, S. and Jiang, H. (2007). Identification and characterization of microRNAs from the bovine adipose tissue and mammary gland. *FEBS Letters* **581**, 981-8.
- Guarner, F. and Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet* **361**, 512-9.
- Guarner, F., Perdigon, G., Corthier, G., Salminen, S., Koletzko, B. and Morelli, L. (2005). Should yoghurt cultures be considered probiotic? *Br J Nutr* **93**, 783-6.
- Guarner, F., and Schaafsma, G. J. (1998). Probiotics. *Int J Food Microbiol* **39**, 237-8.
- Gurr, M. I. (1987). Nutritional aspects of fermented milk products. *FEMS Microbiology Reviews* **46**, 337-42.
- Haruki, Y. (1990). Structure characterization and antitumor activity of a pectic polysaccharide from the roots of *Angelica acutiloba*. *planta Medica* **56**, 182-186.
- Havenaar, R. and J.H.J. Huis in't Veld. (1992). Probiotics: a general view. In: The Lactic Acid Bacteria. Volume 1. The Lactic Acid Bacteria in Health and Disease, B.J.B Wood, Ed., Elsevier Applied Science, London, pp. 151-170
- Hayaloglu, A. A., M. Guven, P. F. F., Fox, P. F. and P. L. H. McSweeney (2005). Influence of Starters on Chemical, Biochemical, and Sensory Changes in Turkish White-Brined Cheese During Ripening *J. Dairy Sci* **88**, 3460-3474.
- Hebert, L. A., M. E. Falkenhain, N. S. Nahman, Jr., F. G. C., and O'Dorisio., T. M. (1999). Combination ACE inhibitor and angiotensin II receptor antagonist therapy in diabetic nephropathy. *Am. J. Nephrol* **19**, 1-6.
- Hedrick, U. P. (1972). "Sturtevant's Edible Plants of the World," Dover Publications.
- Hekmat, S., and McMahon, D. J. (1992). Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in ice cream for use as a probiotic food. *J Dairy Sci* **75**, 1415-22.
- Heller, K. J. (2001). Probiotic bacteria in fermented foods: product characteristics and starter organisms. *Am J Clin Nutr* **73**, 374S-379S.
- Hemme, D., Nardi, M. B., and Jette, D. (1980). Galactosidases et phosphogalactosidases de *Streptococcus thermophilus*. *Le lait* **60**, 595-618.

- Heng, W.J. (2008). Inhibitory potentials of *Illicium verum*-treated yoghurt against key enzymes linked to diabetes and hypertension. 3rd Year Biochemistry Project Report, Division Biochemistry, Institute of Biological Science, University Malaya.
- Hood, S. K., and Zottola, M. L. (1998). Effects of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. *Journal of Food Science* **53**, 1514-1516.
- How, W.T. (2008). Changes in sensory, chemical and microbiological properties of Herbal yoghurt during storage. 3rd Year Biochemistry Project Report, Division Biochemistry, Institute of Biological Science, University Malaya.
- Hsu, H. Y., and Chen, Y. P. (1986). "Oriental Materia Medica: a concise guide, Oriental Healing Arts Institute," Long Beach, CA.
- Huang, X., Yang M, Wu X, and J, Y. (2003). Study on protective action of *lycium barbarum* polysaccharides on DNA impairments of testicle cells in mice. *J Hygiene Res.* **32**, 599- 601.
- Huang, Y., and Adams, M. (2004). In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *Int J Food Microbiol* **91**, 253- 60
- Hugenholts, J., and Loojesteijn, P. (1999). Uncoupling of growth and exopolysaccharide expression in *lactococcus lactis* subsp. cremoris Ropy352 strain. *J.DairySci* **83**, 633-640
- Hugenholtz, J., M. Starrenburg, I. Boels, W. Sybesma, A.C. Chaves, Mertens, A., and Wageningen, M. K. (1999). "Metabolic engineering of lactic acid bacteria for the improvement of fermented dairy products " Centre for Food Sciences and NIZO food research, PO Box 20, 6710 BA, The Netherlands.
- Hughes, D. B. and Hoover, D. G. (1991). Bifidobacteria their potential for use in American dairy products. *Food Technol* **45, 74, 76, 83**, 78-80.
- Huxley, A. (1992). "The New RHS Dictionary of Gardening," MacMillan Press.
- Isolauri, E., Laiho, K., Hoppu, U., Ouwehand, A. C. and Salminen, S. (2002). Probiotics: On-going research on atopic individuals. *British Journal of Nutrition* **88**, 19-27.
- Isolauri, E., Sutas, Y., Kaukaanp.a.a, P., , H. and Salminen, S. (2001). Probiotics: Effects on immunity. *American Journal of Clinical Nutrition.* **73**, 444-450.
- Israili, Z. H. and Hall, W. D. (1992). Cough and angioneurotic edema associated with angiotensin-converting enzyme inhibitor therapy: a review of the literature and pathophysiology. *Ann Intern Med.* **117**, 234-242.

