CHAPTER 1: INTRODUCTION

Yogurt is recognized as a popular healthy food and as the most important delivery vehicle for probiotic organisms such as *Lactobacillus acidophilus*, *Bifidobacterium* ssp. and *L. casei* Yogurt is basically the fermentation product of lactic acid in milk by lactic acid producing bacteria (LAB) *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*. It has both the exceptional nutritional and therapeutical values. This fits in nicely to the current consumer's preference for foods with functional properties (Metchinkoff, 1901). Fermented dairy products are considered to have functional properties because probiotic bacteria may be added to the regular fermentation cultures to enhance health and therapeutic benefits such as enhancing immune system, producing anti-carcinogenic substances, maintain normal intestinal micro-flora, reduce lactose intolerance, reduce serum cholesterol, improve nutritional value of food, improvement inflammatory bowel diseases and suppression of *Helicobater pylori* infection (Kurmaann & Rasic, 1991; Shah 2004). Patients with any of these conditions could benefit from the consumption of yogurt considered mediated by the gut microflora, bowel transit time, and enhancement of gastrointestinal innate and adaptive immune responses (Dave and Shah, 1997).

Amongst the bacterial chronic infection of the gastrointestinal system, *H. pylori* plays an important etiological agent of chronic gastritis, peptic ulceration and gastric cancer in human (Blaser, 1992; Parsonnet, 1993). It is estimated that one-half of the world's population is infected with *H. pylori* (Go, 2002) and the eradication of *H. pylori* is considered a crucial factor to decrease the rate of relapse of gastric and duodenal ulcers (Marshall *et al.*, 1988). *H. pylori* carriage rates are about 80–90% in developing countries with a high risk of gastric cancer (more than 90%) and antibiotic resistance

(Lacy, 2001). Antibiotics produce undesirable side effects and non-compliance among the patients (Broutet, 2003) in the long run. The failure of antibiotics to treat infection as a result of increased microbial resistance (Megraud, 2004), and the fear that new, more successful, antibiotics will not be developed has caused a profound doubt on therapeutic effectiveness of antibiotics. In addition, the consumer's preferences for dietary supplements to maintain gastrointestinal health have motivated further research into alternative approaches to suppress *H. pylori* infection. For instance, the growth of *H. pylori in vitro* may be suppressed by *L. acidophilus* and *Bifidobacterium* (Midolo, 1995; Coconnier, 1998). This may explain the therapeutic effects of probiotic yogurt, possibly by *in vivo* suppression of the growth of *H. pylori* in infected clinical patients (Sheu, 2002; Wang, 2004).

The use of natural food additives and the consumption of health-promoting substances has become a popular trend amongst health conscious consumers (Varga, 2006). Cinnamon (*Cinnamon zeylanicum*), licorice (*Glycyrrhiza glabra)* and garlic (*Allium sativum*) were selected in the present study due to their well-known benefits for human health and wide application as flavoring ingredients worldwide (Cutler, 1995). These plants also contain bioactive compounds with anti-bacterial activity against *H. pylori* growth (Tabak, 1999; Arora and Kaur, 1999). Their antimicrobial activities against several pathogenic bacteria (Fukai, 2002; Harris, 2001; Shaik Mahboob *et. al*, 2005;) also mean that their presence in yogurt would help to prevent the growth of pathogenic bacteria.

C. zeylanicum has a long history both as a spice and as a medicine (Toussant-Samat, 1992). *Cinnamaldehyde 2- hydroxycinnamaldehyde,* and *eugenol* are the main components of bark extract of cinnamon and are active against many pathogenic bacteria (e.g. *H. pylori*), fungi and viruses (Tabak, 1999; Shaik Mahboob, 2005). The root of licorice (*Glycyrrhiza glabra)* has a sweet flavor and it has been used for

medicinal purposes for at least 4000 years (Fiore *et al.*, 2005). The extract of this medicinal plant is used as the basis of anti-ulcer medicines for treatment of peptic ulcer and mouth ulcer (Das, 1989). Anti *H. pylori* activities of licorice flavonoids may be attributed to glycyrrhizin (Fukai, 2002) since it is a main bioactive compound in licorice (Kaefer and Milner, 2008). *A. sativum* has a wide spectrum of actions including antibacterial activity and beneficial effects on the cardiovascular and immune systems (Harris, 2001). The antibacterial activity of garlic is widely attributed to its most important component called "allicin" (Miron *et al*. 2000). Epidemiological studies showed a negative association between stomach cancer, which is strongly correlated with *H. pylori* infection, and the consumption of *Allium* vegetables (O'Gara, 2000). There is also *in vitro* evidence for anti *H. pylori* effects of aqueous garlic extracts (Cellini, 1996; Jonkers, 1999).

The consumption of yogurt has increased dramatically during the last two decades due to its nutritional and therapeutic benefits for consumers. Most viability and anti *H. pylori* studies have been conducted on herbs only or natural plain bio-yogurts and very few studies have looked at yogurt containing herb(s). In the present study, the ability of yogurt bacteria to grow and survive in the presence of cinnamon, licorice or garlic was assessed. Changes in pH, titratable acidity, antioxidant activity and the viable count of *S. thermophilus* and *Lactobacillus* ssp. were monitored during the manufacture and refrigerated storage of yogurts. Effects of yogurt- containing herbs were also investigated on the growth of *H. pylori in vitro*.

CHAPTER 2: LITERATURE REVIEW

2.1 Yogurt

Fermented dairy products form a major part of diet of millions of people around the world. Among the fermented milk products, yogurt is by far the most important vehicle for the delivery of probiotic organisms (Anelie and Bennie, 2001). Yogurt is basically a fermented dairy product made by adding bacterial cultures (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) to milk, which causes the transformation of the milk's sugar (lactose), into lactic acid (Dave and Shah, 1998). Yogurt is thus a nutritious food (rich in protein, calcium, riboflavin, vitamin B6, vitamin B12) by virtue of the nutrients in milk and the fact that it is more digestible than milk (Shah, 2007). Several studies emphasize the development of healthy product called bio-yogurt which is basically fermented milk products containing added live strains of *Lactobacillus acidophilus* and *Bifidobacterium* cells (known as AB culture). This type of yogurt is considered to provide additional health benefits (antibacterial and anti inflammatory properties; Shah, 2006) when consumed at minimum values ranging between 10^6 and 10^8cfu/g (Playne *et al.*, 2003; Kailasapathy *et al.*, 2007).

2.1.1 Health benefits of yogurt

Yogurt is a very good source of calcium, phosphorus, riboflavin-vitamin B2 and iodine. Yogurt is also a good source of vitamin B12, pantothenic acid-vitamin B5, zinc, potassium, protein and molybdenum (Meyer *et al.*, 2006). These nutrients by themselves make yogurt a health-supportive food.

a) *Yogurt improves the bioavailability of other nutrients*

Yogurt increases the absorption of calcium and B-vitamins. Folate is a good example of a B-vitamin that some LAB species synthesize (Crittenden, 2003). The lactic acid in the yogurt aids in the digestion of the milk calcium, making it easier to be absorbed (Adolfsson *et al.,* 2004).

b) *Yogurt can decrease yeast infections*

Eating yogurt containing live and active cultures daily was shown to reduce the amount of yeast colonies in the vagina and decrease the incidence of vaginal yeast infections (Shah, 2000). *L. acidophilus* is a vital part of normal vaginal microflora and its production of lactic acid prevent overgrowth of pathogenic bacteria like *Candida*. The production of hydrogen peroxide as well as lactic acid by *Lactobacillus* also helps to keep the desirable pH (3.8-4.5) for healthy vaginal flora (Laye *et al.,* 1993).

c) *Yogurt is a rich source of calcium*

Yogurt is also a rich source of calcium, a mineral that discourages excess growth of the cells lining the colon, which can place a person at high risk for colon cancer (Peter, 1992). Calcium also binds cancer producing bile acids and protects them from irritating the colon wall (Jacobs, 1991). This could well explain people consuming diets high in calcium (e.g. in Scandinavian countries) have lower rates of colorectal cancer (Slattery *et al.,* 1990). An average intake of 1,200 milligrams of calcium a day is associated with a 75 percent reduction of colorectal cancer (Smith *et al*., 1985). Most yogurts have higher bioavailable calcium because the live-active cultures in yogurt increase the absorption of calcium by fermenting the milk sugar, and get more calcium into the body than the same volume of milk can (Cheng *et al.,* 2005).

d) *Yogurt is an excellent source of protein*

The protein content of yogurt may be higher than the milk protein because of the frequent addition of nonfat dry milk during processing and concentration, which increases the protein content of the final product (The National Yogurt Association, 2001). Besides being a rich source of proteins, the proteolytic activity of the yogurt bacteria during fermentation makes milk proteins easier to digest. For this reason, the proteins in yogurt are often called "predigested" (Shahani and Chandan, 1979).

e) *Boost the Body's Ability to Build Bone*

Calcium plays important role in the formation and mineralization of bone. Yogurt also contains lactoferrin, an iron-binding protein that boosts the growth and activity of osteoblasts and decreases the formation of osteoclasts (Cornish *et al.,* 2004). The activities of both types of proteins ensure optimal bone development during growth, and help to prevent or slows down osteoporosis during old age (Oskar, 2004).

2.2 The human gastrointestinal tract and its microbiota

The gastrointestinal tract (GI) is a very diverse and metabolically active organ in the human body. Apart from digestion of food, GI maintains its beneficial microbiota against harmful and opportunistic microorganism (Srikanth and McCormick, 2008), which is vital for host health and reduction of disease risks. In particular, the intestinal microbiota is involved in the fermentation of microbial growth. Another important role of microbiota is production of vitamin B and K in the gut (Hooper *et al.,* 2002). Stomach is the first segment of GI, which accommodates ingested food. Only few microorganisms can survive the harsh, strongly acidic and peristaltic nature of the stomach. These include acid-tolerant lactobacilli, yeasts and bacteria, (especially *Helicobacter pylori;* Montecucco and Rappuoli, 2001). There is a high extent of

changeability between the stomach, small intestine and colon in terms of numbers and bacterial population types. This is owing to different passage times, secretions and nutrient availability (Lambert and Hull, 1996; Gulliams, 1999). Microorganisms also interact with and influence their surroundings to ensure the survival against competitors. This is accomplished through among others, increasing anaerobicity as well as the production of toxic compounds (e.g. acid or antimicrobial substances), which benefits the host or having devastating effects on pathogenic bacteria (Fooks and Gibson, 2002).

There are at least four different microhabitats along the length of GI tract, i.e. the surface of epithelium cells, the vaults of the ileum and colon, the mucus gel covering the epithelium, and the lumen of the intestine (Freter, 1992). Several factors such as high-stress, processed food and eating habits of modern-day living can influence gastrointestinal microbiota possibly via changing the microhabitats (Roderick *et al.*, 1999). In addition, the balance between beneficial and harmful microorganism (gut homeostasis) can be disturbed by factors including chronic illness, immune suppression, and the use of broad-spectrum antibiotics against infection (June and Sarkis, 2009).

2.3 Probiotics

2.3.1 Introduction

Probiotic foods, including dairy products, have been classically defined as ‘foods containing live micro-organisms believed to actively enhance health by improving the balance of micro-flora in the gut’ (Fuller, 1992). Currently, they are defined as ‘microbial cells preparations or components of microbial cells that have a beneficial effect on health and well being of the host’ (Gardiner *et al*, 2002). In this regard, a probiotic yogurt is a type of milk product that contains probiotic bacteria as health promoting components.

A number of genera of bacteria (and yeast) are used as probiotics, including *Lactobacillus, Leuconostoc, Pediococcus, Bifidobacterium* and *Enterococcus*. Probiotics from of the genera *Lactobacillus* and *Bifidobacterium* have a long and safe history in the produce of dairy products and these bacteria are in fact isolated from the gastrointestinal microflora (D'Ammimo *et al.,* 2007). Probiotic bacteria with desirable properties and well-documented clinical effects include *L. johnsonii La1*, *L. rhamnosus GG* (ATCC 53103), *L. casei Shirota, L. acidophilus NCFB* 1478, *B. animalis Bb12* and *L. reuteri* (Shah, 2004 and 2007).

To provide health benefits, it is important that dairy products with health claims meet the criterion of a minimum concentration of probiotic bacteria of 10^6 recommended colony forming units (CFU) ml^{-1} or g^{-1} at the expiry date, which ensures the supply of minimum therapeutic dose of 10^8-10^9 cfu ml^{-1} (Kailasapathy *et al.,* 2007).

2.3.2 Therapeutic value of probiotics

The consumption of probiotic products is helpful in keeping good health, and in combating intestinal disorders and other diseases (Metchnikoff, 2004). Oral administration of *Lactobacillus* and *Bifidobacterium* are able to restore the normal balance of microbial populations in the intestine (Shah, 2006) and provide therapeutic and beneficial effects (see Table 2.1).

Table 2.1 Claimed therapeutic and beneficial effects of probiotic bacteria in human (Fuller, 1989; Shah, 2007)

Maintenance of normal intestinal microflora
Reduction on lactose intolerance
Reduction of serum cholesterol
Stimulation of the immune system
Anticarcinogenic and antimutagenic activity
Enhancement of nutritional value of foods
Controlling gastrointestinal infections (e.g. diarrhea)
Improvement in inflammatory bowel disease
Suppression of *Helicobacter pylori* infection
Prevention of urogenital infection

2.3.2.1 Improvement of lactose metabolism

One of the health benefits of probiotic organism is to relief the symptoms of lactose malabsorption. The failure to digest lactose adequately by some people is because of the deficiency in intestinal *ß*-galactosidase production (Voet, 2004), which is important in the hydrolysis of lactose into its component monosaccharides, i.e. glucose and galactose. Lactic acid bacteria used as starter culture in milk and fermentation, such as *L. acidophilus* and *B. bifidum* produce *ß-D*-galactosidase, which resulted in reducing lactose intolerance in people who lack this enzyme (Analie and Bennie, 2001).

2.3.2.2 Reduction in serum cholesterol level

The elevated level of serum cholesterol is a major factor for cardiovascular disease. The consumption of fermented milk was reported to significantly reduce serum cholesterol (Ouwehand *et al*., 2002; Liong and Shah, 2006). Probiotic bacteria are able to de-conjugate bile salts which does not absorb lipid as readily as its conjugated counterpart, which lead to a reduction in cholesterol level (Shah, 2007). *L. acidophilus* may also consume cholesterol during growth and this makes it less available for absorption into the blood stream (Klaver & Meer, 1993).

