INFLUENCE OF *ALLIUM SATIVUM* OR *CINNAMOMUM VERUM* ON FERMENTATION OF MILK AND THEIR EFFECTS ON THE PHYSICOCHEMICAL, BIOCHEMICAL, MICROBIOLOGICAL AND FUNCTIONAL PROPERTIES OF PROBIOTIC YOGURT

SHORI, AMAL BAKR

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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SHORI, AMAL BAKR

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INSTITUTE OF BIOLOGICAL SCIENCES
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DEDICATION

I dedicate this PhD to my mother, father and family for their endless support

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Regisration/Matric No.: **SHC090050**

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ABSTRACT

The present study investigated the effects of *Allium sativum* or *Cinnamomum verum* water extract on fermentation of milk and subsequent changes in yogurt bacteria growth and fermentation products on the organoleptic, nutritional and functional values of yogurt. Three types of milk (cow, camel and goat) were incubated (41°C) with starter culture in the presence of *A. sativum* or *C. verum* water extract until pH of yogurt was reduced to 4.5. The presence of *A. sativum* or *C. verum* water extract in cow, camel and goat milk did not affect pH reduction during fermentation whereas titratable acidity (TA) increased only in *A. sativum*-cow milk yogurt. Both herbal extracts enhanced proteolytic activity only in cow milk during fermentation but the viable cell counts (VCC) increased ($p<0.05$) in presence of these herbs in all the three types of milk. Further increase ($p<0.05$) in TA occurred only in *A. sativum*-cow milk yogurt during storage (4°C). *A. sativum* or *C. verum* had no influence on proximate composition for all treatments during storage. The VCC of *S. thermophilus* in cow- or camel- milk yogurt was not affected by either *A. sativum* or *C. verum* during storage but the reverse was true for *B. bifidum*. The VCC of *Lactobacillus* spp was higher ($p<0.05$) in herbal- than plain-camel milk yogurt. The presence of *C. verum* in the three types of yogurt enhanced the survival of LAB more than the presence of *A. sativum* after simulated gastrointestinal digestion (SGD). The extent of proteolysis was higher ($p<0.05$) in presence of *A. sativum* than presence of *C. verum* during storage with cow-milk yogurt being more susceptible than camel-milk yogurt. All yogurt showed the highest inhibitory activity of ACE-I on day 7 of storage. The presence of both herbal extracts in the three types of yogurt showed some influence on the extent of proteolysis after SGD but not on ACE-I inhibition activity. Both herbal- cow- and camel-milk yogurt showed increased ($p<0.05$) α-amylase and α-glucosidase inhibitory activities, antioxidant activity and phenolic contents during 21 days of storage. SGD of herbal- yogurt has no effect on α-amylase and α-glucosidase inhibitory activities and total phenolic content of the three types. However, SGD increased antioxidant activity in all fresh *C. verum*-yogurt after SGD. The presence of *A. sativum* or *C. verum* in cow- and camel- milk yogurt showed improved water holding capacity, susceptibility to syneresis and exopolysaccharides content. However, both herbs affected yogurt rheology properties by showing lesser ability to resist deformation upon applied shear and exhibited shear thinning behaviour. The addition of *A. sativum* and *C. verum* did not affect the organoleptic properties of cow- and camel- milk yogurt although *A.*

sativum reduced the aroma score in the former. The presence of *A. sativum* or *C. verum* in milk during yogurt bacteria fermentation can enhance microbial growth and metabolism resulting in an increase in VCC, nutrient digestibility, rheological characteristics and functional properties. The potential stability of yogurt to exposure to digestive enzymes was also enhanced in the present of *A. sativum* or *C. verum*.

ABSTRAK

Kajian ini mengkaji kesan ekstrak air *Allium sativum* atau *Cinnamomum verum* ke atas penapaian susu dan perubahan berikutnya dalam pertumbuhan bakteria yogurt dan produk penapaian (protein, peptida, oligosaccharides dan asid organik) terhadap organolepsis, nilai pemakanan dan fungsi yogurt. Tiga jenis susu (susu lembu, unta dan kambing) telah dieram (41 ° C) dengan kultur pemula dalam kehadiran ekstrak air *A. sativum* atau *C. verum* sehingga pH yogurt menurun ke 4.5. Kehadiran *A. sativum* atau *C. verum* ekstrak air dalam susu lembu, unta dan kambing tidak menjejaskan pengurangan pH semasa penapaian manakala keasidan tertitrat (TA) meningkat hanya dalam yogurt susu lembu-*A. sativum*. Kehadiran kedua-dua ekstrak herba peningkatan aktiviti proteolisis hanya dalam susu lembu semasa penapaian tetapi bilangan sel yang hidup (VCC) meningkat ($p<0.05$) dalam kehadiran herba-herba ini dalam kesemua tiga jenis susu. Peningkatan lanjut TA hanya berlaku di dalam yogurt susu lembu-*A. sativum* (0.2%; $p <0.05$) berbanding yogurt biasa (kawalan) sepanjang 21 hari penyimpanan sejuk (4°C). *A. sativum* atau *C. verum* tidak mempengaruhi komposisi proksimat bagi semua rawatan semasa penyimpanan. VCC *S. thermophilus* dalam yogurt susu lembu atau unta tidak terjejas samada oleh *A. sativum* atau *C. verum* semasa penyimpanan tetapi sebaliknya adalah benar untuk *B. bifidum.* VCC Lactobacillus spp adalah lebih tinggi ($p<0.05$) dalam yogurt susu unta-herba daripada yogurt susu unta biasa. Tambahan pula, kehadiran *C. verum* dalam tiga jenis yogurt meningkatkan survival LAB lebih daripada dalam kehadiran *A. sativum* selepas penghadaman simulasi gastrousus (SGD). Sejauh proteolysis adalah lebih tinggi ($p <0.05$) dalam kehadiran *A. sativum* daripada dalam kehadiran *C. verum* semasa penyimpanan dengan yogurt susu lembu menjadi lebih mudah terubah daripada yogurt susu unta. Semua yogurt menunjukkan aktiviti perencatan tertinggi ACE-I (40-70%). pada hari ketujuh penyimpanan. Kehadiran kedua-dua ekstrak herba dalam tiga jenis yogurt menunjukkan pengaruh tertentu ke atas takat proteolysis selepas SGD tetapi tidak terhadap perencatan aktiviti ACE-I. Kedua-dua yogurt susu lembu-herba dan yogurt susu unta herba menunjukkan peningkatan ($p <0.05$) α-amilase (30-58%) dan α-glucosidase (9-18%) aktiviti perencatan, aktiviti antioksidan (20% - 65%) dan kandungan fenolik (37 -78 GAE μg / ml) selama 21 hari penyimpanan. SGD yogurt-herba tidak mempunyai kesan ke atas perencatan α-amilase dan α-glukosidase dan kandungan jumlah fenol. Walau bagaimanapun SGD meningkatkan aktiviti antioksida dalam kesemua yogurt-C. verum- selepas penghadaman gastrik. Kehadiran *A. sativum*