- Jackson, K. A. and Savaiano, D. A. (2001). Lactose maldigestion, calcium intake and osteoporosis in African-, Asian-, and Hispanic-Americans. *J Am Coll Nutr* **20**, 198S-207S.
- Jaiarj, P., Khoohaswan P, Wongkrajang Y, Peungvicha P, Suriyawong P, Saraya MLS, and O., R. (1999). Anticough and antimicrobial activities of *Psidium guajava* Linn. leaf extract. *J Ethnopharmacology* **67**, 203-212.
- Jaiarj, P., Wongkrajang Y, Thongpraditchote S, Peungvicha P, Bunyapraphatsara N, and N., O. (2000). Guava leaf extract and topical haemostasis. *Phytother Res.* **14**, 388-391.
- Jaubert , A. and Martin, P. (1992), Reverse-phase HPLC analysis of goat caseins. Identification of α_{s1} and α_{s2} genetic variants. *Lait* **72**, 235–247
- Jian-Hui, L., Yong-Ming, BAO, J.-J., and SONG, L.-J. A. (2003). *Codonopsis pilosula* (Franch) Nannf total alkaloids potentiate neurite outgrowth induced by nerve growth factor in PC12 cells. *Acta Pharmacol Sin.* **24**, 913-917.
- Kailasapathy, K. and Iyer, C. (2005). Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under in vitro acidic and bile salt conditions and in yogurt. *Journal of Food Science* **7**, M18-M23.
- Kailasapathy, K., and Rybka, S. (1997). *L. acidophilus* and *Bifidobacterium* spp.-their therapeutic potential and survival in yogurt. *Aust J Dairy Technol* **52**, 28-35.
- Kamath, J., Rahul, N., Kumar, C. A., and Lakshmi, S. M., (2008). *Psidium guajava* L: A review. *International Journal of Green Farmacy*, **2**, 9-12.
- Kanbe, M. (1992). "Functions of Fermented Milk: Challenges for the health sciences," Elsevier Appl. Sci. Publ, London.
- Keogh, M. K., and O’Kennedy, B. T. (1998). Rheology of stirred yogurt as affected by added milk fat, protein and hydrocolloids. *Journal of Food Science* **63**, 108-112.
- Kunji, E.R.S., Mierau, I., Hagting, A., Poolman, B. And Konings, W.N. (1996) The proteolytic systems of lactic acid bacteria. *Antonie Van Leeuwenhoek*. **70**, 187-221.
- Klaenhammer, T. R., and Sanozky, R. B. (1985). Conjugal transfer from *Streptococcus lactis* ME2 of plasmids encoding phage resistance, nisin resistance and lactose-fermenting ability: Evidence for a high-frequency conjugative plasmid responsible for abortive infection of virulent bacteriophage. *Journal of General Microbiology* **131**, 1531-1541.
- Klaver, F. A. M. and van der Meer, R. (1993). The assumed assimilation of cholesterol by lactobacilli and *Bifidobacterium bifidum* is due to their bile salt-deconjugating activity. *Appl. Environ. Microbial.*, **59**, 1120-4.

- Kneifel, W., Ulberth, F., Erhard, F. and Jaros, D. (1992). Aroma profiles and sensory properties of yogurt and yogurt-related products. I. Screening of commercially available starter cultures. *Milchwissenschaft*, **47**, 362-365.
- Kneifel, W., Jaros, D. and Erhad, F. (1993). Microflora and acidification properties of yogurt and yogurt-related products fermented with commercially available starter cultures. *International journal of food microbiology* **18**, 179-89.
- Kneifel, W., Rajal, A. and Kulbe, K. D. (2000). *In vitro* growth behaviour of probiotic bacteria in culture media of prebiotic importance. *Microbial Ecology in Health and Disease* **12**, 27-34.
- Konoshima, T. and Takasaki, M. (2002). Cancer chemopreventive effects of natural sweeteners and related compounds. *Pure Appl. Chem* **74**, 1309-1316.
- Kunkel, G. (1984). "Plants for human consumption an annotated checklist of the edible phanerogams and ferns," Königstein, Germany.
- Kurmann, J. A. and Rasic., J. L. (1991). "The health potential of products containing *bifidobacteria*. In: Robinson," Elsevier Science Publishers, Ltd., London.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Laffiner, G., Lescut, D. and Vincent, P. (1996). A case control study of drinking water and dairy products *Science* **274**, 262-5.
- Laroia, S. and J. H. Martin, (1991). Methods for enumerating and propagating bifidobacteria. *Cult. Dairy Prod. J.* **26**:32–33.
- Law, B. A. (1981). The formation of aroma and flavor compounds in fermented dairy products. *Dairy Sci.* **43**, 143-154.
- Law, J. and Haandrikman, A. (1997). Proteolytic enzymes of lactic acid bacteria *International Dairy Journal* **7**, (1-11).
- Lewis, S. J., and Freedman, A. R. (1998). Review article: The use of biotherapeutic agents in the prevention and treatment of gastrointestinal disease. *Alimentary Pharmacology and Therapeutics* **12**, 807-822.
- Lawrence, R. C., L. K. Creamer, and J. Gilles. 1987. Texture development during cheese ripening. *J. Dairy Sci.* **70**, 1748–1760.
- Leclerc, P.-L., Gauthier, S. F., Bachelard, H., Santure, M. and Roy, D. (2002) Antihypertensive activity of casein-enriched milk fermented by *Lactobacillus helveticus*. *Int. Dairy J.* **12**, 995–1004.
- Li, J. M. (1997). "Principles and methods of biochemical experiments," Beijing University Press, Beijing.