2.2.2.3 Immune system stimulation

Probiotics could indirectly influence the body's immune function by altering the activity of the intestinal microflora (Marteau *et al*., 1997). Probiotic cultures produce interferon and stimulate cytokines as represented by TNF-α (tumor necrosis factor) and IL-6 and IL-10 (interleukines 6 or 10; Morita et al., 2002). Manipulation of immune system by *L. acidophilus* and *Bifidobacterium*, in particular IgA levels and non-specific immunity has been studied (Robert *et al.,* 2007). Ingestion of bio-yogurt stimulates and enhances cytokine production in blood cells (MacFarlane and Cummings, 2002).

2.3.2.4 Anticarcinogenic and antimutagenic activity

L. acidophilus and *Bifidobacterium* ssp. may reduce certain enzymes level (e.g. ß-glucuronidase, azoreductase, and nitroreductase), responsible for procarcinogens activation and accordingly decrease the risk of tumor development (Yoon *et al*., 2000). Short chain fatty acids produced by *L. acidophilus* and *Bifidobacterium, L. plantarum* and *L. rhamnosus* are also reported to inhibit the production of carcinogenic products by reducing enzyme activities (Cenci *et al*., 2002).

The anti-carcinogenic effect of probiotic organism against mutagens was suggested a culminated effects of blocking the adhesion site, production of discouraging materials and competition for nutrients (Orrhage *et al.,* 1994). Probiotic bacteria also reduce faecal enzymatic activities including ß-glucuronidase, azoreductase and nitroreductase, which are involved in activation of mutagens (Goldin and Gorbach, 1984).

2.3.2.5 Controlling gastrointestinal infections

Probiotic strains can prevent commonly known food borne pathogens and traveler's diarrhea (Lim *et al.,* 1993; Hilton *et al.,* 1997) caused by bacteria, especially the enterotoxigenic *E. coli*. This is made possible by the production of antimicrobial substances such as bacteriocins, organic acids, hydrogen peroxide and short-chain fatty acids (Gillore *et al.,* 2008). In addition to competition for nutrients and occupying attachment sites, probiotics make harsh antimicrobial microenvironment for pathogenic bacteria (Maire *et al.,* 2006).

2.3.2.6 Suppression of *Helicobacter pylori* infection

Lactobacillus preparations may be useful as adjunct to the treatment and prevention of gastritis because *Lactobacilli* are acid-tolerant and able to persist in the stomach longer than other bacteria (Dionyssios *et al.,* 2005). Probiotics cannot eliminate *H. pylori* from stomach but can reduce the severity of the infection in human (De Vrese, and Schrezenmeir, 2002). *Lactobacilli* as comensal bacteria in normal stomach vary between the ranges of 0 to 10^3/ml (Gotteland *et al.,* 2006). Kabir *et al.,* (1997) found that *L. acidophilus* had a strong inhibitory effect on the colonization of *H. pylori* to the epithelial lining cells. Inhibition of *H. pylori* growth occurred due to production of organic acid by predominant *L. acidophilus* (Wang *et al.,* 2004). *L. casei Shirota* together with *L. acidophilus* inhibits the growth of *H. pylori* (Cats *et al.,* 2003). Supplementing yogurt with *Lactobacillus acidophilus* and *Bifidobacterium* could improve the eradication rate of *H. pylori* (Sheu *et al.,* 2002).

2.3.3 Important criteria for probiotic lactic acid bacteria

The use of various strains of lactic acid bacteria as probiotics in foods or dietary supplements is gaining interest as a mean to improve the health of humans. Vitamin production is one of the numbers of functional characteristics associated with probiotic bacteria and gastrointestinal microbiota (Holzapfel and Schilinger, 2002). For instance, LAB and *Bifidobacterium* ssp. have been reported to produce vitamins such as folate, cobalamin, vitamin K_2, riboflavin and thiamine (Tamime, 2005). Therefore, the use of these cultures in food fermentation potentially offers ways not only to improve the nutritional profile of the food but also to deliver microorganisms to the gut, where they can synthesize such vitamins *in vivo*. The desirable properties of probiotic and lactic acid bacteria are summarized in Table 2.2. The basis for selection of probiotic microorganisms can be summarized to safety, functional aspects (survival, adherence,

colonization, antimicrobial production, immune stimulation, and prevention of pathogens) and technological aspects (growth in milk or other food base, sensory properties, stability, and viability) (Salemin *et al.*, 1996; Tannock, 1997).

Table 2. 2 Desirable properties of probiotic lactic acid bacteria (Adopted from Salemin *et al.*, 1996; Tannock, 1997)

Probiotic strain characteristic	**Functional and technological properties**
Human origin	Species-dependent health effects and maintained viability; applicability to fermented foods
Acid and bile stability	Survival in the intestine
Adherence to human intestinal cells	Competitive exclusion of pathogens: immune modulation
Colonization of the human intestinal tract	Multiplication in the intestinal tract at least temporarily, immune modulation
Production of antimicrobial substances	Pathogen inactivation in the intestine, normalization of gut flora
Antagonism against pathogenic bacteria	Prevention of dental decay, prevention of pathogen adhesion
Safety in food and clinical use	Accurate strain identification, documented safety
Clinically validated and documented health effects	Dose-response data to minimum effective dosage in different products
Shelf life and stability during processing and storage	All of the above properties should be maintained during processing and storage, especially adherence, antimicrobial activity, anticarcinogenic properties

2.4. Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) have a long history of application in fermented foods because of their beneficial influence on nutritional, organoleptic and shelf-life characteristics (Wood and Holzapfel, 1995; Leroy and De Vuyst, 2004). LAB are a group of Gram-positive bacteria, which cause rapid acidification of the raw material through the production of organic acids, mostly lactic acid. Their production of acetic acid, ethanol, aroma compounds, bacteriocins, polysaccharides and several enzymes are

of prime importance (Giraffa *et al.,* 2010). The genera *Lactobacillus, Leuconostoc, Pediococcus,* and *Streptococcus* are the core of LAB group (Vandenbergh, 1993). LAB and their probio-active cellular substances exert numerous beneficial effects in the gastrointestinal tract. LAB prevents adherence, establishment and replication of several enteric mucosal pathogens through several antimicrobial mechanisms (Naidu *et al.,* 1999). LAB also releases various enzymes into the intestinal lumen and exerts potential synergistic effects on digestion and alleviates symptoms of intestinal malabsoption (Kumar *et al.,* 2010). Consumption of LAB fermented dairy products may elicit antitumor effects. These effects are attributed to the inhibition of mutagenic activity; decrease in several enzymes implicated in the generation of carcinogens, mutagens, or tumor-promoting agents and suppression of tumors (Hammes and Tichaczec, 1994; Kumar *et al.,* 2010). Specific cellular components in LAB strains seem to induce strong adjuvant effects including modulation of cell-mediated immune responses, activation of reticulo-endothelial system, augmentation of cytokine pathways and regulation of interleukins, and tumor necrosis factors (Naidu *et al.,* 1999).

2.4.1 Antibacterial components from lactic acid bacteria

Fermentation reduces the amount of available carbohydrates and results in a range of small molecular mass organic molecules that show antimicrobial activity, the most common being lactic, acetic, and propionic acid (Ouwehand and Vesterlund, 2004). Except for the production of these inhibitory primary metabolites, many other antimicrobial components can be formed by different lactic acid bacteria (Savadogo *et al.,* 2004).

2.4.1.1 Organic acids

Lactic acid bacteria produce over 90% of lactic and acetic acid. Of the two acids, acetic acid is the stronger inhibitor and has a wide range of inhibitory activity, inhibiting yeasts, molds, and bacteria (Ouwehand and Vesterlund, 2004). Lowering of pH due to lactic acid or acetic acid produced by these bacteria in the gut has a bacteriocidal or bacteriostatic effect.

2.4.1.2 Hydrogen peroxide

In the presence of oxygen, lactic acid bacteria are able to generate hydrogen peroxide (H_2O_2) through the action of flavoprotein-containing oxidases, NADH oxidases, and superoxide dismutase (Ouwehand and Vesterlund, 2004). The bactericidal effect of hydrogen peroxide is because of its strong oxidizing effect on the bacterial cell; sulfhydryl groups of cell proteins and membrane lipids can be oxidized (Lindgren and Dobrogosz, 1990).

2.4.1.3 Carbon dioxide

Carbon dioxide has a dual antimicrobial effect by virtue of its intrinsic antimicrobial activity and creation of an anaerobic environment during CO_2 formation. At low concentrations carbon dioxide can encourage the growth of some organisms, whereas at higher concentrations it inhibits (Roland *et al.*, 2002).

2.4.1.4 Bacteriocins

The term “bacteriocin” introduced by Jacob *et al.* (1953) refers to protein antibiotics of relative high molecular weight, which act against the same or closely related species by adsorption to receptors on the target cells. Bacteriocins have potential anti *H. pylori* activity. They are small, heat resistant and dialyzable peptidic structures

with antimicrobial activities, which are synthesized by several bacterial species including lactic acid bacteria (Kim *et al*, 2003). Bacteriocin production in LAB is associated to its growth and mainly occurs throughout the growth phase and finishes at the end of the growth (Neysens *et al.*, 2003). Production of bacteriocins can be affected by type and level of the carbon, nitrogen and phosphate sources, cations surfactants and inhibitors (Savadogo *et al.*, 2006).

2.4.1.5 Adhesion inhibitors

Adhesion to a surface is important to bacteria in most environments. It enables them to colonize environments under conditions where they otherwise would be washed away. Due to secretion of fluids and peristaltic movements *bifidobacteria* are able to block the adhesion and invasion of many pathogens (Coconnier *et al.*, 1993; Bernet et *al.*, 1994; Aissi *et al.*, 2001). Thus consumption of probiotics especially *Bifidobacteria* and *Lactobacilli* are found to suppress the colonization of the gut by pathogenic bacteria (Wang *et al.*, 2004).

2.5 Herbs

2.5.1 Introduction

Spices and herbs have been used for thousands of years in many parts of the world to enhance the flavor and aroma of foods. Being antioxidants and anti-inflammatory agents, spices thus have profound antibacterial and antimicrobial properties (Lai and Roy, 2004). Spices and herbs have been added to foods since early times, not only as flavoring agents, but also for their therapeutic or medicinal value and food preservatives (Cutler, 1995). Herbal plants contain phytochemicals, which is variety of chemical substances that act upon the body. Certain spices and herbs prolong

the storage life of foods by preventing rancidity through their antioxidant activity or through bacteriostatic or bactericidal activity (Shana *et al.*, 2007).

Antimicrobial activities of plant extracts are known to act against some types of human pathogens, and food borne pathogens (Hara-Kudo *et al.*, 2004; Brandi *et al.*, 2006). Spices contain antioxidant vitamins, ascorbic acid (vitamin C) and tocopherols (vitamin E) in addition to other, very potent antioxidants, such as phenols, thiols (as sulphur compounds) and carotenoids (Yang *et al.*, 2004; Sharma *et al.*, 2005). The phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo *et al.*, 2004) and contribute to their antioxidant and pharmaceutical properties (Cai *et al.*, 2004; Shan *et al.*, 2005; Wu *et al.*, 2006).

2.5.2 *Cinnamon zeylanicum*

Figure 2.1 *Cinnamon zeylanicum* Bark

The name cinnamon (*Cinnamon zeylanicum)* is derived from a Greek word meaning “sweet wood”. Cinnamon used in food preparation is derived from the inner bark of the cinnamon tree, an evergreen tree of the family *Lauraceae*. Cinnamon has a long history both as a spice and as a medicine. In addition of being famous for its aroma and flavor, cinnamon is an excellent source of manganese and a very good source of dietary fiber, calcium and iron (Wood, 1988).

The antimicrobial activity of cinnamon has been studied extensively (Friedman *et al.,* 2002). Among the spices and herbs that Zaika *et al.* (1988) studied, cinnamon had the strongest antimicrobial activity. The main antimicrobial components of bark extract of cinnamon are eugenol and cinnamaldehyde (Friedman *et al.,* 2002). Both compounds are active against many pathogenic bacteria (Suresh, 1992; Shashidar, 2002) and viruses (Pacheco *et al.,* 1993). Phytochemicals in cinnamon, called chalcone polymers can increase glucose metabolism in the cells by 20 times or more and are powerful antioxidants. Cinnamon also contains flavonoid called anthocyanins, which improve capillary function and act as antioxidants (Wilkonson, 2008).

2.5.2.1 Medicinal value of cinnamon

Cinnamon was used widely as an ingredient of medicines for sore throats and coughs in medieval time. Cinnamon is effective to overcome gastrointestinal disorders such as indigestion, stomach cramps, intestinal spasms, nausea, and treat diarrhea (Charles, 1998). The cinnamon anti-inflammatory, anti-spasmodic and anti-clotting properties are attributed to its cinnamaldehyde components (Puangpronpitag and Sittiwet, 2009). Cinnamon extracts are active against *Candida albicans*, which is responsible for vaginal yeast infection, and also *Helicobacter pylori*, the bacterium responsible for stomach ulcers (Tabak *et al.,* 1999). Cinnamaldehyde also inhibited nitric oxide production, which has been implicated in the inflammatory disease process (Lee *et al.,* 2005). Crude cinnamon extracts also inhibited the growth of cultured tumor cells and this effect was suggested to be due to procyanidins and eugenol in the bark extract (Shahidar, 2002). Cinnamon is also useful as a food preservative to inhibit the growth of common food-borne bacteria such as *Salmonella* and *E. coli* (Suresh, 1992). Inclusion of cinnamon in the diet of patients with Type-2 diabetes has been shown to reduce risk factors associated with diabetes (Khan *et al.,* 2003; Anderson, 2008; Baker,

2008). In particular, a 40-day study involving 60 people with type 2 diabetes showed that cinnamon reduced blood sugar levels (18-29%), triglycerides (23-30%), total cholesterol (12-26%) and LDL cholesterol (7-27%) after daily consumption of 1-6 g cinnamon (Diabetes care, 2003).

2.5.3 Glycyrrhiza glabra

Figure 2.2 Licorice Plant, Root and Fruit

Licorice is the dry root of *Glycyrrhiza glabra* from family of *Fabaceae*. Licorice is a hardy herb or under plant, which grows to about 2m in height. The roots are long, cylindrical, thick and multi-branched (Langmead and Rampton, 2001) and have very sweet, moist and soothing properties. It was widely used to cover the unpleasant flavor of other medications (Jose *et al.,* 2010). The first report of medicinal use comes from Greeks, who recommended it for the treatment of gastric and peptic ulcers, but licorice root has been used in both Eastern and Western medicine to treat a variety of illnesses ranging from the common cold to liver disease (Langmead and Rampton, 2001). Chemical analyses revealed the presence of a wide variety of bioactive phenolic components in licorice and these have attracted attention as a potential source of chemical leads (Hibata, 2000). Licorice root contains a compound called "anethole", an aromatic, unsaturated ether compound that is also found in anise, fennel and other herbs.