atau *C. verum* dalam yogurt susu lembu dan unta menunjukkan peningkatan dalam kapasiti penakungan air (WHC), kecenderungan syneresis (STS) dan kandungan exopolysaccharides (ESP). Walau bagaimanapun, kedua-dua herba mempengaruhi sifat reologi yogurt dengan menunjukkan kurangnya kebolehan merintang deformasi apabila ricihan dikenakan dan tingkah laku penipisan ricih. Penambahan *A. sativum* dan *C. verum* tidak menjejaskan sifat organolepsis yogurt susu lembu dan susu unta walaupun *A. sativum* mengurangkan skor aroma dalam susu lembu. Kehadiran *A. sativum* atau *C. verum* dalam susu semasa penapaian oleh bakteria yogurt boleh meningkatkan pertumbuhan dan metabolisme mikrob yang mengakibatkan peningkatan dalam VCC, kebolehadaman nutrien, ciri-ciri reologi dan sifat-sifat berfungsi. Kemungkinan kestabilan yogurt semasa pendedahan kepada enzim pencernaan telah juga dipertingkatkan dalam kehadiran *A. sativum* atau *C. verum.*

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List of Abbreviations

EPS = exopolysaccharides

TSC = total solids content

NaCl = sodium Chloride

KCl = potassium chloride

$CaCl_2$ = calcium Chloride

$NaHCO_3$ = sodium bicarbonate

Na_2CO_3 = disodium carbonate

WHC = water holding capacity

STS = Susceptibility to syneresis

G′ = elastic modulus

G″ = viscous modulus

Hz = hertz

% = Percentage

ºC = degree Celcius

& = and

Etc = et cetera

Min = minute

Mg = milligram

L = liter

ml = milliliter

µl = microliter

µg = microgram

nm = nanometer

OPA = O-phthalaldehyde

HCl = hydrochloric acid

LAB = lactic Acid Bacteria

NaOH = Sodium hydroxide

TPC = total phenolic content

DPPH = 1, 1-diphenyl-2-picryhydrazyl

dH_2O = distilled water

SGD = simulated gastrointestinal digestion

TA = total acidity

µ = micro

µm = micrometre

ANOVA = analysis of variance

BSA = bovine serum albumin

cfu = colony forming unit

g = gram

mm = millimetre

mM = millimolar

MW = molecular weight

Pa = Pascal

pH = hydrogen ion concentration

rpm = revolution per minute

ssp. = subspecies

UV = ultra violet

w/v = weight per volume

1.0 Introduction

1.1 Background

Diabetes mellitus (DM) is a disease characterized by high blood sugar level (glucose) that results from the failure of body to produce enough insulin (DMT1) or unable to respond properly to the insulin that had been produced by the pancreas (DMT2; Diamond, 2003). The global prevalence of diabetes mellitus for all age groups was estimated to be 2.8% in 2000 and is estimated to rise to 4.4% in 2030 (Wild *et al*., 2004). The major part of this increase is expected to occur in the third world countries with the number of diabetics to increase by 35% by 2025 among those aged 20 years or above. Hyperglycemia is a metabolic disorder (circulation of blood glucose level is excessive in the blood plasma) resulting from defects in insulin secretion, insulin action, or both. The function of insulin is to lower the level of blood glucose, which occurred especially after eating. Chronic hyperglycemia is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, heart, nerves, kidneys and blood vessels and has been shown to be also linked with hypertension (Ranade *et al*., 2001). However, metabolic control can be improved through diet and physical activity with or without anti-diabetes drugs which showed significant decrease in the risk of complications (Lindstrom *et al.*, 2006).

Postprandial hyperglycaemia is a serious health problem in type 2 diabetes patients occurs due to exaggerated rise in blood sugar following a meal (Cervera *et al*., 2008). Pancreatic α-amylase (EC 3.2.1.1) is a important enzyme in the digestive system and catalyses the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and various oligosaccharides. This is followed by the action of α-glucosidase (EC 3.2.1.20) to further breaks down the disaccharides into simpler sugars readily to be absorped in the intestine. Therefore, the inhibition of α-amylase and α-glucosidase enzymes involved in the digestion of carbohydrates with natural compounds (such as

medicinal herbs) can significantly decline the postprandial increase of blood glucose after a mixed carbohydrate diet (Kim *et al*., 2006; Ranilla *et al*., 2010). This can be more practical and economical strategy in the management of postprandial blood glucose level in type 2 diabetes patients with minimal side effects than treatments with drugs such as acarbose that showed to causes gastrointestinal side-effects such as flatulence and diarrhea (78% and 14% of patients respectively; Kwon *et al*., 2006; Ali *et al*., 2006; Kresge, 2011).

Type-2 diabetes and hypertension are interconnected metabolic disorders that strongly lead to atherosclerotic cardiovascular disease (CVD) and to renal failure (Sowers and Bakris, 2000). It is reported that hypertension is likely two times higher in subjects with diabetes than those without (Sowers and Bakris, 2000). The action of the angiotensin converting enzyme-I (ACE-I) is known to be one of the most important intermediary factors for controlling hypertension (Hernadez-Ledesma *et al*. 2004). ACE-I converts angiotensin I to angiotensin II, a potent vasoconstrictor and stimulator of aldosterone secretion by the adrenal gland. ACE-I inhibition is therefore considered a helpful therapeutic approach in the management of hypertension in both diabetic and non-diabetic patients (Crook, 2007). Interest in this approach increased tremendously with the discovery of several functional foods capable of modulating the physiological effects (Gumienna *et al*., 2009; Darmawan *et al*., 2010) in the prevention (Darmawan *et al*., 2010) and cure of diseases (Korhonen and Pihlanto, 2003; Gobbetti *et al*., 2004). Biotechnological research on food has played important role in the development and creation of products enriched with nutrients (Miyoshi, 2006) and therapeutic benefits (Nevala *et al.* 2002; Chen *et al*., 2009).

Yogurt is a fermented milk product often regarded as a nutritious food because of the fermentative action of lactic acid bacteria (LAB) and their metabolites

(Lourens-Hattingh and Viljoen, 2001; Alimentarius, 2008). In addition, yogurt enhances digestion (Gibson *et al*., 2004), absorption of vitamins and minerals and can be taken daily to boost the body health (Isolauri *et al*., 2001).