- Lina, W.-H., Chin-Fa Hwangb, Li-Wei Chenc, and Tsenb, H.-Y. (2006). Viable counts, characteristic evaluation for commercial lactic acid bacteria products *Food Microbiology*. **23**, 74-81.
- Liong, M. T., and Shah, N. P. (2005). Acid and bile tolerance and cholesterol removal ability of Lactobacilli strains. *Journal of Dairy Science*. **88**, 55-66.
- Liong, M. T., and Shah, N. P. (2006). Effects of *Lactobacillus casei* symbiotic on serum lipoprotein, intestinal microflora, and organic acids in rats. *Journal of Dairy Science*. **89**, 1390-1399.
- Liu, L., Wang, J.H., Hou, N., Hu, Y. and Chen, J.W. (1990). Studies on the pharmacological effects and mechanisms of *Codonopsis pilosula* (CP) and its efficacious chemical ingredients on preventing the gastric mucosal damage of rats IV. The effects of the fraction-2 of extract of CP on gastric mucosal barrier. *Pharmacol. Clin. Chi. Med.* **6**, 11-14.
- Livia, A. (1982). Effect of Fermentation on Proteins of Swedish Fermented Milk Products. *J. Dairy Sci* **65**, 1696--1704.
- Loones, A. (1989). " Modification de la composition du lait durant la fermentation du yoghourt.," Libbey Eurotext, Paris.
- Lourens-Hattingh, A., and Viljeon, C. B. (2001). Yoghurt as probiotic carrier food. *International Dairy Journal* **11**, 1-17.
- Lozoya, X., Bercerril G, and M., M. (1990). "Intraluminal perfusion model of *in vitro* guinea pig ileum as a model of study of the antidiarrheic properties of the guava (*Psidium guajava*)," Arch Invest Med, Mex.
- Lozoya, X., Meckes, M., Abou-Zaid, M., Tortoriello, J., Nozzolillo, C., and Amason, J. T. (1994). Quercetin glycosides in *Psidium guajava* L. leaves and determination of spasmolytic principle. *Arch. Med. Res* **25**, 11-15.
- Lucey, J. A., Tamehana, M., Singh, H., and Munro, P. A. (1998). Acomparision of the formation, rheological properties and microstructure of acid skim milk gels made with a bacterial culture or glucono-lactone. *Food Res. Int* **31**, 147-155.
- Lutterodt, G. D. (1989). Inhibition of gastrointestinal release of acetylcholine by quercetin as a possible mode of action of *Psidium guajava* leaf extracts in the treatment of acute diarrhoeal diseases. *J Ethnopharmacology* **25**, 235-247.
- Lutterodt, G. D., and Maleque, A. (1988). Effects on mice locomotor activity of a narcotic-like principle from *Psidium guajava* leaves. . *J Ethnopharmacology* **24**, 219-231.
- Lu CX, and Cheng BQ. (1991). Radiosensitizing effects of Lycium barbarum polysaccharide for Lewis lung cancer. *Zhong Xi Yi Jie He Za Zhi* **11**.611-2, 582.
- Macfarlane , G. T., and J.H. Cummings (1999). Probiotics and prebiotics: can regulating the activities of intestinal bacteria benefit health. *BMJ* **318**, 999-1003.

- Marshall, V. M. (1996). Bioyogurt: How healthy? *Dairy Industries International*, **61**, 28-29.
- Marshall, V. M. E., and Tammine, A. Y. (1997). "Physiology and biochemistry of fermented milks in Microbiology and Biochemistry of Cheese and fermented Milks," 2nd/Ed. Blackie Academic & professional, London.
- Marteau, P., Flourie, B., Pochart, P., Chastang, C., Desjeux, J.-F., and Rambaud, J.-C. (1990). Effect of the microbial lactase (EC 3.2.1.23) activity in yoghurt on the intestinal absorption of lactose: an *in vivo* study on lactase deficient humans. *Br. J. Nutr.* **64**, 71-79.
- Marteau, P., Minekus, M., Havenaar, R., and Huis in't Veld, J. H. (1997). Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: Validation and the effects of bile. *Journal of Dairy Science* **80**, 1031-1037.
- Maruyama, S., H. Mitachi, H. Tanaka, N. Tomizuka, and H. Suzuki (1987). Studies on the active site and antihypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. *Agric. Biol. Chem.* **51**, 1581-1586.
- Matsumoto, K., R. Kasai, K. Ohtani, and O. Tanaka (1990). Minor cucurbitane glycosides from fruits of *Momordica grosvenori* (Cucurbitaceae). *Chem. Pharm. Bull* **38**, 2030-2032.
- Matsumoto, M., and Benno, Y. (2004). Consumption of *Bifidobacterium lactis* LKM512 yogurt reduces gut mutagenicity by increasing gut polyamine contents in healthy adult subjects. *Mutat Res.* **568**, 147-53.
- Matsumoto, M., and Benno, Y. (2006). Anti-inflammatory metabolite production in the gut from the consumption of probiotic yogurt containing *Bifidobacterium animalis* subsp. *lactis* LKM512. *Biosci Biotechnol Biochem.* **70**, 1287-92.
- Matsumoto, M., Hara, K., and Benno, Y. (2007). The influence of the immunostimulation by bacterial cell components derived from altered large intestinal microbiota on probiotic anti-inflammatory benefits. *FEMS Immunol Med Microbiol.* **49**, 387-90.
- Matsumoto, M., Ohishi, H., and Benno, Y. (2001). Impact of LKM512 yogurt on improvement of intestinal environment of the elderly. *FEMS Immunol Med Microbiol.* **31**, 181-6.
- Matsumura, A., Saito, T., Arakuni, M., Kitazawa, H., Kawai, Y., and Itoh, T. (1999). New binding assay and preparative trial of cell-surface lectin from *Lactobacillus acidophilus* group lactic acid bacteria. *J Dairy Sci.* **82**, 2525-9.
- McGee, Harold. (2005). On food and cooking. National Dairy Council, New York.
- Meckes, M., Calzada, F., Tortoriello, J., González, J. L., and Martinez, M. (1996). Terpenoids isolated from *Psidium guajava* hexane extract with depressant activity on central nervous system. *Phytother. Res.*, **10**:600-603.