The major constituent of licorice is glycyrrhizin a compound that is about 50 times sweeter than sugar (Bensky, 2004) and flavonoids such as liquiritin, isoliquiritin and their aglycones. These phytochemicals are perceived as the active principles responsible for its pharmacological efficacy (Zhang and Ye, 2009).

2.5.3.1 Medicinal value of licorice

Licorice is used as a medicine and dietary supplement since it has a wide-ranging therapeutic property. These include relief of rheumatic, bronchitis and sore throat, as well as the source of anti-ulcer medicines for treatment of peptic ulcer (Kim *et al.,* 2000). Licorice is the most used herb in Kampo medicines (traditional Chinese medicines) and for cough and cold in addition to relieving the symptoms of eczema such as itching, swelling and redness (Olukoga and Donaldson, 2000). Other uses of the plant include the treatment of sex-hormone imbalances and menopausal symptoms in women (Yan Wu and Fisher, 1997). Licorice root contains various active compounds of which, glabridin and glabrene (components of *Glycyrrhiza glabra*) are thought having the most medicinal potential (Fukai *et al.,* 2002). Glycyrrhizin has anti-inflammatory as it inhibits two enzymes, lipoxygenase and cyclooxygenase that promote inflammation (Isbrucker and Burdock, 2006). Glycyrrhizin also exhibited inhibitory activity against *H. pylori* growth *in vitro* (Hibata, 2000).

Polyphenols components in licorice have antioxidants activities and these may protect cells against damage caused by free radicals (Fuhrman *et al.,* 1997). The antioxidants in licorice may even have the potential to kill cancer cells (Isbrucker and Burdock, 2006).

2.5.4 Allium sativum

Figure 2.3 *Allium Sativum* (Garlic)

Garlic (*Allium sativum*) belongs to *Liliaceae* family and is widely used with a long medicinal history (Ali *et al.,* 2000). Garlic is high in flavonols and organosulfur compounds with antioxidant, anti-inflammatory and antimicrobial properties. After chopping or crushing the garlic, allinase enzyme converts alliin (a cysteine-sulphoxide) in garlic to allicin (allyl 2-propene thiosulfinate) (Banerjee *et al.,* 2003; Benkeblia, 2004), which are responsible for many of garlic's medicinal effects. This compound may also be metabolized to a number of additional organosulphur compounds (Khanum *et al.,* 2004). Allicin is the main active antimicrobial components of garlic (Stoll and Seebeck, 1951). The antibacterial properties of garlic can be eliminated by inhibition of the allinase enzyme and prevention of allicin formation (Wilson and Adams, 2007). The antibacterial effect of garlic is due to the interaction of sulphur compounds (e.g. allicin) with sulphur (thiol) groups of microbial enzymes (such as trypsin and other proteases), leading to an inhibition of microbial growth (Jonkers *et al.,* 1999; Bakri and Douglas, 2005). Many Gram-positive and Gram-negative bacteria can be inhibited by garlic, and some strains were inhibited much more strongly by allicin or garlic extract compared to antibiotics (Lai and Roy, 2004; Bakri and Douglas, 2005). In addition, garlic can exert a differential inhibition between useful intestinal microflora and harmful enterobacteria (Ruiza *et al.*, 2010).

2.5.4.1 Medicinal value of garlic

Garlic has a wide range of actions including antibacterial, antiviral, antifungal and antiprotozoal (Harris *et al.,* 2001). In addition garlic also has positive effects on the cardiovascular and immune systems. Thus it is not surprising since garlic has been used for centuries in various cultures to battle infectious disease (Lanzotti, 2006). The anticarcinogenic effects of garlic may well originate from the organosulfur compounds responsible for its scent and flavor (Wargovich, 2006). Garlic with the high content of flavanols, mainly kaempferol, was suggested crucial in the detoxification of carcinogenic compounds (Bilyk and Sapers, 1985).

Anti *H. pylori* effects of aqueous garlic extract (as low as 40 µg/mL) has been demonstrated (Cellini *et al.,* 1996; Sivam *et al.,* 1997; Jonkers *et al,* 1999). Direct intragastric effects of garlic may also be possible because garlic antimicrobial activity was shown not only unaffected by the acid environments but also may be enhanced by the gastric juice (Njuma *et al.,* 2009). Epidemiological studies show a negative relationship between stomach cancer, which is strongly correlated with *H. pylori* infection, and the consumption of *Allium* vegetables (Browna *et al.,* 2002).

2.6 *Helicobacter Pylori*

2.6.1 Disease and prevalence

Helicobacter pylori is associated with chronic gastritis (Kashiwagi, 2003; Weck *et al.,* 2009), peptic ulceration (Blaser, 1993), gastric malignancy (Weck *et al.,* 2009), and possibly a risk factor for coronary heart and cardiovascular diseases (Redeen *et al.,* 2009). Warren and Marshal first identified this bacterium in 1983 from endoscopic biopsy specimens of human gastric mucosa. Epidemiological studies have established an up to 6-fold increased risk of developing adenocarcinoma in patients infected with *H.*

pylori and the involvement between *H. pylori* and gastric cancer rank was comparable with association between smoking and lung cancer (Fox and Wang, 2001). *H. pylori* was also classified as a Type I carcinogen for humans by the IARC/WHO (Plummer, 2004). *H. pylori* established a chronic infection, represented by an antrally predominant chronic active gastritis in the majority of infected patients. Many of these infected patients infected with *H. pylori* have regular abdominal symptoms without ulcer disease (McCarthy, 1995). Inflammation of the duodenum often occurs with *H. pylori* infection, and duodenal ulcers develop in as many as 16% of infected individuals. *H pylori* infection was also reported associated with more than 90% of duodenal ulcers and the majority of gastric ulcers (Kenneth and McColl, 2010). In patients with long-standing *H. pylori* infection, persistent inflammation can lead to chronic atrophic gastritis, which is a recognized indication for gastric ulcer disease and gastric adenocarcinoma (Fox and Wang, 2007).

The s-shaped, microaerophilic, *H. pylori* is a human specific bacterium and is able to invade and lives in the stomach and duodenum (Goodwin and Armstrong, 1990), colonize the gastric epithelial surface and can survive in acidic environment of the stomach, despite the stomach high acidity (Parsonnet *et al.,* 1994). These organisms may also be found transiently in saliva, feces, and areas of gastric metaplasia of the small intestine. The density of *H. pylori* infection correlates with the extent of inflammation in the stomach and in the duodenal bulb (Tytgat, 1994). Some strongest evidence which relate the bacterium to duodenal ulcer showed that the relapse rates after *H. pylori* has been eradicated is much less (2.6–7%) than compared with patients in whom the bacterium is not eradicated (58–67%; Pakodi *et al.*, 2000).

H. pylori infects over half of the world's population and its carriage rates are about 70-90% in developing countries and 25-50% in developed countries (Marshall, 1994; Go, 2002). The prevalence is however unbalanced between rural developing areas

(more than 80%) and urban developed areas (less than 40%). As a result, *H. pylori* cause an enormous burden of morbidity and is the reason for prescription of numerous courses of antibiotics (Malfertheiner, 2000).

2.6.2 Characteristics and growth requirements of the genus *Helicobacter pylori*

The genus *Helicobacter pylori* includes spiral or curved bacilli ranging from 0.3 to 1.0 μm in width and 1.5 to 10.0 μm in length. *Helicobacters* are Gram-negative, non-spore forming rods that may form spheroid or coccoid bodies with prolonged culture (Bode *et al.,* 1993). These bacteria are motile and possess multiple monopolar-sheathed flagella. Some differences between *H. pylori* and the closely related genus Campylobacter are the strong urease activity as well as the presence of a flagella sheath and bulb in *H. pylori* (Kusters *et al.,* 2006).

H. pylori grows between 30˚C and 37˚C but not at lower temperature (25˚C). This organism possesses a respiratory type of metabolism (Dubois, 1995), which means for an optimum growth, *H. pylo*ri requires a humid atmosphere at 37˚C with reduced levels of oxygen (5 to 10%) and increased level of carbon dioxide (5 to 12%). Atmospheric hydrogen (as much as 5 to 10%) may either require or stimulates the growth of the bacterium. The bacterium natural habitat is the acidic gastric mucosa and can survive short exposure to pH of <4, but growth occurs only at the pH range of 5.5 to 8.0, with best growth at neutral pH (Kusters *et al.,* 2006).

Two main requirements for a successful isolation of *H. pylori* are the utilization of fresh media and the maintenance of adequate humidity throughout incubation period (Hachem *et al.,* 1995). Brain heart infusion broth (BHIB) supplemented with 10% horse or sheep blood was reported to be the best medium to maintain the viability of *H. pylori* for 3 days (Sgouras *et al.,* 2004). Isolation of *H. pylori* from samples other than gastric samples was reported to be difficult (Goodman and Correa, 1995). In fact, no *in vivo*

isolation of *H. pylori* from environmental samples such as water and stool specimens using traditional cultures has been reported. The reason of *H. pylori* difficult isolation is due to its exposure to environmental conditions changes in morphology, metabolism, and growth patterns (Kusters *et al.*, 2006).

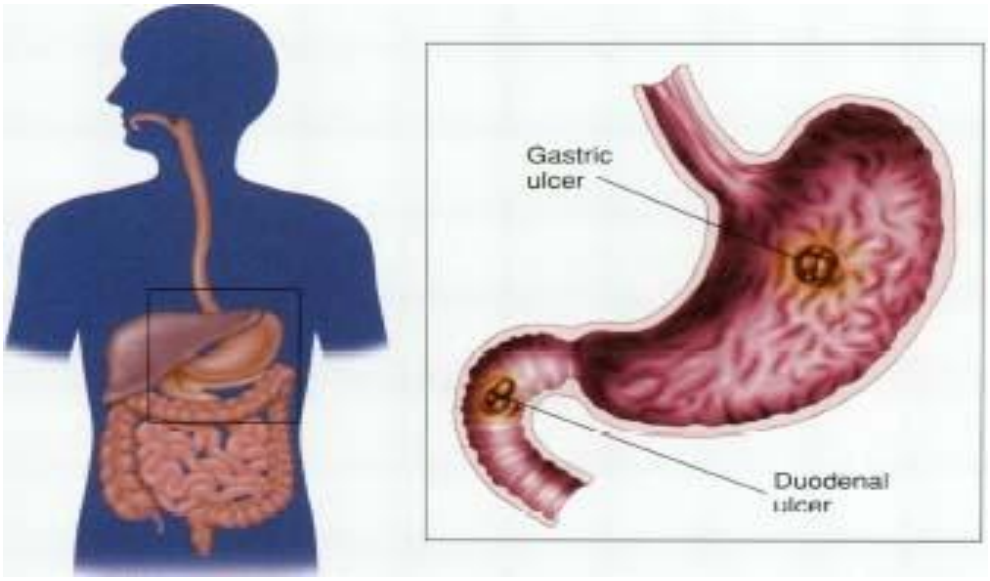

Figure 2.4 Peptic and duodenal ulcers in human.
Peptic ulcers may occur in the stomach (gastric ulcers) or in the first part of the small intestine (duodenal ulcers). Nearly all peptic ulcers are the result of infection with *H. pylori* bacteria.

2.6.3 Mode of transmission

The mode of transmission of *H. pylori* is still unidentified, but foodborne and waterborne pathways have been considered as the epidemiological patterns of the infection. Both fecal-oral and oral-oral modes of interhuman transmission are likely to represent the major routes of spreading of *H. pylori* (McCallion, 1996; Dunn *et al.*, 1997). Fecal-oral transmission is highly possible since the pathogen has been detected in human feces (Nilsson *et al.,* 1996). The lack of hot running water and hygienic drinking water, crowded living conditions (Nouraie *et al.,* 2009), poor social and economic development, poor hygienic practices, and unsanitary prepared food has been associated with an increased chance of *H. pylori* infection (Mendall, 1992). The

improvement of general hygienic conditions reduces the occurrence of the infection (Fujimoto *et al.*, 2007).

Table 2. 3 Biochemical characteristics of genus *H. pylori* (Adopted from Fox and Lee, 1997)

Characteristics	**Reaction**
Catalase production	+
Nitrate reduction	—
Alkaline phosphatase	+
Urease	+
Hippurate hydrolysis	—
Resistancy (R) or suitability (S) to nalidixix acid antibiotic	R
Resistancy or suitability to cephalotin antibiotic	S
Resistancy or suitability to metronidazole antibiotic	S

2.6.3 Mechanism of adhesion to the stomach cells

Helicobacter genus has expanded extremely in the last decade. Both microbial and host factors seem to be responsible for attachment of *H. pylori* to the stomach of infected patients. The genetic diversity of humans, besides genetic of *H. pylori* could provide mixtures of adhesion and receptors (Cover and Blaser, 1996; Dorrell, 1998). *H. pylori* possess about 1600 coding genes, where a supergene family of 32 genes encoding recognized outer membrane proteins (OMPs) (Tomb *et al*., 1997). This remarkable microorganism also expresses lipopolysaccharide (LPS) with unusual properties. The O-antigen of LPS in most *H. pylori* strains expresses the Lewis blood group antigens Lewis X (Le^x), Le^y, H type 1, Le^a, Le^b, i-antigen, sialyl Le^x and blood group A. This limited diversity in O-antigen expression is unusual and recommends a role for specific *H. pylori* Lewis epitopes in pathogenesis. *H. pylori* LPS Lewis antigens play a role in colonization and adhesion but not in autoimmunity (Ilver *et al*., 1998; Ben *et al*., 2000). Virulence factors identified in *H. pylori* for its gastric colonization, tissue damage, and

survival are listed in Table 2.4. Adhesions, urease and flagella are the most essential factors for *H. pylori* colonization to gastric mucosa.

The curved morphology of this bacterium and a right-handed body with a bunch of unipolar flagella in one end cause screw-like movement and enable the bacterium to penetrate the mucin layer and colonize the gastric mucus (Lee, 1996). Amongst the factors involved in *H. pylori* colonization, urease activity and chemotactic motility using flagella are identified to be the most important (Velenzuela *et al.,* 2003).