Cow milk represents 85% of the milk consumed in the world with goat and sheep milk constituting a much smaller proportion of 10%. Other dairy animals (buffalo, yak, mare, and camel) despite being scarce have their own unique roles in the survival of mankind. Yogurt manufacture from cow's milk is the most widely used in many countries. Other milk types such as camel and goat are becoming increasingly available because of their local availability, unique taste and therapeutic values (Östman *et al*., 2001). Camel milk, besides being part of the staple diet in parts of Africa and Asia, is also considered as health promoting (Anonymous, 2003). It is common practice in these regions to recommend consumption of camel milk either in fresh or sour state (Abdelgadir *et al*., 2008) for the general treatment of DM (Yagil and Van, 2000). The health benefits associated with camel milk consumption was suggested (Agrawal *et al.*, 2005) due to the presence of high concentration of insulin/ insulin like protein (~52 units/L). Camel milk has all required nutrients and the chemical composition is comparable to that of cows' milk (Yagil and Van, 2000). However, camel milk has special properties not found in cow milk including lower cholesterol (Abdelgadir *et al*., 2008), higher antibacterial and antiviral properties (El-Agamy *et al*., 2009), higher vitamin C content (Kamal *et al*., 2007; Al-Hashem, 2009), higher levels of immunoglobulin, lactoferrin, lysozyme, lactoperoxidase and peptidoglycan recognition protein (Agrawal *et al.*, 2004; El-Said *et al*., 2010) No allergic responses were reported in camel milk due to low amount of whey protein (β-lactoglobulin) which is one of the main allergens in cow's milk (Shabo *et al*., 2005; Al-Alawi and Laleye, 2011). On the other hand, goat milk has special nutritional

properties that make it preferable to consumers (Haenlein, 2004). It has fat rich in medium-chain triglycerides (MCT). Its digestibility is better than cow milk by virtue of higher proportion of short to medium fatty acids as well as reduced dimensions of casein micelles and fat globules (Park *et al*., 2007). Goat milk contains free taurine, one of the final metabolic products of sulphur-containing amino acids (Park *et al*., 2007) which affect several biological functions including as modulator of growth (Minervini *et al*., 2009) and of neuronal activity (Jiang *et al*., 2004); conjugation of bile salts (Chesney and Hedberg, 2010); regulation of osteoblast metabolism (Menzies, 2002); protection of cells against various types of injury and prevention of cardiovascular damage (Warskulat *et al*., 2007) and treatment of fatty liver of children (Pugh, 2002; Menzies, 2002). In comparison to cow milk, regular consumption of goat milk significantly increased blood serum level of vitamin and haemoglobin, improves the body weight and mineralization of skeleton (Banu *et al*., 2007).

The food biotechnology industry has developed number of commercial products containing a single probiotic strain or bacterial associations of various complexities. Bio-yogurt is a yogurt containing probiotic bacteria which are live microorganisms including *Lactobacilli*, *Streptococci* and *Bifidobacteria*. These bacteria transit the gastrointestinal tract and help to maintain or create a favourable microbial condition to provide healthy digestive function and provide therapeutic benefits for the consumer (Tannock *et al*., 2000).

The traditionally prepared yogurt may be improved by the inclusion of ingredients such as soya protein, vegetables, sweet potato, pumpkin and plum (Joo *et al.,* 2001, Park *et al.,* 2003) to enhance the flavour as well as the nutritional quality (Shori and Baba, 2011, Amirdivani and Baba, 2011). Plants such as *Allium sativum* and *Cinnamomum verum* have unique propositional values to be considered as

addition in yogurt making. For example, increase glucose uptake and glycogen synthesis (Jarvill-Taylor *et al.,* 2001; Qin *et al.,* 2004) and improve glucose and lipid profiles of type 2 diabetes patients (Khan *et al*., 2003). They also have a variety of pharmacological properties including hepato-protective (Perez Alvarez *et al.,* 2001), anti-malarial (Wiesner *et al.,* 2001), antioxidant (Lee *et al.*, 2009), anti-hyperglycemic activities (Ziegenfuss *et al*., 2006; Sukandar *et al.,* 2010) and Antihypertensive (Preuss *et al*. 2006; Sukandar *et al*., 2010).

1.2 Problem statement

A profound understanding in the relationship between food and health is integral in the development of the concept of functional foods (Bhat and Bhat, 2010). According to this concept foods meant not only to satisfy hunger and to deliver essential nutrients but also to prevent the development of nutrition-related diseases and to improve physical and mental well-being of consumers. The increasing demand for functional foods can be explained by certain factors like increasing cost of healthcare, the steady increase in life expectancy and the intention of older people for enhanced quality of their later years (Roberfroid, 2002; Kotilainen *et al*., 2006). Diabetes and hypertension are two chronic diseases fast developing in Saudi Arabia as in other developing countries associated to unhealthy eating habits and lifestyle. The consumption of *Allium sativum* and *Cinnamomum verum* are known effective at controlling the development of these two metabolic syndromes (Harauma and Moriguchi, 2006; Ziegenfuss *et al*., 2006; Nickavar and Yousefian, 2009; Ponnusamy and Pari, 2011). Fermented dairy products play a functional role either directly through interaction with consumed microorganisms (probiotic effect) or indirectly as a result of action of microbial metabolites like vitamins, proteins, peptides, oligosaccharides and organic acids generated during the fermentation process (Bhat

and Bhat, 2011). Yogurt is fermented milk and it contains milk nutritious properties, healthy bacteria and fermentation products with anti-diabetic and anti-hypertensive properties (Östman *et al*., 2001; Papadimitriou *et al*., 2007; Donkor *et al*., 2007). Much of these properties are by virtue of the proteolytic system of lactic acid bacteria to the liberation of bioactive peptides (Pripp *et al*., 2006). Since yogurt is readily available in Saudi Arabia it would be advantageous to make yogurt more effective in anti-diabetic and anti-hypertensive properties. It is hypothesised that the presence of *A. sativum* or *C. verum* would alter lactic acid bacteria fermentation of milk by manipulating the proteolytic system of these bacteria and thus modifies fermentation products compositions. Differences in the chemical composition between cow, camel, and goat milk present unique opportunity to study the differences in yogurt bacteria growth and metabolism, and their responses to phytochemicals that lead to altered fermentation and subsequent yogurt quality.

1.3 Objectives of study

In the present study the functional properties of yogurt prepared from cow, camel and goat milks were investigated in the presence of water extracts two versatile herbs; *A. sativum* and *C. verum*.

The specific objectives were:

1) To determine the effects of *C. verum* or *A. sativum* water extract on the viable cell counts of LAB and *Bifidobacterium bifidum* during milk fermentation.

2) To measure the influence of *A. sativum* or *C. verum* water extract on post-acidification and proximate composition of yogurt during 21 days of refrigerated storage.