- Meisel, H. (1998). Overview on Milk Protein-derived Peptide. *Int. Dairy Journal* **8**, 363-373.
- Meisel, H., and Bockelmann, W. (1999). Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Antonie van Leeuwenhoek* **76**, 207-215.
- Meisel, H., and E. Schlimme (1990). Milk proteins: Precursors of bioactive peptides. *Trends in Food Science & Technology* **1**, 41-43.
- Meisle, H., Geopfert, A., and Gunther, S. (1997). Occurence of ACE inhibitory peptides in milk products. *Milchwissenschaft* **52**, 307-311.
- Metchinkoff, E. (1908). "The Prolongation of Life " Putmans Sons, New York.
- Meydani, S. N., and Ha, W.-K. (2000). Immunologic effects of yogurt. *The American Journal of clinical Nutrition* **71**, 861-872.
- Meyer, A. L., Elmadfa, I., Herbacek, I., and Micksche, M. (2007). Probiotic, as well as conventional yogurt, can enhance the stimulated production of proinflammatory cytokines. *J Hum Nutr Diet.* **20**, 590-8.
- Meyer, A. L., Micksche, M., Herbacek, I., and Elmadfa, I. (2006). Daily intake of probiotic as well as conventional yogurt has a stimulating effect on cellular immunity in young healthy women. *Ann Nutr Metab.* **50**, 282-9.
- Midori, T., Takao, K., Yuji, M., Masaki, S., Hoyoku, N., Harukuni, T., Kazuhiro, M., Ryoji, K., and Kazuo, Y. (2003). Anticarcinogenic activity of natural sweeteners, cucurbitane glycosides, from *Momordica grosvenori*. *Cancer Letters* **198**, 37-42.
- Miller, D. D. (1989). Calcium in the diet, food sources, recommended intakes and nutritional bioavailability. *Adv. Food Nut. Res* **33**, 103-154.
- Mishra, A. K., and Kuila, R. K. (1991). Bifidus milk: potential for developing countries. *Indian Dairyman.* **43**, 390.
- Mistry, V. V. and Kasperson, K. M. (1998). Influence of Salt on the Quality of Reduced Fat Cheddar Cheese, *Journal of Dairy Science* . **81**,1214-1221
- Mital, B. K., and Garg, S. K. (1992). Acidophilus milk products: manufacture and therapeutics. *Food Rev. Int.* **8** 347.
- Mitsuoka, T. (1992). "The human gastrointestinal Trac. In: Lactic Acid Bacteria in Health and Disease " Elsevier Applied Science, London.
- Mitsuoka, T. (1992). "The human gastrointestinal tract.," Cambridge: Elsevier Science Publisher Ltd, .
- Mizutani Kenji, Yuda Masamichi, tanaka Osamu, and Saruwatad Yuh-ichiro (1983). Tangshenosides I and II from Chuan-RADIXRadix Codonopsis, the root of *Codonopsis tangshen* [M]. *Oliv.Chem.Pharm.*

- Modler, H. W. (1990). *Bifidobacteria* and bifidogenic factors. *Can. Inst. Food Sci. Technol. J.* **231**, 29-41.
- Montero Marin, A., Limia Sanchez, A., Franco Vargas, E., and Belmonte Cortes, S. (2006). Study of nutrition and health claims on labelling of fermented milk products. *Nutr Hosp* **21**, 338-45.
- Morales, M. A., Tortoriello, J., Meckes, M., Paz, D., and Lozoya, X. (1994). Calcium-antagonist effect of quercetin and its relation with the spasmolytic properties of *Psidium guajava*. *L. Arch. Med. Res* **25**, 17-21.
- Mottar, J., and Bassier, A. (1989). Effect of Heat-induced Association of Whey Proteins and Casein Micelles on Yogurt Texture. *J of Dairy Science* **72**, 2247-2256.
- Mowrey, D. B. (1990). "The scientific validation of herbal medicine," McGraw-Hill, New York.
- Moyler, D. A. (1994). "Spices - recent advances," Elsevier, Amsterdam.
- Moyler, D. A., and Heath, H. B. (1986). "Liquid CO₂ extraction of essential oils. In Flavors and Fragrances: A World Perspective," ICEO, Washington, DC.
- Nacoulma-Ouédraogo, O. G. (1996). Plantes médicinales et pratiques médicales traditionnelles au Burkina Faso: cas du plateau central. Université de Ouagadougou, 62 pages.
- Nadathur, S. R., and Bakalinsky, A. T. (1995). Antimutagenicity of an acetone extract of yogurt. *Mutat. Res. Environ. Mutat* **334**, 213-224.
- Naidu, A. S., Bidlark, W. R., and Clemens, R. A. (1999). Probiotic spectra of lactic acid bacteria (LAB). *Critical Reviews of Food Science and Nutrition* **38**, 13-126.
- Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., and Takano, T. (1995)^a. Purification and characterization of angiotensin I-converting enzyme inhibitors from a sour milk. *Journal of Dairy Science* **78**, 777-783.
- Nakamura, Y., Yamamoto, N., Sakai, K., and Takano, T. (1995)^b. Antihypertensive effects of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J Dairy Sci* **78**, 1253-7.
- Nihal, E. M., Soda, E. L., Desmazeaud, M. J., and Ismail, A. (1986). Peptide hydrolases from the *Thermobacterium* group of *Lactobacilli*. *Le Lait* **66**, 445-451.
- Nilson, K. M., Vakil, J. R., and Shahani, K. M. (1965). B-complex vitamin content of cheddar cheese. *Journal of nutrition* **86**, 362-8.
- Noe, J. E. (1997). *Angelica sinensis*: a monograph. *Journal of Naturopathic Medicine* **7**, 66-72.