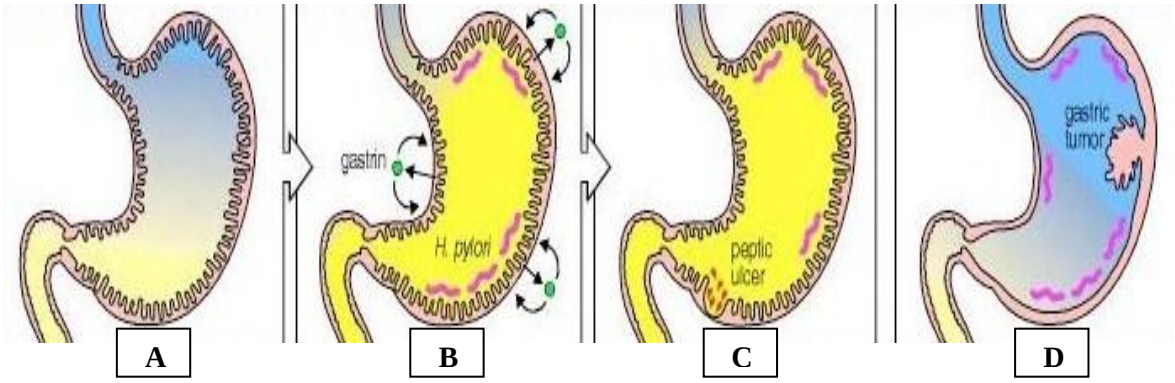

Figure 2.5 Gastric ulcer developments:
A. In normal stomach, acid secretions maintain a low pH to aid food digestion.
B. In response to *H. pylori* infections, gastrin is secreted by G cells and stimulates excess acid production.
C. Continued excess stomach acid causes tissue damage and ulceration**.**
D. A chronic inflammation develops, leading to atrophy of stomach wall and malignant outgrowths.

Urease is one of the key enzymes in *H. pylori* pathogenesis. Urease hydrolyzes urea to ammonia and carbon dioxide, neutralize the acidic microenvironment and increase the pH of the stomach, which is necessary for bacterial survival in the acidic stomach (Perez, 1992). This high level of ammonium ions can be toxic to the gastric epithelial cells and continued production of ammonia cause cell inflammation or death (Lytton *et al.,* 2005).

Motile bacteria with polar flagella swim to chemical attractants and away from repellents. Bacteria sense the concentration of attractant such as amino acids and sugars,

and swim toward them. *H. pylori* also have the ability to sense and move toward urea (ureataxis), bicarbonate ion and sodium ion at the concentration of 1 to 10 mM (Mizote, 1997). Since urea is synthesized in liver, circulated by the blood stream (Neithercut, 1993), and secreted into the gastric juice, a concentration of urea is formed in the gastric mucus layer, and which can be sensed by *H. pylori*. The concentration gradient of urea is kept elevated throughout the infection, since bacterial urease is constantly hydrolyzing urea to produce ammonia and protect the bacteria from attack by gastric acid (Akada *et al.*, 2000).

2.6.4 Therapy

The treatment of *H. pylori* infection seeks to eradicate the bacterium. Eradication of *H. pylori* is considered successful when the tests for organism become negative, one month after completion of the course of antimicrobial treatment. Repeat testing need to be done at least 4 weeks after finishing treatment of the bacterium (Katelaris and Jones, 1997). Triple therapy or combination of antibiotics such as amoxicillin, clarythromicin, bismuth salts and nitroimidazoles (metronidazole or tinidazole) together with a proton-pump inhibitor (e.g. Omeprazol, lanzoprazol) for 1-2 weeks has been shown to be essential to eliminate *H. pylori* from stomach (Taylor and Parsonnet, 1995).

Apart from patients compliance due to the many tablets which they have to ingest, the disadvantages with this regimen lie in being expensive, causing side effects (e.g. vomiting, diarrhea, abdominal pain, etc.) and, the most important, encouraging the development of resistant strains (Dobrilla, 1993). It was reported that antibiotic therapy fails in about 20% of patients (Parente *et al.*, 2003; Kusters *et al.*, 2006) due to antibiotic resistance, especially those already treated with metronidazole. On the other hand, these regimens give cure rates at about >90% of patients (Megraud, 2004). The

increase in antibiotic resistance call for effort to develop low cost, large-scale and search for non-antibiotic substances, which are effective and safe in terms of gastrointestinal protection from *H. pylori* infection.

Table 2. 4 Virulence factors identified in H. pylori (Adopted from *et al.*, 1987 & 1988; Higashi *et a.l*, 2002)

Virulence factor	**Effect**
Colonizing	
Flagella	Active movements through mucin
Urease	Neutralization of acid
Adhesions	Anchoring *H. pylori* to epithelium
Tissue damaging	
Proteolytic enzymes	Glucosulfatase degrades mucin
120-kDa cytotoxin (Gac A)	Related to ulcer and severe gastritis
Vacuolating cytotoxin (Vac A)	Damage of the epithelium
Urease	Toxic effect on epithelial cells, disrupting cell tight junctions
Phospholipase A	Digest phospholipids in cell membranes
Alcohol dehydrogenase	Gastric mucosal injury
Survival	
Intracellular surveillance	Prevent killing in phagocytes
Superoxide dismutase	Prevent phagocytosis and killing
Catalase	Prevent phagocytosis and killing
Coccoid forms	Dormant form
Heat shock proteins	
Urease	Sheathing antigen
Other	
Lipopolysaccharide	Low biological activity
Lewis X/Y blood group homology	Autoimmunity

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Medicinal herbs

Dried stem bark of cinnamon (*Cinnamon zeylanicum*), root of licorice (*Glycyrrhiza glabra)*, and commercially prepared garlic (*Allium sativum*) powder (McCormicK, Australia) were purchased from local supermarket.

3.1.2 Yogurt bacteria and probiotic mixture

Capsules containing *Lactobacillus acidophilus NCFM, L. delbrueckii* ssp. *bulgaricus, L. casei, L. rhamnosus, Bifidobacterium bifidum, B. infantis, and B. longum* (Bio-Life Sdn Bhd, Malaysia) and sachets containing *L. acidophilus LA-5, Bifidobacterium Bb-12, L.casei LC-10, and Streptococcus thermophilus Th-4* (Nn Yogurt Mix, Malaysia) were used in the preparation of starter culture.

3.2 Methods

3.2.1 Herbs

3.2.1.1 Preparation of water extract of herbs

The plant materials were oven dried at 45˚C followed by grinding using a kitchen blender. Each powdered herbs (10g) were soaked in 100ml of distilled water, and was left over-night in a warm (70˚C) water bath under constant shaking (50 rpm). The suspension was then centrifuged (2000 rpm, 15 min, at 4˚C) and the supernatants collected and sterilized through 0.22μm filter (Sartorius, Germany).

3.2.2 Yogurt

3.2.2.1 Preparation of starter culture

Fresh and pasteurized low fat milk (Dutch Lady) was pre-heated to 41˚C. The contents of a capsule of Bio-Life and a sachet of Nn Yogurt Mix were poured into the pre-heated milk and the bacteria-milk mixture was mixed thoroughly. The mixture was incubated for 12 h at 41˚C (Amirdivani and Baba, 2011). The yogurt formed was kept refrigerated at 4°C and used within 24 hours as starter culture for preparing yogurt.

3.2.2.2 Herbal yogurt preparation

Starter culture (5g) and herbal water extract (60ml; see section 3.2.1.1) were added into homogenized and pasteurized low fat (1.5% fat) milk (1L). The fat content was corrected by adding 2% (w/v) skim milk powder (Amirdivani and Baba, 2011). The mixture was aliquoted into 100ml plastic cups. Incubation was carried out at 41˚C and pH was read at predetermined incubation times. The fermentation was terminated when the pH of yogurt reached 4.5.

3.2.2.3 Time Interval specifications

The 0 hour time represents the moment that mixtures of starter culture-milk with or without herbal water extract were placed in the water bath (41˚C). The 0 day yogurt represents yogurts after overnight refrigeration, whereas, days 7, 14, 21 and 28 represent yogurt after 7, 14, 21 and 28 days of refrigerated storage respectively.

3.2.3 Measurement of pH and Titratable Acidity (TA)

Changes of pH and TA were monitored every hour during fermentation and once a week during refrigerated storage. Samples of yogurt (1g) were mixed with distilled water (1ml) and the pH of the mixture was determined using pH meter (Mettler-Toledo 320, Shanghai). The pH meter was routinely calibrated with standard buffers pH 4.0 and 7.0.

The TA of the yogurt was determined by titrating mixture of 1g yogurt and 9ml distilled water with 0.1*N* NaOH using a 0.1% phenolphthalein (3-5 drops) as color indicator. NaOH was added slowly with constant swirling of the yogurt sample until the indicator changed to a constant pink color. The amount of acid produced during fermentation was calculated as follows:

Lactic Acid (%) = Dilution factor (10) x V NaOH x 0.1N x 0.009 x 100%

where V is volume of NaOH required to neutralize the acid.

3.2.4 Preparation of yogurt water extract

Yogurt samples (10ml) were homogenized with 2.5ml of dH_2O, and the homogenates were acidified to pH 4.0 using 1M HCl followed by incubation at 45 ˚C for 10 minutes. The homogenates were then centrifuged (4˚C, 10000 rpm; 10 min) and the supernatants were harvested and then neutralized to pH 7.0 by the addition of 0.5M NaOH. The neutralized supernatants were centrifuged (1000 rpm; 10 min; 4˚C) in order to remove precipitated proteins. The clear supernatants (yogurt water extracts) were harvested and used for further analysis.

3.2.5 Measurement of 1, 1-diphenyl-2-pycrylhydrazyl (DPPH)

Each herbal-yogurt water extracts (250µl) was added to 3ml of 60µM DPPH in ethanol and the decline in absorbance was measured at 517 nm. The absorbance readings were compared with the controls, which contained 250µl of dH_2O instead of the yogurt water extracts (Shetty *et. al*, 2007). The antioxidant activity inhibition was calculated as follows:

$$\%inhibition = \frac{[A_{517}control\ \_\ A_{517}extract]}{[A_{517}control]} \times 100$$

3.2.6 Organoleptic assessment

Sensory evaluation of yogurts was carried out after 1 day of refrigerated storage at 4˚C by an assessment team consisting of 12 untrained panelists (mean age was 25). The samples were placed in uniform disposable plastic cups. The sample size was large enough (~5g) so that panelists could re-taste the products if they desired. The panelists were asked to evaluate appearance, flavor, texture, aroma, sweetness, sourness and overall taste of the samples. Scoring was performed on a hedonic scale of 1-10 ranging from 1 for "dislike extremely" to 10 for "like extremely" (Larmond, 1987). Two commercial yogurts (strawberry and kiwi flavored) were used for comparison.

3.2.7 Enumeration of viable cell (CFU) in yogurt

3.2.7.1 *Lactobacillus* ssp.

Enumeration of *Lactobacilli* species was carried out using pour plate method. Yogurt samples (1ml) were aseptically mixed with 9ml of 0.15% sterile buffered peptone water (Oxoid, UK). The sample was thoroughly stirred and the mixture was serially repeated (1:10 dilution) 6 times using peptone water (Oxoid, UK) as the diluents. Empty petri dishes were inoculated with 1ml of diluted yogurt, followed by the

addition of 15ml autoclaved melted (45˚C) MRS agar into the petri dishes. The contents were mixed thoroughly by gentle tilting and swirling the dishes. The plates were then inverted once the agar has solidified and the dishes were incubated anaerobically (Revco Ultima) at 37˚C for 24-48 hours (Kailasapathy *et. al*, 2007).

3.2.7.2 *Streptococcus thermophilus*

Streptococci were enumerated by spread count method. Melted autoclaved (45˚C) M17 agar (Oxoid, UK) was initially placed into a petri dish followed by cooling of agar to room temperature to allow solidification. The agar was then inoculated by spreading the surface evenly with 0.1ml of diluted yogurt with a sterile glass hockey shaped spreader. The colonies formed (colony forming unit, cfu) were counted after 24-48 hour incubation aerobically at 37˚C. Viable microbial count was calculated as follows:

$$\text{cfu/ml} = (\text{cfu/plate}) \times \text{dilution factor}$$

3.2.7.3 Observing yogurt viable cells by Scanning Electron Microscopy (SEM)

Lactobacillus ssp. and *Streptococcus thermophilus* were observed with scanning electron microscopy (SEM) method. Preparation of the sample was carried out by spreading the pure culture of *Lactobacillus* ssp. and *S. thermophilus* on the glass slide separately and stained the surface of smear by Gram-staining method (see appendix 2, section 2.9). The heat-fix sample was put into the vacuum chamber and it was subsequently covered with a thin coating of metal to increase electrical conductivity. The microbes were observed using the magnification of x4900 and x11800 (Figure 4.8).

3.2.8 *Helicobacter pylori* bacteria

3.2.8.1 Bacterial isolation

In the present study, 3 clinical isolates of *H. pylori* from stomach biopsies were obtained from the Endoscopic Unit of University of Malaya Medical Centre, Kuala Lumpur, Malaysia. Bacterial cells were identified according to colony morphology, Gram staining characteristics, microaerophilic growth (at 37°C) and oxidase, catalase, and urease assays (Goodwin and Armstrong, 1990). The growth of *H. pylori* was maintained under microaerophilic conditions in anaerobic jars with CampyPakPlus (MGC Anaeropack, Microaero) at 37°C for 3–5 days.

Oxidase test

Paper disk was impregnated with oxidase reagent. The *H. pylori* colonies were smeared on the paper disk with sterile applicator. Development of blue color at the inoculation site within10 seconds was taken as positive.

Catalase test

Sample of *H. pylori* was transferred using a sterile applicator from the center of the colony to the surface of a glass slide and one drop of 3% hydrogen peroxide was added. Formation of gas bubbles was considered as positive.

Urease test

Fresh *H. pylori* colonies were placed in 0.5ml Christensens's urea broth using a sterile applicator. The broth was then incubated at 37°C. The tube was examined after 30 minutes and a color change from yellow to pink was taken as positive.

3.2.8.2 Preservation of strains

Colonies from pure culture of *H. pylori* isolated on blood agar (Oxoid, UK) were suspended in 1ml of brain heart infusion broth (BHIB) (Oxoid, UK) containing 15% (v/v) glycerol. The vials were screw-capped and then stored in a -70º C freezer until required for further testing.

3.2.9 Measurement of *H. pylori* growth: Disk Diffusion Method (DDM)

Growth inhibition was performed by the filter paper disk diffusion method (Zaika, 1988) on Columbia agar supplemented with 5% sheep blood (BML, Malaysia). Each aqueous plant extracts (25 µl) and freeze-dried herbal-yogurt extracts (0.04 g/µl) from day 0, 7 and 14 of refrigerated yogurt were placed on standard 6 mm disks (Whatman, UK). The disks were then placed on Columbia sheep blood agar plate (maximum 5 disk onto the surface of the agar), which had been inoculated earlier with 0.1ml *H. pylori* suspension at the density equivalent to McFarland no. 3 (10^{-8}-10^{-9} cfu/ml) in brain heart infusion broth (BHIB). The growth was maintained under microaerophilic conditions in an anaerobic jar with a gas generator system (MGC Anaeropack, Microaero, Japan) at 37˚C for 3-5 days (Tabak *et al.,* 1996; Vale and Vitor, 2007). The agar plates were then examined and the diameter of the zone of inhibition measured using a metric ruler.