3) To study the effects of *A. sativum* and *C. verum* water extracts on sensory, physical and rheological properties of yogurt during 21 days of refrigerated storage and evaluate the organoleptic properties of these yogurt.

4) To evaluate the viability of LAB and *B. bifidum* in *C. verum*- or *A. sativum*- yogurt during 21 days of refrigerated storage and the survival of these bacteria after simulated gastrointestinal digestion.

5) To evaluate the extent of proteolysis in *A. sativum*- or *C. verum*- yogurt and *in vitro* ACE inhibitory activity during 21 days of refrigerated storage and after simulated gastrointestinal digestion.

6) To determine the effects of refrigerated storage of *A. sativum*- or *C. verum*- yogurt on antioxidant activity and *in vitro* inhibition of enzymes related to type 2 diabetes before and after simulated gastrointestinal digestion.

1.4 Significant of study

This study would provide more information on how differential effects of yogurt bacteria on cow, camel and goat milk fermentation by these two herbs (*A. sativum* and *C. verum*) can be capitalised to increase the availability of suitable fermented functional foods for arid countries like Saudi Arabia.

1.5 Organization of Chapters

Chapter 2.0 of this thesis contains a thorough literature review of recent scientific reports on the proposed study. The effects of inclusion of *C. verum* or *A. sativum* water extract in cow, goat and camel milk on the acidification, proteolysis and viability of LAB and probiotics during fermentation until the pH reach 4.5 is described in Chapter 3.0. Chapter 4.0 focus on study the influence of *A. sativum* or *C. verum* water extract on the proximate composition of yogurt made from cow, camel

and goat milk during 21 days of refrigerated storage. Chapter 5.0 evaluates sensory-, physical and rheological properties of set-type *A. sativum*- and *C. verum*- yogurt made from cow or camel milk during 21 days of refrigerated storage. Chapter 6.0 examines the viability of *Streptococcus thermophilus, Lactobacillus* spp and *Bifidobacterium bifidum* in *C. verum*- and *A. sativum*- yogurt made from camel or cow milk during 21 days of refrigerated storage. The effect of survival of above-mentioned bacteria present in *C. verum*- and *A. sativum*- yogurt made from cow, camel and goat milk after simulated gastrointestinal digestion is discussed in Chapter 7.0. Proteolytic activity and *in vitro* angiotensin-converting enzyme inhibitory peptides in *A. sativum*- or *C. verum*- yogurt made from cow or camel milk during 21 days of refrigerated storage were investigated in Chapter 8.0. Chapter 9.0 reports on the changes in *in vitro* angiotensin-converting enzyme inhibitory peptides of *A. sativum*- and *C. verum*- yogurt made from cow, camel and goat milk and the extent of milk protein proteolysis in yogurt after stimulated gastrointestinal digestion. Chapter 10.0 describes the effect of *A. sativum* or *C. verum* on cow and camel milk yogurt antioxidant activity and inhibition of α-amylase and α-glucosidase activities using *in vitro* models. Chapter 11.0 investigates anti-diabetic enzymes and antioxidant activity of *A. sativum*- and *C. verum*- yogurt made from cow, camel and goat milk after simulated gastrointestinal digestion. The overall conclusions and future research studies are included in Chapter 12.0, and all references are listed in Chapter 13.0.

2.0 Literature review

2.1 Aims and scope of the literature review

The aim of this literature review is to present the current application of fermented milk such as yogurt and medicinal plants in the treatment and management of various diseases, in particular type 2 diabetes mellitus and hypertension.

This literature review begins with an overview of the historical of functional food. This will then be followed by a discussion of yogurt as functional dairy product with known the role of LAB and probiotic bacteria in yogurt. An attempt on the overview of general procedures used in the lactic acid fermentation of milk is made. Attention is focused on the changes in milk components after fermentation such as proteolysis of milk protein, some milk-protein-derived peptides with antihypertensive effects and the effect of fermentation on rheological and physical properties of yogurt. This will then be followed by a brief discussion of diabetes mellitus and the importance of inhibition of α-amylase and α-glucosidase. A general overview of human digestive system and the process of food digestion in the body will be described. Current use of traditional medicinal plants and/or their products is briefly made followed by a thorough consideration on *Cinnamomum verum* and *Allium sativum* (the focus of this study), their uses in traditional medicine as well as the findings of various studies on the therapeutic uses. Finally, the phenolic compounds present in plants as well as their role as natural foods will be discussed.

2.2 What are functional foods?

Foods are functional when they provide additional properties other than nutritive values. However, added physiologic benefits to foods are now being examined intensively which may either a state of well-being and health and/or to the reduction of the risk of a disease. Functional foods have no universally accepted

definition. The concept was first developed in Japan in the 1980s when the Ministry of Health and Welfare introduced a controlling system to approve certain foods with recognized health benefits in hopes of developing the health of the nation's aging population (Yamada *et al*., 2008). These foods are also called "Foods for Specified Health Use" (FOSHU, Yamada *et al*., 2008). Functional foods may also be defined as "any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Hasler, 2002). The International Life Sciences Institute defines them as further refinement i.e. "foods that, by virtue of the presence of physiologically-active components, provide a health benefit beyond basic nutrition" emphasise the importance of therapeutical values inherent in functional foods. Functional foods must not be consumed as medicine but rather as foods that are "whole, fortified, enriched or enhanced" but more importantly, states that such foods must be consumed as "part of a varied diet on a regular basis, at effective levels " for consumers to reap their potential health benefits" (American Dietetic Association, 1999). Nutraceuticals is another term commonly used with functional foods. This term created in 1991 by the Foundation for Innovation in Medicine refers to nearly any bioactive component that provides a health benefit.

A food can be assumed to be functional if it follows one of the next criteria (Ramchandran and Shah, 2009):

a) It comprises a food component (being nutrient or not) which have positive effects targeted one or a limited number of function(s) in the body.

b) It has physiological or psychological properties further than the traditional nutritional effect.

The component that makes the food "functional" can be 'either an essential macronutrient with particular physiological properties or an essential micronutrient for body needs on a daily basis. In addition, some of food components may not recorded as essential, such as some oligosaccharides, or they have no nutritive value, such as live microorganisms or plant chemicals' (Nakakuki, 2002). The main types of functional foods are indicated in Table 2.1.

Table 2.1 Different types of functional foods.

Type	Description	Some examples
Fortified products	Increasing the content of existing nutrients	Grain products fortified with folic acid, fruit juices fortified with additional vitamin C
Enriched products	Adding new nutrients or components not usually present in a certain food	Fruit juices enriched with calcium, foods with probiotics and prebiotics
Altered products	Replace existing components with beneficial components	Low-fat foods with fat replacers
Enhanced commodities	Changes in the raw commodities that have altered nutrient composition	High lysine corn, carotenoid containing potatoes, lycopene enhanced tomatoes

(Source: Spence, 2006).