- Oh, W., Lee, C., Lee, M. S., Bae, E. Y., Sohn, C. B., Oh, H., Kim, B. Y., and Ahn, J. S. (2005). Antidiabetic effects of extracts from *Psidium guajava*. *J. Ethnopharmacol* **96**, 411-5.
- Ong, L., A. Henriksson, and N.P. Shah (2005). Proteolytic pattern and organic acid profiles of probiotic Cheddar cheese as influenced by probiotic strains of *Lactobacillus acidophilus*, *Lb. paracasei*, *Lb. casei* or *Bifidobacterium* sp. *International Dairy Journal* 1-12.
- Ong, L., Henriksson, A., and Shah, N. P. (2006). Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* and *Bifidobacterium* spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal* **16**, 446-456.
- Osman, A. M., Younes, M. E., and Sheta, A. E. (1974). Triterpenoids of the leaves of *Psidium guajava*. *Phytochemistry* **13**, 2015-2016.
- Ott, A., Germond, J. E., Baumgartner, M., and Chaintreau, A. (1999). Aroma comparisons of traditional and mild yogurt. Headspace- GC quantitation of volatiles and origin of R-diketones. *J. Agric. Food Chem* **47**, 2379-2385.
- Owen, P. L., and Johns, T. (1999). Xanthine oxidase inhibitory activity of northeastern North American plant remedies used for gout. *J. Ethnopharmacology* **64**, 149-160.
- Park, Y. W. and Jin, Y. K. (1998). Proteolytic patterns of Caciotta and Monterey Jack hard goat milk cheeses as evaluated by SDS-PAGE and densitometric analyses. *Small Ruminant Research* **28**, 263-272.
- Petrillo, E., W., J. and Ondetti, M. A. (1982). Angiotensin converting enzyme inhibitors: medicinal chemistry and biological actions. *Med.Res. Rev.* **2**, 1-41.
- Pette, J. W. and Lolkema, H. N. (1950). Acid production and aroma formation in yoghurt. *Milk Dairy J.* **4**, 261-273.
- Phadungath, C. (2005)^a. Casein micelle structure: a concise review, Songklanakarin *J. Sci. Technol* **27**, 201-21.
- Phadungath, C. (2005)^b. Cream cheese products: A review, Songklanakarin *J. Sci. Technol.* **27**, 191-199.
- Pihlanto-Leppä^{la}, A., T. Rokka, and H. Korhonen, (1998). Angiotensin I converting enzyme inhibitory peptides from bovine milk proteins. *Int. Dairy J.* (**8**) 325–331.
- Pinto, M. G. V, Franz, C. M. A. P, Schillinger, U., & Holzapfel, W. H. (2006). *Lactobacillus* spp. with in vitro probiotic properties from human faeces and traditional fermented products. *International Journal of Food Microbiology*, **109**, 205-214.
- Pripp, A. H., R. Sørensen, L. Stepaniak, and T. Sørhaug, (2006). Relationship between proteolysis and angiotensin-I–converting enzyme inhibition in different cheeses. *LWT Food Sci. Technol.* **39**,677–683.

- Qadan, F., Thewaini, A. J., Ali, D. A., Afifi, R., Elkhawad, A., and Matalaka, K. Z. (2005). The antimicrobial activities of *Psidium guajava* and *Juglans regia* leaf extracts to acne-developing organisms. *Am J Chin Med.* **33**, 197-204.
- Qi Zongshao, Li Shufang, and Wu Jiping, (1986). Chemical Analysis on *Lycium Barbarum* Fruit and Leaves. *Zhong Yao Tong Bao* (Chinese Herb News). 11(3):41.
- Qian, H., and Nihorimbere, V. (2004). Antioxidant power of phytochemicals from *Psidium guajava* leaf. *J Zhejiang Univ Sci. B* **6**, 676-83.
- Rajagopal, S. N. and W. E. Sandine, (1990). Associative Growth and Proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in Skim Milk, *Journal of Dairy Science*, 4(**73**): 894-899
- Rajiv, I. D., and Nagendra P. Shah (1997). Viability of Yoghurt and Probiotic Bacteria in Yoghurts Made from Commercial Starter Cultures. *Int. Dairy Journal* **7**, 31-41.
- Ramchandran, L., and Shah, N. P. (2008). Proteolytic profiles and angiotensin-I converting enzyme and alpha-glucosidase inhibitory activities of selected lactic acid bacteria. *J Food Sci* **73**, M75-81.
- Rao, C. V., Sanders, M. E., Indranie, C., Simi, B. and Reddy, B. S. (1999). Prevention of indices of colon carcinogenesis by the probiotic *Lactobacillus acidophilus* NCFMTM in rats. *Intl. J. Oncol* **14**, 939-944.
- Rao, D. R., Pulsani, S. R. and Chawan, C. B. (1986). "Natural inhibitors of carcinogenesis: fermented milk products, in: B.S. Reddy and L.A. Cohen (Eds.), Diet, Nutrition and Cancer: A Critical Evaluation," CRC Press, Boca Raton.
- Rapp, M. (1969). On the ability of lactic acid bacteria to degrade proteins *Milchwissenschaft* **24**, 208-211.
- Rasic, J. L. and Kurmann, J. A. (1978). "Fermented fresh milk products and their cultures. I. Yoghurt, scientific grounds, technology, manufacture and preparations," Technical Dairy Publishing House, Copenhagen, Denmark.
- Re, L., Barocci S, Capitani C, Vivani C, Ricci M, Rinaldi L, Paolucci G, Scarpant, onio A, Leon-Fernandez OS, and MA., M. (1999). Effects of some natural extracts on the acetylcholine release at the mouse neuromuscular junction. *Pharmacological Research.* **39**, 239-245.
- Reid, G. (1999). The scientific basis for probiotic strains of *Lactobacillus*. *Appl Environ Microbiol* **65**, 3763- 6.
- Reid, G. (2001). Probiotic *Lactobacillus* dose required to restore and maintain a normal vaginal flora. *FEMS Immunol and Med Microbiol* **32**, 37 - 41.
- Reid, G. (2002). Probiotics for urogenital health. *Nutr Clin Care* **5**, 3 - 7.