3.2.10 Measurement of *H. pylori* growth: Minimum Inhibitory Concentration (MIC) test

The minimum inhibitory concentration (MIC) was the lowest concentration of each extracts, which completely inhibited visible bacterial growth at 37˚C after 72 h incubation. This was determined by initially mixing various volumes (0.25-3 ml) of herbal-yogurt extracts or plant extracts with heated (50˚C) Mueller Hinton (MH) blood

agar supplemented with aged (>2 week old) defibrinated sheep blood (5% v/v), prior to the inoculation with *H. pylori* suspension (Barer, 1988). The MIC was determined by identifying the lowest concentration of the extracts in the plate with no visible bacterial growth. The growth of each plate was compared with positive control plates.

3.2.10.1 Inoculum preparation

The inoculum is prepared using direct colony suspension method. At least 5-10 isolated colonies from a 72 hours- old *H. pylori* blood agar plate subculture were touched with a flame-sterilized wire loop and transferred to individual tubes containing 2-3ml buffered peptone water. The turbidity of the solution was adjusted to McFarland no.2 (10^7 to 10^8 cfu/ml) using McFarland machine (BioMérieux, France).

3.2.10.2 Inoculation of agar plates

The surface of the MH agar containing herbal-yogurt and herbal extract (see section 3.2.10) should be in a dry condition prior to inoculation. The *H. pylori* inoculums (1-3µl per spot) (see section 3.2.10.1) were applied directly onto the agar plates using a pipette (Eppendorf®, Canada). The agar plates were left at room temperature until the inoculums were absorbed into the agar and the surface of agar has dried. The agar plates were incubated in inverted position at 35º C for 72 hours in microaerobic atmosphere produced by a gas generating system (MGC Anaeropack, Microaero, Japan). Growth control plates which contain no plant extracts or no herbal-yogurt extracts were then included in each experiment as positive control. The respective dilution plates were then checked for in the order from lowest to the highest concentration.

3.3 Statistical analysis

Data were collected from triplicate batches of yogurt. Each yogurt batch was refrigerated up to 28 days and the samples for analysis were taken weekly (i.e. on day 0, 7, 14, 21 and 28). Data were analyzed using one-way ANOVA by SPSS®, version 15.0 and Microsoft® Excel 2007. Means were compared using Duncan's multiple range tests among the days, and a significant deviation was recognized by ANOVA at $p<0.05$.

CHAPTER 4: RESULTS

4.1 Effect of herbal yogurt on the changes of pH

The changes of pH in the plain- and herbal-yogurts during fermentation and storage at 4˚C are shown in Figures 4.1 and 4.2. A decline in pH occurred during fermentation and 28 days of refrigeration for all types of yogurt. The presence of garlic in yogurt lowered the pH compared to plain-yogurt throughout the storage period but the effect was not significant (*p>0.05)*.

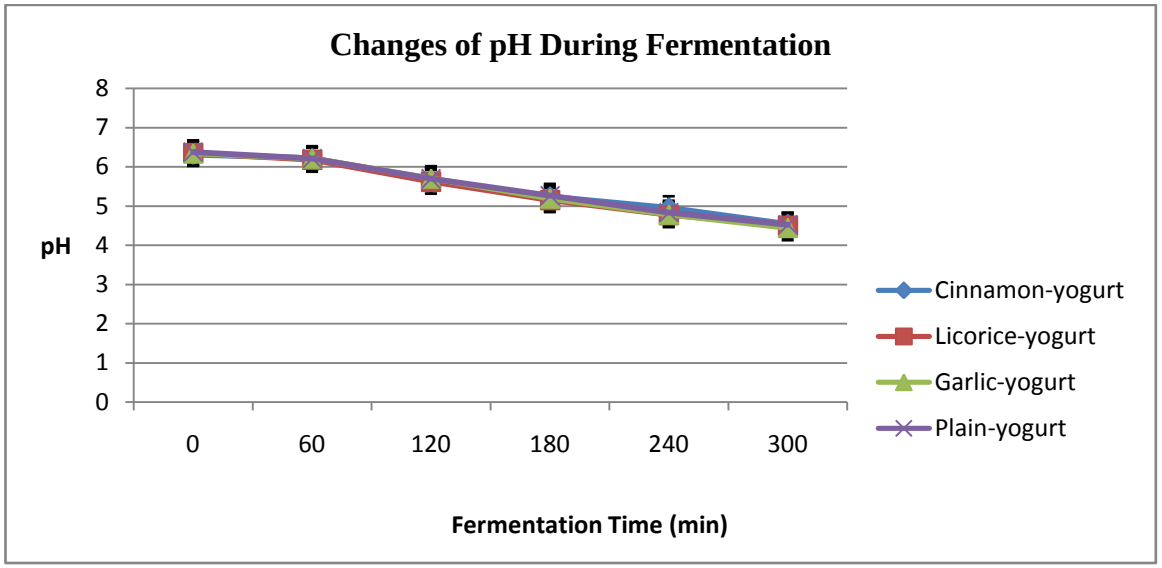

Figure 4. 1 Effects of herbs on the changes of pH in yogurt during fermentation at 41˚C

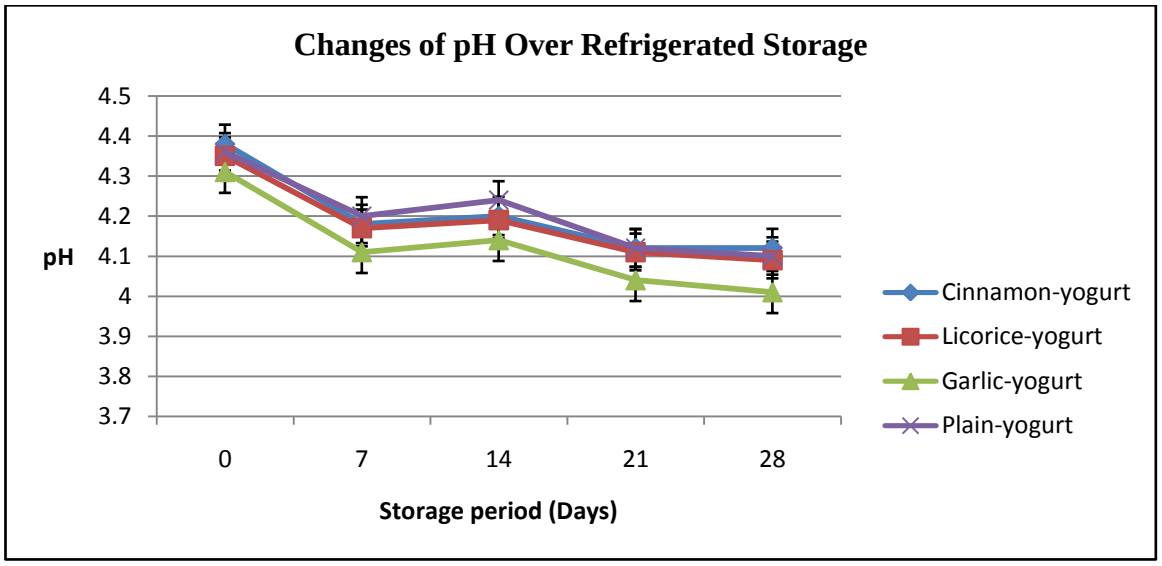

Figure 4. 2 Effects of herbs on the changes of pH in yogurt during 28 days of storage at 4˚C

4.2 Effect of herbal yogurt on the changes of TA

Titratable acidity (TA) measures the accumulation of organic acids produced by the fermentation of yogurt. TA of yogurt during the fermentation and refrigerated storage are as shown in Figures 4.3 and 4.4 respectively. The presence of herbs tend to increase TA of yogurts but the effect was not significant ($p>0.05$) compared to plain-yogurt.

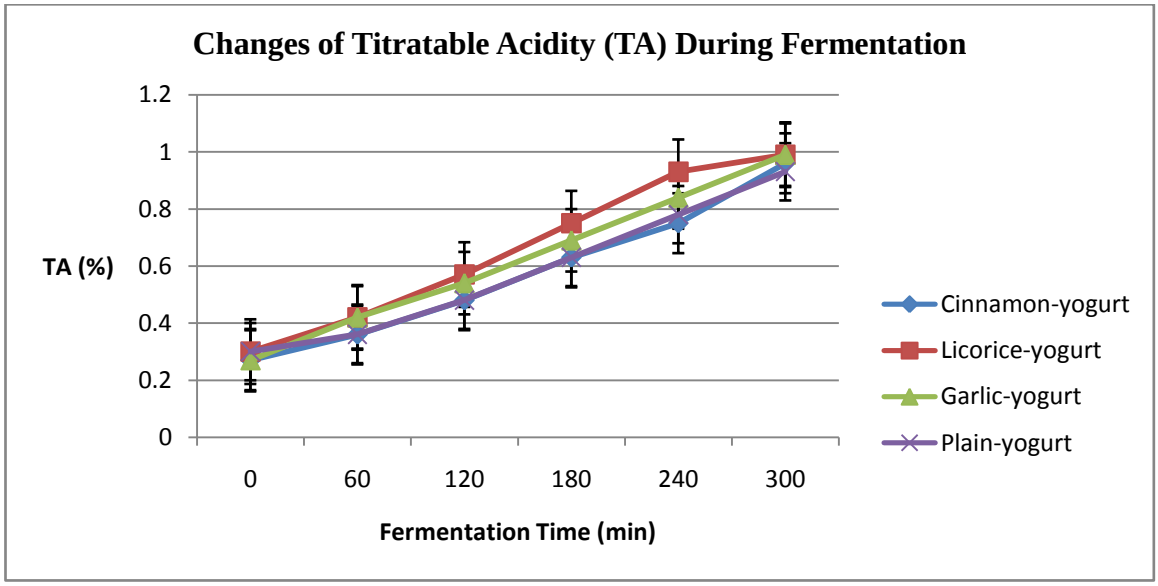

Figure 4. 3 Effects of herbs on the changes of TA in yogurt during the first 5 hours of fermentation at 41˚C

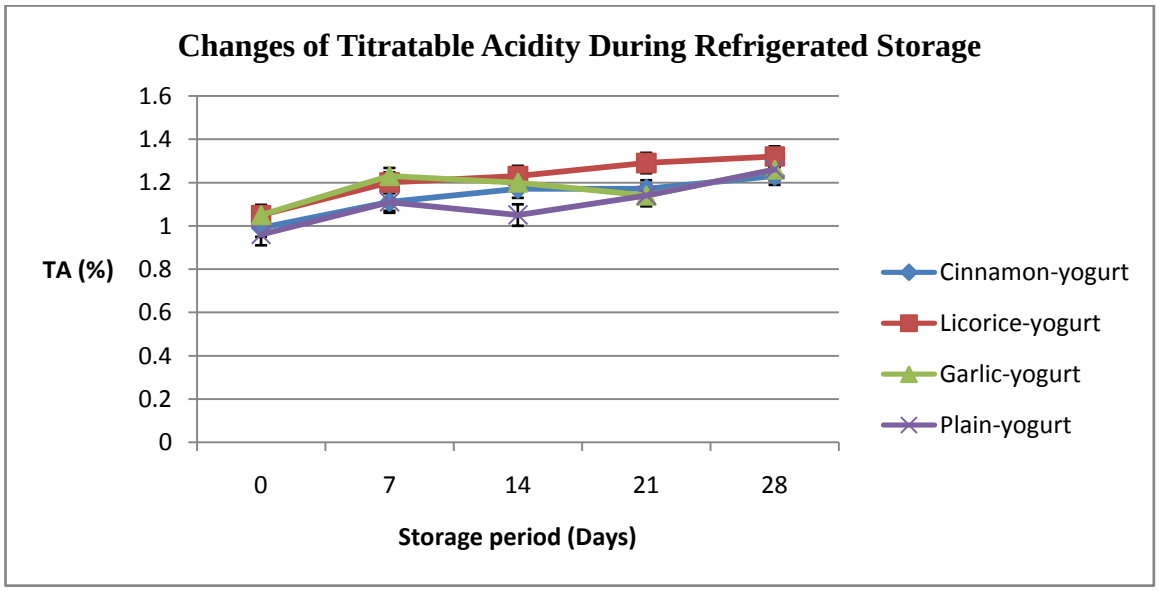

Figure 4. 4 Effects of herbs on the changes of TA in yogurt during 28 days of storage at 4˚C

4.3 Antioxidant activity in herbal yogurts

Figure 4.5 shows the level of antioxidant activity in yogurt at different stages of refrigerated storage. The highest antioxidant activity (% inhibition of DPPH) was recorded on day 7 for cinnamon-yogurt (30.2%) followed by licorice-yogurt (23.86%) and plain-yogurt (21.8%). The addition of garlic into the yogurt resulted in a lower antioxidant activities compared to plain-yogurt ($p<0.05$). The addition of cinnamon or licorice increased the antioxidant activity of yogurts compared to plain-yogurt ($p<0.05$) at all storage periods.