2.2.1 Functional dairy products

Dairy products are established as healthy natural products and they form one of the four major food groups (the other three being protein, fruits and vegetables and grains) that make up a balanced diet (Ramchandran and Shah, 2009). Regular consumption of certain dairy products has beneficial effects in the prevention of disease (Bozanic *et al*., 2001) because they contain a number of active compounds with putative roles in both nutrition and health protection (Table 2.2).

Table 2.2 Dairy components and ingredients in functional foods and their health claims.

Component	Sources	Claim areas
Minerals	Calcium Casein peptides	Optimum growth and development, dental health, osteoporosis
Fatty acids	Conjugated linoleic acid (CLA)	Heart disease, cancer prevention, weight control
Prebiotics/carbohydrates	Galactooligosaccharides Lactulose Lactose	Digestion, pathogen prevention, lactose intolerance, immunity and gut flora balance
Probiotics	Lactic acid bacteria Bifidobacteria	Immunity, heart disease, digestion, vitamin production, remission of inflammatory bowel disease, antitumor activity, alleviation of diarrhea and prevention of allergy
Proteins/peptides	Whey proteins, caseins, lactoferrin, immunoglobulins, glycoproteins, specific peptides	Growth, antibacterial activity, dental health, immunomodulation and hypertension regulation (angiotensin inhibitors)

(Source: Shortt *et al*., 2004).

2.2.1.1 Composition of camel, goat and cow milk

Milk is whitish liquid composed of water, lactose, fats, proteins and various vitamins and minerals. About 85% of the dairy cows consumed in the world with goat and sheep milk constituting a much smaller proportion of 10%. Other dairy animals (buffalo, yak, mare, and camel) despite being scarce have their own unique roles in the survival of mankind. The nutritional utilization is markedly high in camel milk followed by goat and cow milk respectively (Shamsia, 2009). Camel milk fulfills the nutritional requirements of minor population in harsh and arid environment. It's different from other ruminant milk as it gives very soft coagulum in acidic environment (Hashim *et al*., 2009). This phenomenon resulted in passing of camel milk rapidly through stomach together with the specific insulin- like protein/insulin

and stays available for absorption in intestine (Agrawal *et al*., 2005). This is in contrast to the digestion of cow's milk which normally form a solid (curd) attributed to the high degree of phosphorylation of the caseins which affects gastric emptying (Wattiaux and Howard, 2000). The coagulation time of camel milk was reported as being 2-3-fold longer than that of cow's and goat's milk, and curd firmness could not be measured (Boudjenah- Haroun *et al*., 2012).

From the nutrition point of view, camel's milk has low cholesterol content because it contains little fat which consists of mainly polyunsaturated fatty acids that are entirely homogenized and provides the milk a smooth white appearance (Shabo *et al*., 2005). The concentration of lactose present in camel milk is 4.8% and easily metabolized by people suffering from lactose intolerance (Hanna, 2001). A possible explanation to this is that camel milk produces less casomorphin which would provoke less intestinal motility and thus would cause lactose to become more exposed to the action of lactase (Cardoso *et al*., 2010). Camel milk contains low amount of β-lactoglobulin (Merin *et al*., 2001; Al-Alawi and Laleye, 2011) and β-casein (Al-Alawi and Laleye, 2011). The present of these two protein components in cow milk are responsible for allergies and due to this camel milk has little or no allergies effects. Camel milk also has high natural minerals (sodium, potassium, iron, copper, zinc and magnesium), vitamin C, B2, A and E (Konuspayeva *et al*., 2008) and large concentrations of insulin (52 micro unit/ml) than cow milk (16.32 micro unit/ml; Singh, 2001).

Camel milk has higher antimicrobial activity than cow milk partially due to higher concentration of lactoferrin in the former (220 mg/L) than in the latter (110 mg/L; Agrawal *et al*., 2005) and higher concentration of lysozyme in camel milk (288

μg /100 ml; El Agamy, 2000) compared to bovine milk (13 μg/ 100 ml; Piccinini *et al*., 2005).

Goat milk has special nutritional properties that make it attractive to consumers (Haenlein, 2004). It has a fat rich in medium-chain triglycerides (MCT) made up of fatty acids. These fatty acids are present up to 15–18% in goat milk, in contrast to only 5–9% in cow milk (Chilliard *et al*., 2006). From a therapeutic point of view, MCT are of special interest because of their particular roles of applying energy to the human metabolism instead of lipids to adipose tissues and their capability to limit and dissolve serum cholesterol (Haenlein, 2004). The higher proportion of short to medium fatty acids as well as reduced dimensions of casein micelles and fat globules in goat than in cow milk (Park *et al*., 2007) made goat milk easy to digest. Fermented goat milk products are perfect for the people allergic to cow milk. Goats' milk have free taurine, one of the final metabolic products of sulphur-containing amino acids (Park *et al*., 2007) which may have several biological functions: excellent source of energy to use in different metabolic processes (Minervini *et al*., 2009) and of neuronal activity (Jiang *et al*., 2004), enhance digestibility (Chesney and Hedberg, 2010), regulation of osteoblast metabolism (Menzies, 2002), immunological and antibacterial characteristics and prevention of cardiovascular damage (Warskulat *et al*., 2007) and treatment of fatty liver of children (Pugh, 2002; Menzies, 2002). In comparison to cow milk, regular consumption of goat milk significantly increased blood serum vitamin and haemoglobin level, increases the body weight, and mineralization of skeleton (Banu *et al*., 2007). Table 2.3 shows the chemical composition of camel, goat and cow milk

Table 2.3 Chemical composition of camel, goat and cow milks.

Component	Camel	Goat	cow
Fat %	3.24	3.21	3.5
Protein (Nx6.38)%	3.35	2.87	3.3
Casein (%)	2.8	2.4	2.6
Lactose %	4.52	4.10	4.6
Ash %	0.80	0.79	0.70
Total Solids %	11.91	11.05	12.2
Energy (kcal/liter)	670	622	620

(Source: Anonymous , 2003; Park *et al*., 2007).

2.2.2 Yogurt as a functional food

The most common functional dairy products are those containing probiotic bacteria, quite frequently enriched with prebiotics, such as yogurt (Saxelin *et al*., 2003). Yogurt is fermented milk obtained by lactic acid bacteria fermentation of milk and is a popular product throughout the world. The highest production and consumption of yogurt are recorded in countries in the Mediterranean, South Asia and central Europe which surround the possible origin of yogurt i.e. in the Middle East (Lore *et al*., 2005; Rahman *et al*., 2009; Shori, 2012).