- Reid, G. (2006). Safe and efficacious probiotics: what are they? *Trends Microbiol* **14**, 348-352.
- Reid, G., and Burton, J. (2002). Use of *Lactobacillus* to prevent infection by pathogenic bacteria. *Microbes Infect* **4**, 319- 24.
- Reid, G., Charbonneau, D., Erb, J., Kochanowski, B., Beuerman, D. and Poehner, R. (2003). Oral use of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized placebo-controlled trial in 64 healthy women. *Fems Immunol Med Microbiol* **35**, 131 -134.
- Reid, G., Saunders, M., Gaskins, H., Gibson, G., Mercenier, A. and Rastall, M. (2003). New,scientific paradigms for probiotics and prebiotics. *J Clin Gastroenterol* **37**, 105 -18.
- Reid, W. (1995). "Quantitative analysis in Columbia School Theory."In Contini-Morava and Goldberg (eds), 115-152.
- Ricke, S. C., and Pillai, S. D. (1999). Conventional and Molecular Methods for Understanding Probiotic, Bacteria Functionality in Gastrointestinal Tracts. *Critical Reviews in Microbiology* **25**, 19-38.
- Robinson, R. K. (1987). Survival of *Lactobacillus acidophilus* in fermented products. *Suid Afrikaanse Tydskrif Vir Suiwelkunde* **19**, 25-27.
- Rohm, H., Lechner, F. and Lehner, M. (1990). Microflora of Australian natural-set yoghurt. *Journal of Food Protection*, 53, 478–480.
- Rollema, H. S. (1992). "Casein association and micelle formation, In Advanced Dairy Chemistry," P.F. Fox/Ed. Elsevier Science Publisher, Ltd., Essex.
- Rowland (1998). Functional food science in gastrointestinal physiology and function. *Br. J. Nutr* **80**, S147- S171.
- Rusoff, L. L. (1987). Calcium--Osteoporosis and Blood Pressure. *J Dairy Sci.* **70**, 407-413.
- Rybka, S., and Kailasaphaty, K. (1996). Media for enumeration of yogurt bacteria. *International Dairy Journal* **6**, 839-850.
- Salminen, S., C. Bouley, M.-C. Boutron-Ruault, C., A. Franck, G.R. Gibson, E. Isolauri, M.C. Moreau, M. Roberfroid, and I. Rowland (1998). Functional food science in gastrointestinal physiology and function. *Br. J. Nutr* **80**, S147-S171.
- Salwa, A. Aly, E.A. Ghalal and Neimat, A. Elewa (2004). Carrot Yoghurt: Sensory, Chemical, Microbiological Properties and Consumer Acceptance. *Pakistan Journal of Nutrition.* 3 (6): 322-330
- Samona, A., and Robinson, R. K. (1994). Effect of Yogurt culture on the survival of *bifidobacteria* in fermented milks. *J. Soc. Dairy Technol.* **47**, 58-60.

- Savaiano, D. A., and Levitt, M. D. (1984). Nutritional and therapeutic aspects of fermented dairy products. *ASDC J Dent Child* **51**, 305-8.
- Schanbacher, F. L., Talhouk, R. S., and Murray, F. A. (1997). Biology and origin of bioactive peptides in milk. *Livestock Production Science* **50**, 105-123.
- Schiffirin, E., and Blum, S. (2002). Interactions between the microbiota and the intestinal mucosa. *Eur J Clin Nutr* **56**, 60-4.
- Schioppa, F., V. Prete, and D. di Montanaro (1981). Addition of *Lactobacillus* to yoghurt. *Riv. Soc. Ital. Scie. Aliment.* **10**, 247-252.
- Schlimme, E., and Meisel, H. (1995). Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Nahrung* **39**, 1-20.
- Schuler-Malyoth, R., A., R., and Müller, F. (1968). Die Mikroorganismen der Bifidusgruppe (syn. *Lactobacillus bifidus*). 2. Mitteilung: Die Technologie der Bifiduskultur im milchverarbeitenden Betrieb. *Milchwissenschaft* **23**, 554-558.
- Seshadri, T. R., and Vasishta, K. (1965). Polyphenols of the leaves of *Psidium guajava*: quercetin, guaijaverin, leucocyanidin and amritoside. *Phytochemistry* **4**, 989-992.
- Shah, N. P. (2000b). Probiotic bacteria: Selective enumeration and survival in dairy foods. *Journal of Dairy Science* **83**, 894-907.
- Shah, N. P., and Jelen, P. (1990). Survival of lactic acid bacteria and their lactases under acidic conditions. *Journal of Food Science* **55**, 506-509.
- Shah, N. P., Lankaputhra, W. E. V., Britz, M., and Kyle, W. S. A. (1995). Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yogurts during refrigerated storage. *International Dairy Journal* **5**, 515-521.
- Shah, N. P., and Ravula, R. R. (2000). Influence of water activity on fermentation, organic acids production and viability of yoghurt and probiotic bacteria. *Australian Journal of Dairy Technology* **55**, 127-131.
- Shahani, K. M., and Chandan, R. C. (1979). The nutritional and healthful aspects of cultured and culture containing dairy foods. *Journal of dairy science* **62**, 1685-94.
- Shan, B., Yizhong Z. Cai, Mei Sun, and Harold Corke (2005). Antioxidant Capacity of 26 Spice Extracts and Characterization of Their Phenolic Constituents. *J. Agric. Food Chem.* **53**, 7749-7759.
- Sharareh, H., and Gregor, R. (2006). Sensory properties of probiotic yogurt is comparable to standard yogurt. *Nutrition Research* **26**, 163-166.
- Shihata, A., and Shah, N. P. (2000). Proteolytic profiles of yogurt and probiotic bacteria. *International Dairy Journal* **10**, 401-408.