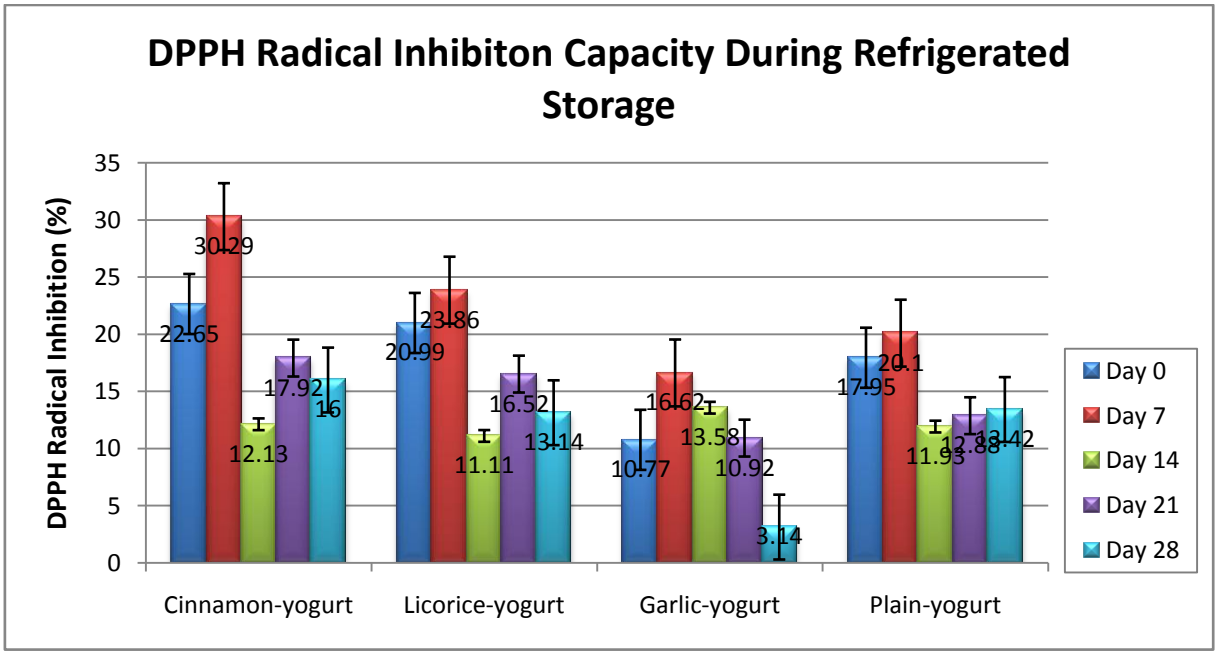

Figure 4. 5 DPPH radical inhibition capacity of water extracts from plain and herbal-yogurts during refrigerated storage

4.4 Viable cell counts in herbal-yogurt

Figures 4.6 and 4.7 show the enumeration of *S. thermophilus* and *Lactobacillus* ssp. respectively in yogurt during refrigerated (4˚C) storage. For fresh yogurts, the presence of herbs lowered *Lactobacillus* ssp. counts ($p>0.05$) in cinnamon- (9.46 x 10^6cfu/ml), licorice- (12.3 x 10^6cfu/ml) yogurts compared to plain-yogurt (12.9 x

10^6cfu/ml). Refrigeration increased (*p>0.05*) viable *Lactobacillus* ssp. counts to 15.8 x 10^6cfu/ml in the plain-yogurt but the presence of cinnamon, licorice or garlic inhibited this increase. Viable *Lactobacillus* ssp. counts were highest in plain-yogurt on day 7 of storage, followed by garlic, licorice and cinnamon. However, the decrease in viable *Lactobacillus* was faster in plain- and garlic-yogurts compared to licorice- and cinnamon-yogurt. Viable *Lactobacillus* ssp. counts were higher in cinnamon- (4.8 x 10^6 cfu/ml; *p>0.05*) and licorice-yogurts (6.4 x 10^6 cfu/ml; *p<0.05*) compared to plain-yogurt (4.0 x 10^6 cfu/ml).

Viable *S. thermophillus* counts in all yogurts also increased by day 7 of refrigeration compared to plain-yogurt (Figure 4.7), but the effect was significant (*p<0.05*) only in the presence of cinnamon and licorice. The reduction of *S. thermophilus* after day 7 was slowest in the plain-yogurt, resulting in higher *S. thermophilus* counts in plain-yogurt (9.2 x 10^7cfu/ml) by day 28 of storage compared to herbal yogurts (ranging from 7.3-8.6 x 10^7cfu/ml).

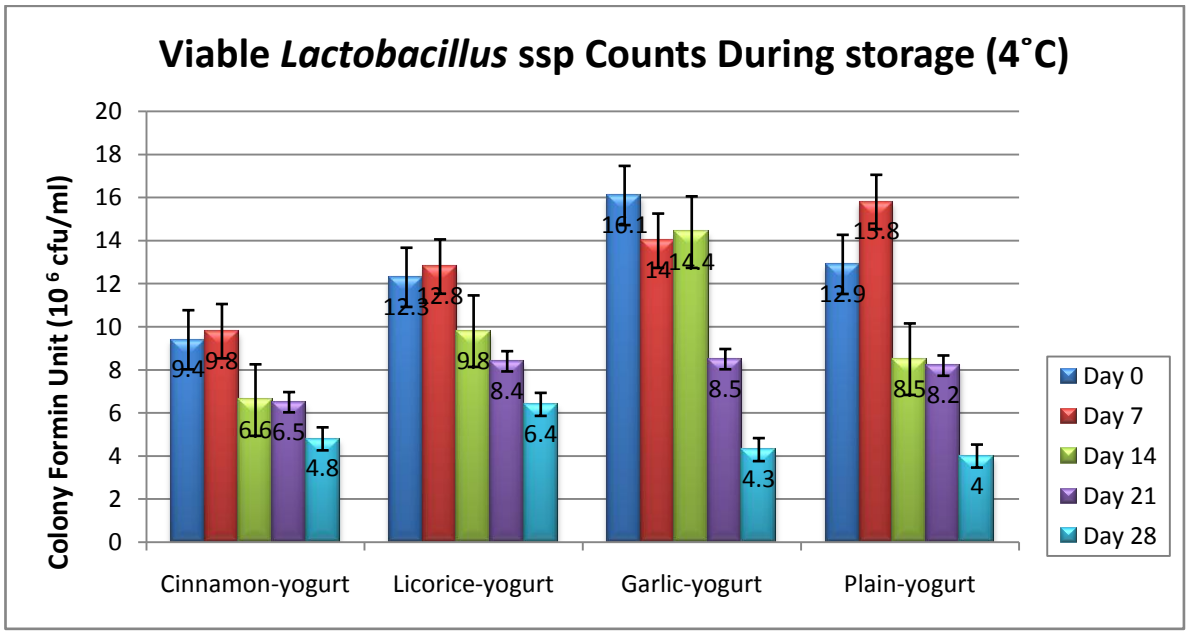

Figure 4.6 Viable cell count (CFU) of *Lactobacillus* ssp. in plain- and herbal-yogurts during refrigerated storage (4°C)

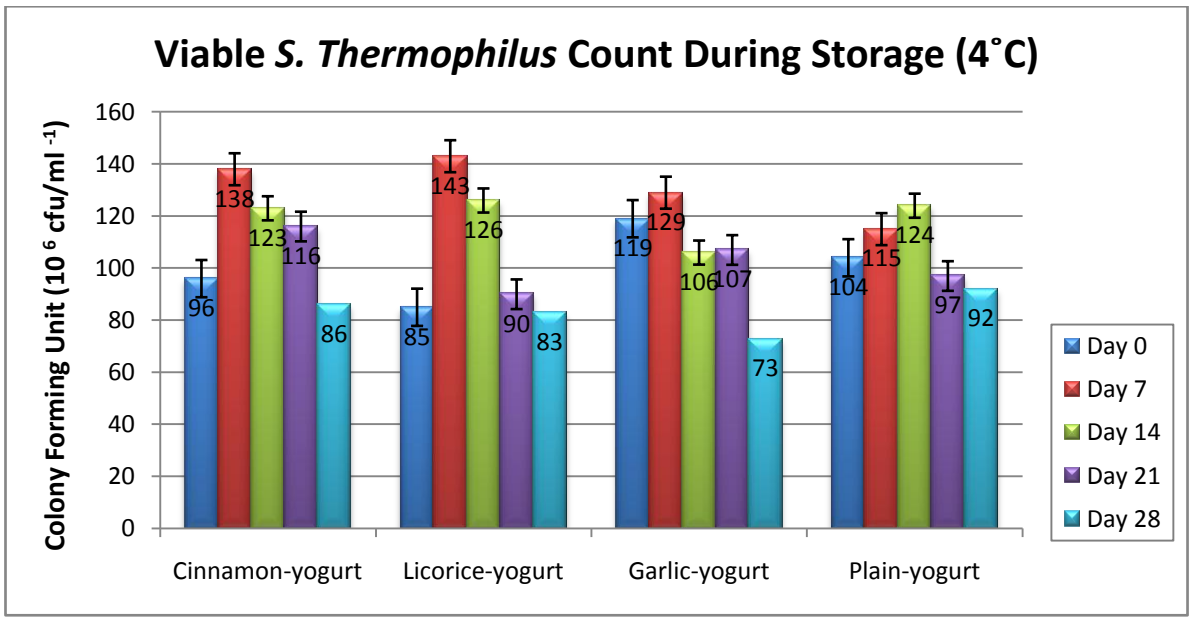

Figure 4.7 Viable cell count (CFU) of *S. thermophilus* in plain- and herbal-yogurts during refrigerated storage (4°C)

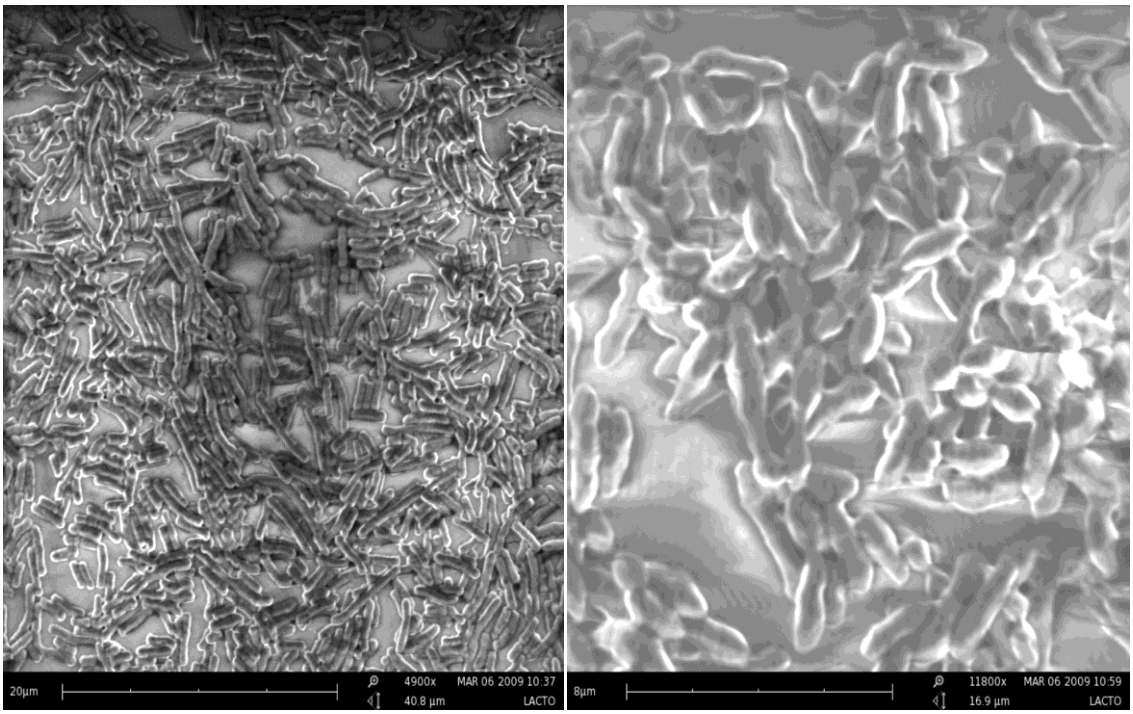

Figure 4.8 Lactic Acid Bacteria (LAB) as observed under electron microscope (magnification from left to right: x4900 and x11800)

4.5 Sensory evaluation of herbal yogurts

The sensory scores of yogurts organoleptic properties are shown in table 4.1. Garlic-yogurt was the least favored for all characteristics evaluated. With regard to taste, plain-yogurt had the highest score (5.8) followed by licorice-(4.5) and cinnamon-(3.8) yogurts. No significant differences ($p>0.05$) in the visual appearance, body and texture, and sourness of the yogurt samples were found. The sweetness tended to reduce in the presence of herbs ($p<0.05$) for licorice and garlic.

Table 4.1 Sensory descriptors and scores* of yogurts

Yogurt type	Visual appearance	Body & texture	Aroma	Sweetness	Sourness	Overall taste
Cinnamon	$5.8^{b}\pm1.6$	$5.7^{b}\pm1.0$	$4.8^{a\ b}\pm1.6$	$3.3^{a}\pm2.0$	$6.0^{b}\pm2.0$	$3.8^{a}\pm2.2$
Licorice	$6.1^{c}\pm1.5$	$6.0^{c}\pm1.4$	$4.5^{b}\pm1.8$	$3.0^{a}\pm1.3$	$6.2^{c}\pm1.3$	$4.5^{b}\pm1.8$
Garlic	$5.5^{b}\pm1.6$	$5.7^{b}\pm1.4$	$3.7^{a}\pm1.8$	$2.7^{a}\pm1.4$	$5.9^{b}\pm2.8$	$3.5^{a}\pm2.0$
Plain	$6.1^{b}\pm1.3$	$5.5^{a\ b\pm1.6}$	$5.5^{a\ b}\pm1.5$	$4.1^{a}\pm2.1$	$6.8^{b}\pm1.4$	$5.8^{b}\pm2.1$

*Data expressed as means ($n=12$) ± standard deviations; means with different letters in the same column differ significantly ($p<0.05$).

4.6 *Helicobacter Pylori*

Three *H. pylori* strains were isolated from biopsies taken from University Malaya Medical Centre (UMMC). Details of sources for these bacterial three strains (UM 1-3) are presented in Table 4.2.

Table 4.2 Strains used in the study, source and patients data

No	Sex	Age (years)	Source
UM-1	M	45	Gastric biopsies
UM-2	F	60	Gastric biopsies
UM-3	F	74	Gastric biopsies

4.6.1 Morphology and identification of *H. pylori*

All bacterial samples were confirmed for *H. pylori* by ensuring they are positive after catalase, urease and oxidase tests (Figures 4.11, 4.12 and 4.13; Goodwin and Armstrong, 1990), and could grow under microaerophilic condition (at 37˚C). The colonies were noted for their small, grey and translucent appearance (Figure 4.9). A typical gram staining of *H. pylori* isolated has been done in this study (see appendix 2, section 2.9), which was Gram-negative as shown in Figure 4.10.