Yogurt is recognized as a healthy food due to the beneficial action of its protein and its rich contents of potassium, calcium, protein and B vitamins (Table 2.4).

Table 2.4 The nutritional value of 100g yogurt

Constituents	Yogurt (low fat and plain)	Constituents	Yogurt (low fat and plain)	Constituents	Yogurt (low fat and plain)
Energy Value (kJ)	220.0				
Major Constituents (g)		Major Constituents (mg)		Vitamins	
Protein	5.00			A (IU)	70-130
Fat	1.00	Orotic Acid	4.00	Thiamine (µg)	37-50
Lactose	5.00	Fumaric Acid	8.00	Riboflavin (µg)	220-260
Galactose	1.50	Succinic Acid	19.00	Pyridoxine (µg)	40-54
Lactic Acid	1.00	Benzoid Acid	7.00	Cyanobalamine (µg)	0.1-0.35
Citric Acid	0.30	Cholesterol	7.00	Ascorbic Acid (µg)	0.1-0.1
Potassium	0.24	Urea	0.02	Tocopherol (µg)	30
Calcium	0.18	Glucose	30.00	Folic Acid (µg)	4
Phosphorus	0.14	5'-UMP	0.50	Nicotinic Acid (µg)	120-130
Chloride	0.18	3'+ 5'-GMP	0.40	Panthothenic Acid (µg)	380
Sodium	0.08	5'-AMP	0.10		
Bacterial mass	0.15	NAD	0.60	Biotin (µg)	1.2-4.0
				Cholinc (µg)	0.6

(Source: Cmckinley, 2005).

Yogurt is formed during the slow fermentation of milk lactose by the thermophilic lactic acid bacteria *S. thermophilus* and *L. delbrueckii ssp. Bulgaricus*. However, these bacteria are not indigenous to humans and cannot colonize the intestine to promote human health. Thus probiotics, mainly *Lactobacillus acidophilus* and *Bifidobacterium* spp. are added to improve the fermentation process for production probiotic yogurt (Donkor *et al*., 2006) and offer many advantages for the consumer. *S. thermophilus* and *L. delbrueckii ssp. Bulgaricus* are required to convert milk to yogurt whereas *L. acidophilus* and *Bifidobacterium* are added to increase the functional and health-promoting properties.

The food biotechnology industry has in recent years developed a huge number of commercial products containing a single probiotic strain or bacterial associations of various complexities (Schiffrin and Blum, 2001; Ranadheera *et al*., 2010). The

development of dairy products with new flavors and products with health benefits has the potential to increase sales and to consumers satisfaction. Dairy products in the marketplace are available to satisfied different consumer groups. For example, fat free dairy products for consumers with cardiovascular problems and lactose free dairy products for lactose intolerant people. In addition, folic acid enriched yogurt taken during initial stages of pregnancy help to prevent neural tube defects such as anencephaly, spina bifida, heart defects, facial clefts, limb deficiencies and urinary tract abnormalities (Boeneke and Aryana, 2007).

The key technological properties of yogurt bacteria in milk fermentation are acidification, flavour production and texture enhancement (Jolly *et al*., 2002; Welman and Maddox, 2003). These properties are strain dependent and they determine the final level of lactic acid, the main product of the metabolic activity of starter cultures as well as the acidification rate during yogurt production (Vinderola *et al*., 2000; Donkor *et al*., 2006). The differences in the acidification activity of different strains are associated to their particular aptitude to assimilate the nutritive compounds of the medium (Badis *et al*., 2004), in addition to other factors such as heat treatment of milk and fermentation conditions. The firmness and the viscosity of yogurt also depend on the composition of the starter culture (Jolly *et al*., 2002: Welman and Maddox, 2003). Some strains are used for their texture-improving properties because they are able to secrete exopolysaccharides (Ramchandran and Shah, 2009). Moreover, gels obtained by using ropy strains were less susceptible to syneresis compared to non-ropy strains (Attia *et al*., 2001). Yogurt aroma is a combination of both volatile compounds initially present in heat-treated milk and secondary metabolites synthesized by the starter culture (Ott *et al*., 2000). The major compounds found in yogurt samples are acetaldehyde, diacetyl, acetone, acetoin, 2-butanone, 2-propanone, ethanol, dimethyl

disulfide, 2, 3-pentanedione and some organic acids (Ott *et al*., 2000; Robinson *et al*., 2002). A wide variation in the levels of flavour compounds has been reported by using different strains of lactic acid bacteria (Ott *et al*., 2000).

Dairy products quality and reproducibility are obtained by using industrial starters. Thus it is important to isolate and characterize the artisanal strains that provide characteristics sensorial properties similar to those of traditional products. New strains of lactic acid bacteria have been isolated from different dairy products such as raw milk (Alonso-Calleja *et al*., 2002 ; Badis *et al*., 2004) fermented milk (Abdelgadir *et al*., 2001 ; Xanthopoulos *et al*., 2001) and cheeses (Durlu-Ozkaya *et al*., 2001 ; Alonso-Calleja *et al*., 2002). In addition, the selection of optimum incubation temperatures affects overall yogurt quality. The high temperature yogurt method which involves incubation for 3 hours at 42°C (short time) is used in yogurt production because it allows faster production of yogurt and is more economical for dairy plants. In contrast, the low temperature method involves incubation for 7-8 hours at 30-37°C (Degeest *et al*., 2002; Güler-Akin *et al*., 2007). Ideally, the incubation time should not be lower than 3 hours to ensure adequate production of aroma substances and to avoid over-acidification. In fact, the longtime incubation method is generally favored for improved physical properties of yogurt and development of more flavor substances (Degeest *et al*., 2002; Güler-Akin *et al*., 2007).

2.2.2.1 Lactic acid bacteria (LAB)

The intestinal microbial population is a dynamic ecosystem of high complexity, consisting of an estimated number of 10^{14} microorganisms including more than 400 bacterial species (Herías *et al*., 2001). It plays a vital role by providing the host with enzymes necessary for assimilation and/or synthesis of certain nutrients,

as well as in the detoxification of harmful dietary compounds (Ruas-Madiedo *et al*., 2002; Güler-Akın and Serdar Akin, 2007). The gastrointestinal microbiota also represents a natural barrier against pathogens (Kolida *et al*., 2006) and stimulates bowel motility and the immune system (Isolauri *et al*., 2001). Lactic acid bacteria and their metabolites play a key role in enhancing microbiological quality and shelf life of fermented dairy products (Lourens-Hattingh and Viljoen, 2001; Leroy and De Vuyst, 2004).