- Shihata, A., and Shah, N. P. (2002). Influence of addition of proteolytic strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* to commercial ABT starter cultures on texture of yoghurt, exopolysaccharide production and survival of bacteria. *International Dairy Journal* **12**, 765-772.
- Sipola M, Finckenberg P, Santisteban J, Korpela R, Vapaatalo H., (2001). Nurminen M-L. Long-term intake of milk peptides attenuates development of hypertension in spontaneously hypertensive rats. *J Phys Pharm* **52**, 745–54.
- Smacchi, E., and Gobetti, M. (1998). Peptides from several Italian cheeses inhibitory to proteolytic enzymes of lactic acid bacteria, *Pseudomonas fluorescens* ATCC 948 and to the angiotensin I-converting enzyme. *Enzyme and microbial technology* **22**, 687-694.
- Smith, T. M., Kolars, J. C., Savaiano, D. A., and Levitt, M. D. (1985). Absorption of calcium from milk and yogurt. *The American Journal of clinical Nutrition* **42**, 1197-1200.
- Sodini, I., Lucas, A., Oliveira, M. N., Remeuf, F., and Corrieu, G. (2002). Effect of milk base and starter culture on acidification, texture and probiotic cell counts in fermented milk processing. *Journal of Dairy Science* **85**, 2479-2488.
- Starr, J. M. (1994). "Ace inhibitors : central actions," raven press.
- Starr-Lawrence, J. M., and Whalley, J. (1994). "ACE-Inhibitors Jeneral Action," Raven press.
- Sary, F. (1983). "Poisonous Plants," Hamlyn , ISBN 0-600-35666-3
- Strus, M., Pakosz K., Gosciniak H., and Przondo- Mordarska A. (2001). Anatgonistic activity of *Lactobacillus* bacteria strains against anaerobic gastrointestinal tract pathogens (*Helicobacter pylori*, *Compylobacter coli*, *Campylobacter jejuni*, *Clostridium difficile*). *Med Doew Mikrobiol* **53**, 133-42.
- Sudarshan, R., Nadathur a, Steven, J., Gould, b., and Alan, T. B. (1995). Antimutagenicity of an acetone extract of yogurt. *Mutation Research* **334**, 213-224.
- Sun, Y., Tang, J., Gu, X., Li, D. (2005). Water-soluble polysaccharides from *Angelica sinensis* (Oliv.) Diels: Preparation, characterization and bioactivity. *International Journal of Biological Macromolecules*, 5 (**36**), 283-289.
- Sung, C. P., Baker, A. P., Holden, D. A., Smith, W. J., and Charkrin, I. w. (1982). Effects of Extract of *Angelica Polymorph* on Reaginic Antibody Production. *Not Known*.
- Swaigood, H. E. (1992). "Chemistry of caseins, In Advanced Dairy Chemistry," P.F. Fox/Ed. Elsevier Science Publisher, Ltd., Essex.
- Takasaki M, Konoshima T, Murata Y, et al. (2003). cucurbitane glycosides, from *Momordica grosvenori*. *Cancer Lett* **198**(1):37-42

- Takeo, E., H. , Yoshida, N. T., T. Shingu, H. Matsuura, Y. Murata, S. Yoshikawa, T. Ishikawa, H. Nakamura, F. Ohsuzu, and H. Kohda (2002). Sweet elements of *Siraitia (Momordica) grosvenori* inhibit oxidative modification of low density lipoprotein. *J. Atherosclerosis Thromb* **9**, 114-120.
- Tamime, A. Y., and Deeth, H. C. (1980). Yogurt: Technology and biochemistry. *J. Food Prot.* **43**, 939-977.
- Tao Maoxuan, and Zhongliang, Z. (1992). "In Vitro Anti-Mutation Effect of *Lycium Barbarum* Polysaccharide (LBP)."
- Tayeb J., Bouillanne C., and Desmazeaud, M.J. (1984). Computerized control of growth with temperature in a mixed culture of lactic acid bacteria. *J. Ferment. Technol.* **62**, 461-470.
- Tramer, J. (1973). Yogurt cultures. *J. Soc. Dairy Technol* **26**, 16-21.
- Tun-hai XU, Ya-juan XU, Ping CHEN, Dong HAN, Hong-feng ZHAO, Yun-shan SI, Dong-ming XU, Yu LI, and NIU, J.-Z. (2007). A New Furospirostanol Saponin, Ophiopuroside B from *Ophiopogon japonicus* (Thunb.) Ker-Gawl *Chemical Research in Chinese Universities* **23**, 742-744.
- Umesaki, Y., and Setoyama, H. (2000). Structure of the intestinal flora responsible for development of the gut immune system in a rodent model. *Microbes and Infection* **2**, 1343-1351.
- Vakil, J. R., and Shahani, K. M. (1970). "Carbohydrate metabolism of lactic acid cultures IV. Glycolytic and HMS pathway enzymes in *Streptococcus lactis* and their sensitivity to antibiotics, biochemical and life science," Springer Netherlands.
- Van Der Zander, K., Bots, M. L., Bak, A. A., Koning, M. M., and de Leeuw, P. W. (2008). Enzymatically hydrolyzed lactotripeptides do not lower blood pressure in mildly hypertensive subjects. *Am J Clin Nutr* **88**, 1697-702.
- Van Vliet, T., and Walstra, P. (1994). Water in casein gels: How to get it out or keep it in. *Journal of Food Engineering* **22**, 75-88.
- Varnam, Alan H., Sutherland, Jane P. (1994). *Milk and Milk Products: Technology, Chemistry and Microbiology*, Chapman & Hall, New York, 451 p.
- Veloso, A. C. A., Natércia Teixeira, and Isabel M.P.L.V.O. Ferreira (2002). Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea-polyacrylamide gel electrophoresis Detection of milk adulterations *Journal of Chromatography* **967**, 209-218.
- Veloso, A. C. A., Natércia Teixeira, António M. Peres, Alvaro Mendonça, and Isabel M.P.L.V.O. Ferreira (2004). Evaluation of cheese authenticity and proteolysis by HPLC and urea-polyacrylamide gel electrophoresis Analytical, Nutritional and Clinical Methods. *Food Chemistry* **87**, 289-295.