Figure 4.9 *H. pylori* translucent colonies

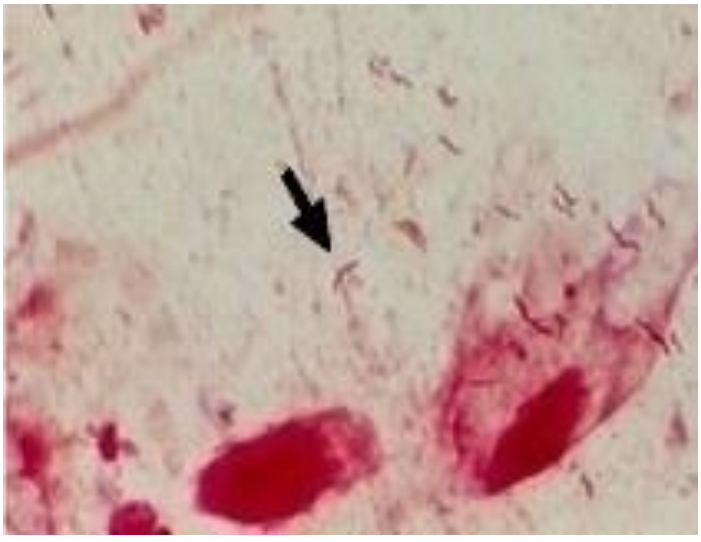

Figure 4.10 Gram-staining from pure *H. pylori* culture
(Magnification: x1000) Adopted from website: http://thunderhouse4-yuri.blogspot.com/

Figure 4.11 Rapid Urease test

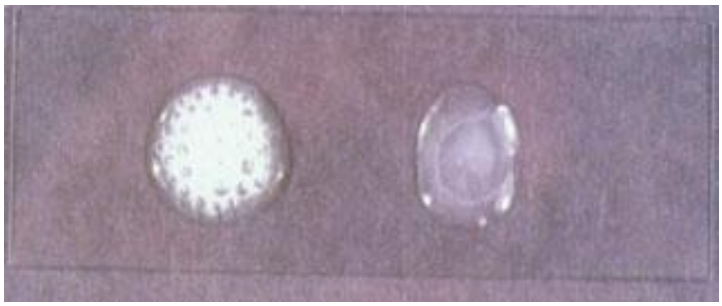

Figure 4.12 Catalase test

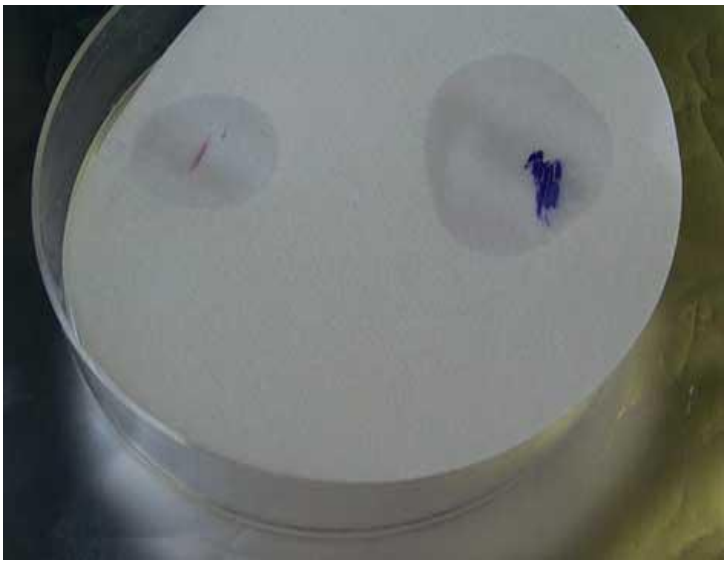

Figure 4.13 Oxidase test

4.7 Effects of extracts of herbs and herbal-yogurts on *H. pylori* growth

The water extract of individual herbs was tested for their inhibitory effect on *H. pylori* growth using disk diffusion method (Table 4.3). Cinnamon water extract exhibited the strongest inhibitory effect on *H. pylori* growth *in vitro* for strains UM-1, UM-2, and UM-3 (22mm, 14mm, and 19.6 mm respectively) in comparison with licorice (11mm, 11mm, and 14mm respectively) and garlic (10.6mm, 10.6mm, and 12.3mm respectively). Water extract of garlic-yogurt (day 14) showed the highest inhibitory effect on UM-1 and UM-2 *H. pylori* strains (10.6mm and 10.3mm respectively). The highest inhibitory effects against all three *H. pylori* strains were recorded mostly by 7-day-old cinnamon-yogurt (11.6mm) and licorice-yogurt (10.3-12.6mm) (Table 4.4).

Table 4.3 Inhibition of *H. pylori* strains growth by various plants water extracts

	Inhibition Zone (mm)		
	Herb Water Extract (mg/ml)[a]		
Isolate Number	Cinnamon	Licorice	Garlic
UM-1	22±0.1	11±0.0	10.6±0.3
UM-2	14±0.5	11±0.0	10.6±0.6
UM-3	19.6±0.5	14±0.1	12.3±0.9

[a]1ml herb = 0.1g herb/ml

Data expressed as means ± standard deviations of triplicate measurements.

Table 4.4 Growth inhibition of *H. pylori* by water extracts from yogurt during 14 days refrigerated storage

		Inhibition Zone (mm)[a]			
		Herbal-Yogurt Water Extract during 14 days storage (mg/ml)			
Isolate Number		Cinnamon-yogurt	Licorice-yogurt	Garlic-yogurt	Plain-yogurt
UM-1	Day 0	9.3	11.3	8.3	10.3
	Day 7	11.6	12.6	9.0	11.6
	Day 14	10.0	11.0	10.6	10.6
UM-2	Day 0	9.6	10.0	8.6	9.3
	Day 7	11.6	10.3	8.3	11.3
	Day 14	11.3	10.6	10.3	10.0
UM-3	Day 0	10.6	10.3	11.0	10.3
	Day 7	11.6	12.0	12.3	11.0
	Day 14	11.6	12.0	12.0	12.3

[a]Diameter of the discs, each containing 0.04g/µl of the freeze-dried supernatant, was 6mm.
Data expressed as means ± standard deviations (n=3).

The MIC for cinnamon water extract was 2, 1 and 1ml for *H. pylori* strains UM-1, UM-2, and UM-3 respectively. The MIC for cinnamon-yogurt water extract was 2ml for all strains studied which was lower than or equal to MIC for plain-yogurt water extract (3, 2, and 3ml for strain UM-1, UM-2 and UM-3 respectively). MIC of cinnamon and licorice water extracts was 3ml for all three isolates. However, owing to strain differences, garlic extract had MIC of 3ml only for UM-3.

Licorice-yogurt water extract showed complete inhibitory effects on isolate UM-1 and UM-2 at concentration of 1ml (Table 4.5), whereas cinnamon-yogurt and garlic-yogurt water extract still showed some evidence of growth at this concentration. Cinnamon-yogurt water extract had MIC of 2ml, whereas, garlic-yogurt water extract had poor inhibition on *H. pylori* since there was still scant growth of *H. pylori* at the highest concentration (3ml) used.

Table 4. 5 Minimum inhibitory concentration (MIC) of herbs and herbal-yogurt water extract on the growth of *H. pylori* isolates[1]

Isolates number	Antimicrobial agent	Volume of herbs or herbal yogurt water extract (ml)[2]					
		0 (blank)	**0.25**	**0.5**	**1**	**2**	**3**
UM-1	Cinnamon	++++	++++	++++	+	—	—
	Licorice	++++	++++	+++	+	+	—
	Garlic	++++	++++	++++	+++	+	+
	Cinnamon-yogurt	++++	++++	++++	++	—	—
	Licorice-yogurt	++++	++++	+++	—	—	—
	Garlic-yogurt	++++	++++	++++	++	+	+
	Plain-yogurt	++++	++++	++++	+++	++	—
UM-2	Cinnamon	++++	++++	+++	—	—	—
	Licorice	++++	++++	+++	+	—	—
	Garlic	++++	++++	++++	++++	+++	+
	Cinnamon-yogurt	++++	++++	++++	+	—	—
	Licorice-yogurt	++++	++++	+++	—	—	—
	Garlic-yogurt	++++	++++	+++	++	++	+
	Plain-yogurt	++++	++++	+++	+	—	—
UM-3	Cinnamon	++++	++++	+++	—	—	—
	Licorice	++++	++++	++++	++	+	—
	Garlic	++++	++++	++++	+++	+	—
	Cinnamon-yogurt	++++	++++	+++	++	—	—
	Licorice-yogurt	++++	++++	+++	+	+	—
	Garlic-yogurt	++++	++++	++++	+++	++	+
	Plain-yogurt	++++	++++	++++	+++	++	+

[1] Note: the extent of *H. pylori* growth in the presence of diluted herbs or herbal-yogurt water extract was described used the "+" or "-": –, no growth; +, scant growth; + +, moderate growth; + + +, extensive growth; + + + +, very extensive growth.

[2] Note: 1ml of herbal extract = 0.1g herb/ml

1ml of yogurt = 0.06g herbal-yogurt extract/ml.

CHAPTER 5: DISCUSSION

Effect of added herbs on the probiotic fermentation of milk may be described in reference to acidification, microbial population, product eating quality, nutritional and therapeutical values. These are discussed further in the following sections.

5.1 Changes in the fermentation characteristics of milk by herbs

Yogurt is formed by the protocooperative action of two homofermentative bacteria, i.e. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. These lactic acid bacteria (LAB) utilize carbon sources (e.g. glucose and galactose) as precursors to obtain energy with the formation of various organic acids as metabolic by-products (Novak and Lubiere, 2000). The differences in the rate of H^+ produced (pH reduction) may be used as indicates for different growth rates of LAB in the yogurt (Adolfsson, 2004).

In the present studies, all yogurts had similar pH reduction patterns during fermentation (Figures 4.1). The initial pH value of cinnamon-, licorice- and garlic-yogurts (6.31, 6.35 and 6.34 respectively) did not differ much from plain-yogurt (6.37), indicating the acidic content of the herbal extracts, if any, were minimal. The phytochemical compounds in these herbs may have masking effect on the milk pH, since Kailasapathy *et al*, (2007) showed differing pH depending on the type of fruit in fruit preparation of yogurt. The effect of herbal preparation on yogurt has not been studied before, therefore the effect of herbs phytochemicals on pH development merit more research in future.

LAB grow optimally under anaerobic condition between 37-41°C. However, these bacteria may be active even at refrigerated temperature resulting in noticeable pH decrease during storage (Shah *et al.,* 1995). Excessive acid production after

fermentation, known as "post acidification", may be attributed to bacterial ß-galactosidase, which is known to remain active at low storage temperature (0-5˚C) (Marshall and Tamime, 1997). The further decrease in the pH of plain- and herbal-yogurts seen in the present studies may regard to occur as a result of accumulation of acetic acid, acetaldehyde, formic acid and lactic acid (Shah, 2007).

The decrease in pH during fermentation was associated with an increase in TA (Figures 4.2 and 4.4). TA are organic acids, metabolically produced by the LAB during fermentation, and these may include lactic acid, acetic acid, propionic acid, citric acid, pyruvic acid, butyric acid and succinic acid (Ostlie *et al.,* 2003). A difference in TA production during fermentation was shown to be attributed to the differential microbial population (Prajapati and Dave, 1994). In certain conditions the increase in TA are associated with increased microbial metabolic activity in the face of bacteriocin instability under stress condition. The bacteriocin production was suggested dependant on the type of stress applied (Leroy *et al.,* 2003). In the present study, certain herbs could create such an environmental stress.

5.2 Eating and nutritional value of herbal-yogurts

5.2.1 Sensory assessment

The organoleptic properties are correlated to TA content (Guler and Multu, 2005; Alakali *et al.,* 2007). Plain-yogurt was the most desirable yogurt compared to herbal-yogurts probably because of its lower TA content than the herbal-yogurts. Apart from TA content, yogurt formed may be characterized physically by its smooth viscous gel structure and oraganoleptically by its taste and flavor (Bodyfelt *et al.,* 1988). Commercial fruit yogurts (strawberry and kiwi) in the present study were used as comparison and scored as the most desirable yogurt for their sensory properties compared to plain- and herbal-yogurts as is also reported in other studies (Kucukoner

and Tarakci, 2004). No significant differences were observed in the sweetness, aroma and visual appearances of herbal-yogurts compare to plain-yogurt (Table 4.1). The differences in sourness and other characteristics (visual appearance, body and texture, aroma, sweetness and overall taste) were also detected between the yogurts. Under normal fermentation condition, the main products of metabolism are lactic acid, acetic acid, acetaldehyde, ethanol and diacetyl, all of which contributed to the specific sour flavor of fermented yogurts (Hammes and Vogel, 1995; Hugenholtz and Kleerebezem, 1999). The concentration of these metabolites were not studied but were expected to change (refer to previous TA discussion) as a result of the influence of added herbal water extracts on LAB fermentation of milk.

Herbs contain phytochemicals and this may play important role in causing undesirable organoleptic properties of herbal-yogurts. This is because most herbs contain a unique richness and diversity of metabolites responsible for their taste and flavor (Rivasseau *et al.,* 2006). Garlic-yogurt was considered by the panelist as the most undesirable in overall taste followed by cinnamon- and licorice-yogurts in comparison to plain-yogurt (Table 4.1). The undesirable taste of garlic-yogurt could be due to the odor from sulfur-containing substrates, such as alliin and other cysteine derivatives (Stoll and Seebeck, 1951). Allicin (diallyl thiosufinate), the first product of alliin through the enzymatic conversion, has a pungent taste and is known to be unstable and easily converted to various types of odorous compounds (Ueda *et al.,* 1990). Although the taste of garlic-yogurt considered by panelists as the most undesirable in the present study, the perception is purely subjective since there are consumers who like the taste and odor of garlic in dairy product such as cheese (Mayer, 2010).

Expolysaccharide (EPS) produced by bacteria, normally produced in response to environmental stress, may also play an important role in visual appearance and texture development of yogurt (Griffin *et al.,* 1996). Although the differences in visual

appearance and texture was not significant in the present study, the texture of yogurt in herbal-yogurts were slightly higher than plain-yogurt. The presence of phytochemicals in herbal water extract may change the medium in which the yogurt bacteria grow. The quality of EPS formed was not detected in the present study. However other studies (Folkenberg *et al.,* 2006; Ruas-Madiedo and De los Reyes-Gavila., 2005) showed correlation between EPS produced by the bacteria and viscosity and texture of yogurt produced. Other study (Marshall and Rawson, 1999) revealed that it may not be the amount of polysaccharide that is important to organoleptic properties, but the type of EPS-producing strains and consequently the interaction of the polymer with the milk proteins during the fermentation.

5.2.2 Antioxidant activities of herbal-yogurts

The stable radical 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) is used broadly for the purpose of evaluating the free radical scavenging activities of antioxidant compounds in plant or fruit extracts and food materials (Soares *et al.*, 1997; Amarowicz *et al.,* 2004). The different antioxidant activity of cinnamon, licorice and garlic can be attributed to the differences in phytochemicals in these herbs. The most common phytochemical with antioxidant activity include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols and polyfunctional organic acids (Hertog *et al.,* 1993).

Antioxidants in plants may be water soluble, fat soluble, insoluble, or bound to cell walls and thus not necessarily freely available to react with DPPH, hence they react at different rates i.e. differing kinetics (Prakash *et al.,* 2001). Each herb in the present study had different antioxidant activity with cinnamon water extract having the highest percentage. Therefore, differences in antioxidant activity of herbal yogurts may be regarded contributed by both the quantitative and qualitative differences bioactive

phytochemicals in the herbal extracts. For instance, the main phytochemicals in *C. zeylanicum* are eugenol and cinnamaldehyde (Shahidar, 2002), whereas *G. glabra* contains flavonoids such as such as liquiritin, isoliquiritin and aglycones (Zhang and Ye, 2009) and *A. sativum*, is rich in flavonols and organosulfur compounds such as allicin enzyme (Wilson and Adams, 2007).