LAB has an essential role in most fermented food for their ability to produce various antimicrobial compounds promoting probiotic properties (Temmerman *et al*., 2002), including antitumoral activity (Brady *et al*., 2000; Ostlie *et al*., 2003), reduction of serum cholesterol (Jackson *et al*., 2002), alleviation of lactose intolerance (De Vrese *et al*., 2001), stimulation of the immune system (Isolauri *et al*., 2001) and stabilization of gut microflora (Saarela *et al*., 2002). LAB strains synthesize short chain fatty acids, vitamins and exopolysaccharides (EPS) which are employed in the manufacture of fermented milk to improve its texture and viscosity (Ruas- Madiedo *et al*., 2002). The bacterial EPS from LAB have been studied not only because of their role in texture, rheology and mouthfeel of fermented dairy products but also due to their immunostimulant properties (Purwandari *et al*., 2007; Table 2.5).

Lactobacillus delbrueckii subsp. *bulgaricus* (*Lb. bulgaricus*) is a thermophilic LAB regularly used with *Streptococcus thermophilus* in the production of yogurt that are less susceptible to syneresis (Ramchandran and Shah, 2009). EPS from *L. bulgaricus* are heteropolysacharides consisting of repeating units of monomers such as glucose, galactose and rhamnose (Briczinski and Roberts, 2002). Other strains are able to produce phosphopolysaccharides (Ramchandran and Shah, 2009). A general feature of *L. bulgaricus* is the production of ESP fractions with two different

molecular weights (Petry *et al*., 2003). *L. bulgaricus* ferments lactose, glucose, fructose and mannose (De Vrese *et al*., 2001) but generally does not grow on galactose. Lactose is the preferred sugar among the sugars fermented by these bacteria. This phenotype is an example of bacterial adaptation to a given growth media since this bacterium is usually found and extensively cultured in milk which has a high concentration of lactose (Chervaux *et al*., 2000). In addition, some LAB strains produce mannitol with several health promoting effects such as *Lactococcus lactis* (Wisselink *et al*., 2002). New sources of nutrients should be more exploited for varying the human diet and also to benefit from new functional ingredients and natural food components.

Table 2.5 Contribution of LAB to functionality of fermented products.

Functional property	Contribution to food functionality
Production of exopolysaccharides, amylase, aroma generation	Safety and/or organoleptic
Bacteriophage resistance, prevention of over acidification in yogurt	Technological
Production of bioactive compounds, nutraceuticals, reduction of toxic compounds and anti-nutritional compounds	Nutritional and health

(Compiled from Leroy and De Vuyst, 2004)

2.2.2.2 Probiotics

The word probiotic, derived from the Greek language, means for life is defined as 'living microbial feed supplements added to the diet and offer beneficial effects on the host by enhancing their intestinal microflora balance' (Fuller, 1989). It is now popularly referred to as being a mono- or mixed culture of live microorganisms (e.g. as dried cells or as a fermented product) which usefully effects the host by enhancing the properties of the native microflora (Huis in't Veld and Havenaar, 1991). By this definition bio-yogurt contains live microorganisms can add beneficial effects in the

gastrointestinal tract of the host and improve the health status (Lourens-Hattingh and Viljoen, 2001). To complement probiotics, "prebiotics" defined as selective non-digestible carbohydrate food sources, are becoming increasingly used in promoting the proliferation of bifidobacteria and lactobacilli (Gibson *et al*., 2004).

Common probiotics in use include members of LAB such as *Lactobacillus* spp., *Bifidobacterium* spp. and selected species of yeasts. These bacteria are added to fermented milk because they help to improve the balance of the intestinal microflora of the host upon ingestion (Saarela *et al*., 2002; Bai *et al*., 2010). In addition these probiotics contribute to the development of the immune system, improvement of normal intestinal morphology and maintaining a chronic and immunological balanced inflammatory response (Tannock, 2004). The growth of these probiotics showed inhibitory activities toward the growth of pathogenic bacteria via the creation of inhibitory compounds such as bacteriocins or reuterin, hydrogen peroxide, reduced pH as a result of accumulation of organic acids and competitive adhesion to the epithelium (Kolida *et al*., 2006). Probiotics also produce enzymes that help in the digestion of food in addition to B-complex vitamins production and neutralization of pathogenic microorganisms responsible for infections and diarrhea (Sanders, 2000; Shah, 2000).

Viability and metabolic activity of the bacteria are important considerations in probiotic inclusion in foods. This is because the bacteria need to survive in the food during shelf life and gastrointestinal digestion i.e. acidic conditions of the stomach and degradation by hydrolytic enzymes and bile salts in the small intestine (Tannock *et al.*, 2000). To ensure health benefits can be delivered by food containing probiotics, products sold with any health claims must meet the standard of a minimum level for probiotic bacteria ranging from 10^6 to 10^7 cfu/ml at the expiry date (Madureira *et al*.,

2011). The viability of probiotic cultures in fermented milk can be affected with several factors include acidity, pH, temperature, the presence of other microorganisms, oxygen content, hydrogen peroxide produced by yogurt bacteria and others (Shah, 2000). The growth of probiotic bacteria in milk is slow because of lack of proteolytic activity and thus the addition of peptides and amino acids such as cysteine in milk improved the survival of probiotic such as bifidobacteria (Shihata and Shah, 2000). Moreover, incubation temperature between 37°C and 40 °C could also be used to develop probiotic growth because this is the optimal temperature for probiotic species growth (Ostlie *et al*., 2003; Güler-Akin and Serdar Akin, 2007).

2.2.2.2.1 Therapeutic value of probiotic

The ingesting of probiotic products is useful in sustaining good health, restoring body vigour in combating intestinal and other disease disorders (Figure 2.1).