- Vermeirssen, V., John Van Camp, and Verstraete, W. (2002). Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. *J. Biochem. Biophys. Method* **51**, 75-87.
- Venizelou, M., Kehagias, C., Samona, A. and Koulouris, S. (2000). Survival of yoghurt characteristic microorganisms in fruit yoghurts prepared under various conditions. *Egyptian Journal of Dairy Science* **28**, 169–182.
- Vinderola, C. G., Gueimonde, M., Delgado, T., Reinheimer, J. A. and de los Reyes Gavilán, C. G. (2000). Characteristics of carbonated fermented milk and survival of probiotic bacteria. *International Dairy Journal*, 3(10), 213-220
- Visser, F. M. W. (1977a). Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 2. Development of bitterness and cheese flavour. *Milk Dairy J* **31**, 247-264.
- Visser, F. M. W. (1977b). Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 3. Protein breakdown: analysis of the soluble nitrogen and amino acid nitrogen fractions. *Milk Dairy J* **31**, 210-239.
- Wai Seng, (2006). Changes in sensory, chemical and microbiological properties of yoghurt in the presence of *Codonopsis pilosula* during storage. 3rd Year Biochemistry Project Report, Division Biochemistry, Institute of Biological Science, University Malaya.
- Wal, J. M. (1998). Immunochemical and molecular characterization of milk allergens. *Allergy* **53**, 114-117.
- Wallowski, I., Rechkemmer, G., and Pool-Zobel, B. L. (1999). Protective role of probiotics and prebiotics in colon cancer. *American Journal of Clinical Nutrition* **73**, 451-455.
- Walstra, P., Geurts, T. J., Noomen, A., Jellema, A., and van Boekel, M. A. J. S. (1999). "Dairy Technology: Principles of Milk Properties and Processes," Marcel Dekker, Inc., New York.
- Wang, H. X., Ng, T. B. (2001). Studies on the anti-mitogenic, anti-phage and hypotensive effects of several ribosome inactivating proteins. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 3 (128), 359-366.
- Wang, K. Y., Li, S. N., Liu, C. S., Perng, D. S., Su, Y. C., Wu, D. C., Jan, C. M., Lai, C. H., Wang, T. N., and Wang, W. M. (2004). Effects of ingesting *Lactobacillus*- and *Bifidobacterium*-containing yogurt in subjects with colonized *Helicobacter pylori*. *Am J Clin Nutr* **80**, 737-41.
- Wang, Z. T., Ng, T. B., Yeung, H. W., and Xu, G. J. (1996). Immunomodulatory effect of a polysaccharide-enriched preparation of *Codonopsis pilosula* roots. *General pharmacology* **27**, 1347-1350.

- Wang, Z. T. (1997). Investigations on the protective action of *Codonopsis pilosula* (Dangshen) extract on experimentally-induced gastric ulcer in rats. *General Pharmacology* **28**, 469-473.
- Watt, J. M., and Branchwizk, M. G. (1969). "The Medicinal and Poisonous Plants of Southern and Eastern Africa," E & S Livingstone, London.
- Wervey, E. J. W., Overbeek, J. Th. G. (1948): *Theory of stability of liophobic colloids*. Elsevier, Amsterdam: 642 p.
- William, R. A. (2004). The Emerging Role of Dairy Proteins and Bioactive Peptides in Nutrition and Health. *Journal of nutrition*, 989S-995S.
- Williams, A. G., Noblea, J., Tammamb, J., Lloydb, D., and Banksa, J. M. (2002). Factors affecting the activity of enzymes involved in peptide and amino acid catabolism in non-starter lactic acid bacteria isolated from Cheddar cheese *Int Dairy J* **12**, 841-852.
- Wohlrab, Y., and Bockelmann, W. (1992). Purification and characterization of a dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Int Dairy J.* **2**, 345-361.
- Wynckel, A., Jaisser, F., Wong, T., Druke, T., and Chanard, J. (1991). Intestinal absorption of calcium from yogurt in lactase-deficient subjects. *Reprod Nutr Dev.* **31**, 411-8.
- Xie C, Xu L, Li X, et al (2001) Studies on chemical constituents in fruit of *Lycium barbarum* L. *Zhongguo Zhong Yao Za Zhi*; 26(5): 323-324.
- Yamamoto, N., Maeno, M., and Takano, T. (1999). Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J Dairy Sci.* **82**, 1388-93.
- Yamamoto, N., and Takano, T. (1999). Antihypertensive peptides derived from milk proteins. *Nahrung* **43**, 159-64.
- Ye, Y. N., H.L. So, E.S.L. Liu, V.Y. Shin and C.H. Cho (2003). Effect of polysaccharides from *Angelica sinensis* on gastric ulcer healing. *life science* **72**, 925-932.
- Ye, Y. N., Koo, M. W. L., Li, Y., Matsui, H., and Cho, C. H. (2001). *Angelica sinensis* modulates migration and proliferation of gastric epithelial cells. *Life Sciences* **68**, 961-968.
- Yeung, H.C. (1985). "Handbook of Chinese Herbs and Formulas," Institute of Chinese Medicine, Los Angeles.
- Yongwen, Z., and Yamada, O. (1996). Polymer Complement activation galactan chain 's in a pectic arabinogalactan (AG II b-1) from the Kitagawa. *Carb roots of Angelica acutiloba* **3**, 149-156.

- Yvonne, F. C., L.H., P., McSweeneyb, and Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge *International Dairy Journal* **13** 841-866.
- Zhao, Y. (2008). Structure and Function of Angiotensin Converting Enzyme and Its Inhibitors. *Chinese Journal of Biotechnology* **24**, 171-176.
- Zhari, I. (1999). "Malaysia herbal monograph." Malaysian Monograph Committee, Kuala Lumpur.
- Zhong, Y., Huang, C. Y., He, T., and Harmsen, H. M. (2006). Effect of probiotics and yogurt on colonic microflora in subjects with lactose intolerance. *Wei Sheng Yan Jiu* **35**, 587-91.
- Zhou, Z. (1991). Influence on Hematopoietic Cells and Colony Stimulating Factors by *Lycium Barbarum* Polysaccharide on Normal Mice. *Chinese Hematological Journal* **12**, 409-411.
- Zhu, M., Phillipson, J. D., Greengrass, P. M., Bowery, N. E., and Cai, Y. (1997). Plant polyphenols: biologically active compounds or non-selective binders to proteins? *Phytochemistry* **44** :441-447.
- Zhu, You-Ping. (1998). Chinese materia medica: chemistry, pharmacology, and applications. Medical, page556.
- Zourari, A., Accolas, J. P., and Demazeaud, M. J. (1992). Metabolism and Biochemical characteristics of yogurt bacteria. *A Rev. Lait*, **72**, 1-34.