An increase in antioxidant activity recorded on day 7 of storage for all yogurts may be attributed to the probiotic microbial growth during refrigerated storage (Shah *et al.*, 1995). This is demonstrated by the probiotic cell counts in the present study, which showed the highest count by day 7 for all yogurts. The degradation of phenolic compounds and formation of free amino acids even at low temperature by the bacteria was suggested to alter the antioxidant activity of yogurt (Blum, 1998; McCue and Shetty, 2005). The changes in antioxidant activity by yogurt bacteria may also be dependent on the phytochemical contents since phenolic antioxidants were not affected by yogurt microflora during yogurt production from soymilk in the presence of kefir culture (McCue and Shetty, 2005). Some lactic acid bacteria possessed significant antioxidant activity, which allow the preservation of phenolics from being oxidized during yogurt fermentation (Kachouri and Hamdi, 2006). Thus, adding herbs in yogurt products provide phenolic compounds that help to deliver antimicrobial and antioxidant activities and make fermented milk more "functional" (Coisson *et al.*, 2005) and possibly also extend the shelf life.

5.2.3 Yogurt fermentation and the predigestion of milk

Yogurt bacteria, such as *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, express functional lactase, the enzyme that breaks down lactose to galactose and glucose (Goodenough and Kleyn, 1976). Fermenting milk converts some of the monosaccharides formed into lactic acid, which then acts as a preservative for the

product. This gives yogurt a mildly sour taste, influences the physical property of casein curd to improve digestibility, and enhances the use of calcium and other minerals by the host (Shahani and Chandan, 1979). In the present studies, the TA content increased during fermentation and storage but the presence of herbs did not affect the herbal-yogurts acidity compared to plain-yogurt, thus cinnamon, licorice and garlic may not affect the carbohydrate breakdown during yogurt formation.

The yogurt bacteria are also highly proteolytic as compared to the probiotic bacteria (*L. acidophilus* and *Bifidobacterium* ssp). Proteolysis occurring in yogurt results from the symbiotic relationship between the two organisms. The proteinase of *L. delbrueckii* ssp. *bulgaricus* hydrolyzes casein to yield polypeptides, which are broken down further by the peptidases of *S. thermopbilus* (Tamime and Robinson, 1985). *S. thermophilus* also metabolize excess amino acids liberated by *L. delbrueckii* ssp. *bulgaricus.* Fermentation of milk by LAB in different types of yogurts resulted in different amount of OPA peptides produced and this reflects the extent of proteolysis as influenced by the presence of herbs. Although the effect of cinnamon, licorice and garlic on proteolysis activity was not examined, interestingly other studies in this lab show that the addition of *M. piperita*, *A. graveolens* and *O. basilicum* increased O-phthalaldehyde (OPA) peptides in herbal-yogurts by 28–36% after 7 days of storage. Herbs could affect microbial growth and enhance proteolysis by producing proteins with antimicrobial activity (Agboola and Radovanovic-Tesic, 2002) in the yogurt. It would be interesting to study this phenomenon in cinnamon, licorice and garlic yogurts in future studies because these yogurts had higher microbial growth than plain-yogurt.

5.3 Functional value of herbal-yogurts

5.3.1 "Gut-friendly" yogurt bacteria

Health conscious consumers seek foods with functional properties in addition to their basic nutrient contents (carbohydrate, lipid, protein, vitamins and minerals). The functional properties in fermented dairy products can be attributed to the probiotic bacteria added to the regular fermentation cultures (Hugunin, 1999). These bacteria provide therapeutic benefits such as modification of the immune system (MacFarlane and Cummings, 2002), reduction in cholesterol (Liong and Shah, 2006), alleviation from lactose intolerance (Voet, 2004), faster relief from diarrhea (Hilton *et al.,* 1997) and the restoration of a healthy intestinal microbiota (Maire *et al.,* 2006). Thus it is of importance to establish whether added herbs can enhance microbial growth during fermentation and also to maintain viable microbes during refrigerated storage.

The initial yogurt bacteria on day 0 of storage for cinnamon-yogurt and licorice-yogurt were lower than plain-yogurt. The incorporation of air into the yogurt during the mixing of herbal extract with milk at the preparation stage may be partially responsible in the initial reduction in viable cell counts by the oxygen sensitive *Lactobacillus* (Talwalkar and Kailasapathy, 2004). Refrigerated storage of yogurt resulted in significant losses in viable cell numbers (Dave and Shah, 1997) as also demonstrated in the present studies for both *Lactobacillus* ssp. and *S. thermophilus* (Figures 4.6 and 4.7). Because of this, yogurt has limited shelf life. The low viability of probiotics in yogurt is mostly attributed to the further reduction of pH in yogurt during post-acidification (Kailasapathy and Rybka, 1997; Shah, 2000). In addition, the hydrogen peroxide produced during manufacture and storage of yogurt may be considered as another important factor responsible for *L. acidophilus* loss (Gilliland and Speck, 1977).

Of immediate interest in the present study is that *L. acidophilus* and *S. thermophilus* retained a satisfactory level of viability throughout the manufacture and storage of plain- and herbal-yogurts (10^6–10^7cfu/ml; see Figures 4.6 and 4.7). This conforms to recommendation made (Dave and Shah, 1997; Vinderola and Reinheimer, 1999) which regarded acceptable level of yogurt probiotic bacteria must be more than 10^6 cfu/ml. The number of *Lactobacillus* species was found to decrease considerably more than *S. thermophilus* during refrigerated storage in both herbal-yogurts and plain-yogurt (Figures 4.6 and 4.7). Refrigerated storage increased viable *Lactobacillus* ssp. counts in the plain-yogurt but the presence of cinnamon, licorice or garlic inhibited this increase in herbal-yogurts (Figure 4.6). On the other hand, added herbs tend to slow down the reduction in viable yogurt bacteria in herbal-yogurts compared to plain-yogurt. One plausible explanation is that yogurt bacteria grew slower during fermentation in response to the antimicrobial activities of the added herbal water extract. This is because they have to initially invest in the production of EPS the function of which to make bacteria less impervious to environmental stress (Sutherland, 1972). Several works on EPS (Ruas-Madiedo *et al.*, 2002; Durlu-Ozkaya *et al.*, 2007) suggest an effective role of this polysaccharide in protecting the microbial cells against phagocytosis, phage attacks, antibiotics, toxic compounds, osmotic stress and bacteriocins, thus slowing down the microbial death in yogurts. Despite lower viable cells in the presence of herbs, the effects of slowing down of microbial death during storage by these herbs could be a positive attribute. A much higher initial herbal-yogurt bacteria counts (e.g 10^9 cfu/ml) may make these yogurts to contain acceptable number of viable cells for extended refrigeration (e.g. >28 days).

The counts of *S. thermophilus* increased in herbal-yogurts after 7 days of refrigerated storage. The increase in the viable cell counts for both yogurt bacteria during the first 7 days coincided with the increase in TA and marked reduction ($p<0.05$)

in pH recorded on day 7 of storage (Figures 4.2 and 4.4). Prolonged storage increased post-acidification (i.e. organic acids accumulation as a result of growth and fermentation; Hood and Zottola, 1998; Shah, 2000). This may explain the consistent reduction in viable cell counts in all yogurts upon extended refrigeration (up to 28 days). Despite lower viable cells, the rate of reduction of viable microbes was slower for herbal-yogurts than that for plain (Figures 4.6 and 4.7).

5.3.2 Anti *H. pylori* activity

World Health Organization (WHO) has classified *Helicobacter pylori* as a class I carcinogen and the eradication of this silent killer with antibiotic combinations has been reported to be beneficial in preventing gastric diseases especially cancer (IARC, 1994). However, the increasing problems of antibiotic resistance, adverse effects and high costing of drug development (Kusters *et al.,* 2006; Megraud, 2004) have lead researchers to explore natural resources especially plant materials as an alternative source of antimicrobials. In the present studies the potential inhibitory effects of cinnamon, licorice and garlic were explored.

All three plant extracts studied showed anti *H. pylori* activity with cinnamon being the most active (Table 4.3). The types of solvents used in the preparation of plant extracts can affect the antimicrobial activities (Taylor, 2004). This implies some bioactive components can be extracted by polar solvents while others by less polar or non-polar solvents (Ogundare *et al.,* 2006). In the present study aqueous extracts for all three herbs were used because of the most direct and economical considerations in the preparation of herbal-yogurts. It was found that the highest inhibitory effects against all three *H. pylori* strains were recorded mostly by day 7 cinnamon-yogurts and licorice-yogurt (Table 4.4).

Inhibitory effects of yogurt on *H. pylori* were also observed (Table 4.4 and 4.5). Probiotics may inhibit *H. pylori* growth by secreting anti bacterial substances (Shah, 2000). Certain *Lactobacilli* are known to synthesize antimicrobial compounds related to the bacteriocin family (Jack *et al.,* 1995; Lesbros-Pantoflickova, 2007). Other known substances secreted by these bacteria are the end products of lactic acid fermentation, such as lactic and acetic acids and hydrogen peroxide (Oliveira *et al.,* 2008). Limited clinical trials have evaluated the effect of probiotics in *H. pylori* colonized adults and children. In most instances probiotics were found unable to eradicate *H. pylori* but rather decrease the density of colonization, thus maintaining lower levels of this pathogen in the stomach (Sheu *et al.,* 2002; Wendakoon *et al.,* 2002). Since the growth of LAB are affected by the presence of herbs, it is anticipated that the anti *H. pylori* effect seen can be either due to changes in LAB growth and/or direct effect of herbs on *H. pylori*.

Probiotic yogurt is reported to have an inhibitory effect on *H. pylori* colonization (Metchnikoff, 2004; Shah, 2006 and 2007) and the administration of yogurt containing *L. acidophilus* LA5 and *B. lactis* Bb12 caused a significant increase in the eradication of *H. pylori* from 72% to 87% without decreases in adverse effects of the infection (Sheu *et al*., 2003). The generally higher anti *H. pylori* effects of herbal-yogurt suggest the formation of more inhibitory factors, possibly by-products from herbal manipulation of yogurts fermentation. Another crucial property of probiotics to check *H. pylori* growth *in vivo* is their ability to adhere to epithelial cells and thereby colonize the stomach (Maire *et al.,* 2006). These observations implicate increased competition for nutrients (MacFarland, 2000), reduced attachment sites on the gastric epithelial cell walls to which pathogens would otherwise bind (Wang *et al.,* 2004) and the increased production of inhibitory compounds (such as lactate, hydrogen peroxide, short-chain fatty acids and bacteriocin-like substances) (Aroutcheva *et al.,* 2001). For instance, the

EPS from lactic acid bacteria (LAB) can alter the adhesion of pathogenic bacteria to intestinal mucus, altering the host's gut microflora (Fooks *et al.,* 1999; Voravuthikunchai *et al.,* 2006). A dominant growth of EPS-producing bacteria in the gastrointestinal tract can be considered to play a significant physiological role in the maintenance of the ecological balance because the lactic acid produced is responsible for low pH level in the tract (Folkenberg *et al.,* 2006). It would be interesting to find out the effects of cinnamon, licorice and garlic on the production of bacterial EPS, as it will not only effect the organoleptic properties, but also the prevention effect on *H. pylori* attachment onto stomach-intestinal wall.

Cinnamon-yogurt completely inhibited *H. pylori* activity at a concentration of 2ml for all three *H. pylori* isolates (Table 4.5). As reported by Tabak *et al,* (1999), chemical compounds in cinnamon such as cinnamaldelyde, eugenol or cavracol are suggested to be responsible as inhibitory factors on *H. pylori* growth. All herbs and herbal-yogurts, except for garlic and garlic-yogurt, at MIC of 3ml could inhibit the growth of *H. pylori*. This suggests garlic and garlic-yogurt are weak inhibitors for *H. pylori* growth. This is quite surprising since garlic is known to exert *in vitro* antimicrobial activity on *H. pylori* (Sivam *et al.,* 1997; O'Gara *et al.,* 2000). This is in contradiction with other studies, which indicate high antioxidant and antimicrobial activity of garlic (Benkeblia, 2004; Banerjee *et al.,* 2003; Bakri and Douglas, 2005; Lai and Roy, 2004). Weak inhibition of *H. pylori* by garlic observed in the present studies could be due to the amount of allicin enzyme and other organosulphur compounds (Tabak *et al.,* 1996) in the garlic extract used. The use of methanolic extract of garlic may be more active than water extract against *H. pylori* and this should be further explored in future *in vitro* studies.

5.4 General Discussion

In the present study, probiotic herbal-yogurts were evaluated for their inhibitory potential *in vitro* against *H. pylori* growth. *H. pylori* is a highly prevelent pathogen which is responsible for chronic gastritis and peptic ulcer and a risk factor for gastric malignancies (Parsonnet, 1993; Kashiwa, 2003). Although the currently used antibiotics-based *H. pylori* eradication treatment is 90% effective (Megraud, 2004) this treatment regime is expensive and causes side effects such as vomiting, diarrhea, and stomach pain, and increase the apportunity for the development of antibiotic resistance (Dobrilla, 1993). Hence, the search and application of low cost and large-scale alternative solutions to prevent or decrease *H. pylori* colonization is of prime importance.

Apart from nutrient contents, yogurt is valued for its high nutrient degradability and therapeutic properties (Adolfsson, *et al.*, 2004). Yogurt bacteria possess numerous health benefits such as attachment to gastric epithelial cells and thus colonize the stomach, competing for nutrients and occupying sites on the gut wall to which pathogens would otherwise bind. Probiotics such as *Lactobacilli* can resist high concentrations of acids thus can survive through passage of highly acidic stomach environment and contain strains that can adhere and survive in the human stomach (Goldin *et al.* 1992). *Lactobacilli* can also produce inhibitory compounds such as lactate, hydrogen peroxide, short-chain fatty acids and bacteriocin-like substances (Wang *et al.*, 2004).

The mixing of herbs with milk prior to fermentation in the making of herbal-yogurts may be recommended because herbs such as cinnamon, licorice and garlic are natural herbal products with a wide range of beneficial and nutritional properties. Probiotic fermentation of milk may be enhanced by herbs leading to generally higher microbial metabolic by-product as reflected by the greater TA contents in herbal-

yogurts. In particular, the inclusion of cinnamon and licorice into yogurt increased both the antioxidant activity of yogurts and antimicrobial activity against *H. pylori* growth. These dual properties may not be universally true for all herbs because garlic reduced the antioxidant activity of yogurt but had poor inhibition on *H. pylori* growth as shown in the present study. The potential role of cinnamon and licorice and manipulation of probiotics into the milk in inhibition of *H. pylori* growth merit more *in vivo* research in future.

5.5 Conclusion

Cinnamon, licorice or garlic did not change yogurt fermentation but sustain the growth of *Lactobacillus* ssp. during 28 days refrigerated storage. The presence of these herbs imparts unfavorable organoleptic characteristics. However, cinnamon-yogurt and licorice-yogurt inhibited the growth of *H. pylori in vitro*. The consumption of cinnamon- and licorice-yogurts can impart unique functional attributes and should be further investigated as promising approach in diet-based management of *H. pylori* infection.