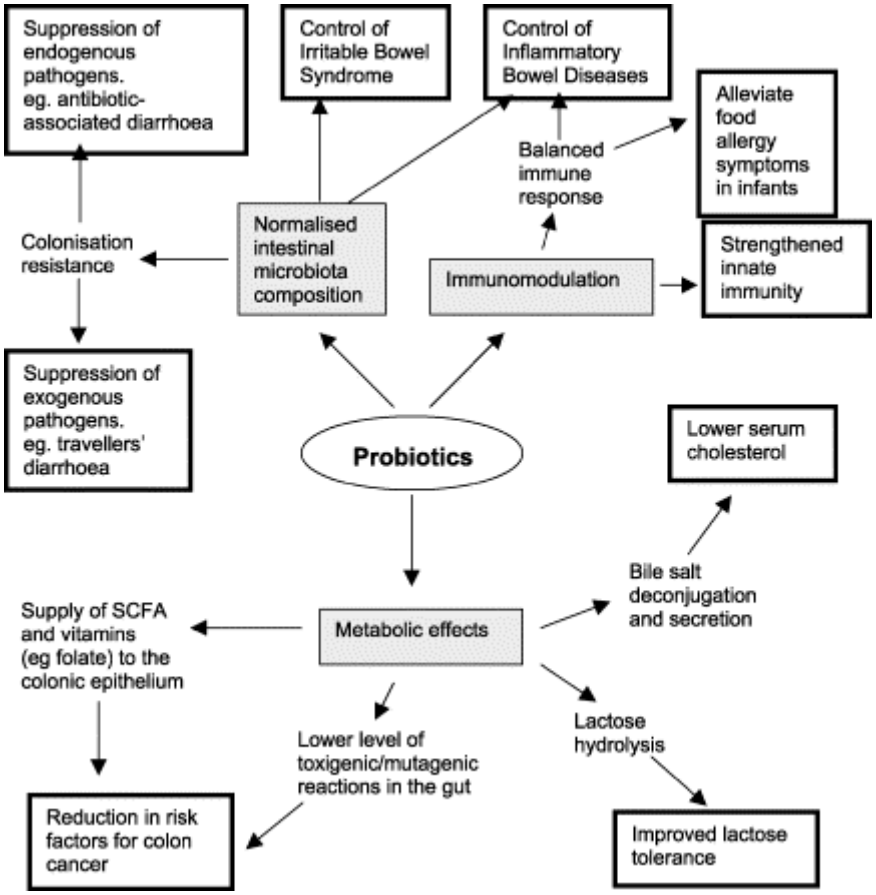


Figure 2.1 Proposed health benefits stemming from probiotic consumption (Saarela *et al*., 2002).

These are further discussed in the following sections.

1) Control of intestinal infections

Probiotic bacteria such as lactobacilli and bifidobacteria have antimicrobial activity (El Agamy, 2000). Both *L. acidophilus* and *B. bifidum* for instance inhibit numerous of the generally known food borne pathogens (Schiffrin and Blum, 2001; Rafter, 2003; Goderska and Czarnecki, 2007). The consumption of milk cultured with *L. acidophilus* or *B. bifidum* or both for preventative control of intestinal infections (Rafter, 2003) can be occurred via:

- Inhibitory/antimicrobial substances production such as hydrogen peroxide, bacteriocins, organic acids, antibiotics and deconjugated bile acids.

- Competitive antagonist's action for example, through competition for adhesion sites and nutrients.

- Immune system stimulation.

The organic acids produced by the probiotics caused reduction in the pH and change the oxidation reduction potential in the intestine which leading to antimicrobial action. In addition, the limited oxygen content in the intestine can help the organic acids to inhibit especially pathogenic gram-negative bacteria type's e.g. coliform bacteria (Nava *et al*., 2005; Ogawa *et al*., 2006; Neal-McKinney *et al*., 2012).

2) Reducing lactose intolerance

The lack of *β-D*-galactosidase in the human intestine results in the inability to digest lactose adequately follows by different degrees of abdominal pain and discomfort (De Vrese *et al*., 2001). LAB used as starter cultures in milk during fermentation and probiotic bacteria such as *L. acidophilus* and *B. bifidum* produce *β-D* –galactosidase that digest lactose which helps consumers having better tolerance for fermented-milk products (De Vrese *et al*., 2001). This utilization is referred to intra-intestinal digestion by *β-D*-galactosidase. Increased digestion of lactose may not only occur by hydrolysis of the lactose before consumption, but also in the digestive tract after ingesting of milk containing *L. acidophilus* (De Vrese *et al*., 2001). Thus the continued utilization of lactose inside the gastrointestinal tract is governed by the survival of the lactobacilli.

3) Reduction in serum cholesterol levels

The consumption of fermented milk could significantly reduce serum cholesterol (Jackson *et al*., 2002). This is good news for hypercholesterolemic persons since substantial decrease in plasma cholesterol level plays a role in reduction heart attacks risk (Agerholm-Larsen *et al.*, 2000; Hitti, 2006). Appreciable amounts of

cholesterol metabolism occur in the intestines before passage to the liver. This could provide some explanation on the association between the presence of certain *L. acidophilus* strains and some *bifidobacteria* species with the ability to reduce cholesterol levels inside the intestine. Cholesterol co-precipitates with de-conjugated bile salts as the pH drops as a result of lactic acid production by LAB (Begley *et al*., 2006). The role of *bifidobacteria* cultures in reducing serum cholesterol is poorly known. Feeding of *bifidobacteria* to rats redced serum cholesterol which may involve HMG-CoA reductase (An *et al*., 2011). Sudha *et al*. (2009) suggested a factor is formed in the milk during fermentation that inhibits cholesterol synthesis in the body. Alternatively, *L. acidophilus* may de-conjugate bile acids into free acids which are excreted faster from the intestinal tract than are conjugated bile acids. Subsequently, the production of fresh bile acids from cholesterol can decrease the total cholesterol level in the body (Begley *et al*., 2006). A third hypothesis is that at lower pH values the production of lactic acid by LAB resulted in co-precipitation of cholesterol with de-conjugated bile salts cause reduction of cholesterol (Sudha *et al*., 2009).

4) Anti-carcinogenic activity

probiotics are known to have antitumour action related to the inhibition of carcinogens and/or inhibition of bacteria that convert pro-carcinogens to carcinogens (Wollowski *et al*., 2001; Rafter, 2003), improvement of the host's immune system (Isolauri *et al*., 2001; Ogawa *et al*., 2006) and/or reduction of the intestinal pH to decrease microbial activity. Studies in rats showed that probiotic bacteria in yogurt and fermented milk inhibited tumor formation and proliferation (Wollowski *et al*., 2001; Rafter, 2003).

5) Prevention of colon cancer

Probiotics have shown capability to reduce risk of colon cancer owing to their ability to bind with heterocylic amines; carcinogenic substances that formed in cooked meat (Wollowski *et al.,* 2001). Most human studies have reported that probiotic may apply anti-carcinogenic effects by reducing the activity of ß-glucuronidase, an enzyme which produces carcinogens in the digestive system (Brady *et al*., 2000). Although human intervention studies demonstrate the reduced presence of biomarkers associated with colon cancer risk. The evidence that probiotics decrease colon cancer occurrence in humans is lacking (Goossens *et al*., 2003). Thus the subject of probiotic uptake and cancer prevention is still open to further investigation.

6) Anti-diarrhea effects

Diarrhea can have many causes and its effects on flushing out the bacteria living in the intestine leaves the body vulnerable to opportunistic harmful bacteria. It is important to replenish the body with probiotics during and after the incidence of diarrhea. The advantages of probiotics in the inhibition and treatment of a range of diarrhea illnesses, such as acute diarrhea caused by rotavirus infections, antibiotic-associated diarrhea, and travelers' diarrhea have been extensively studied (Reid *et al*., 2003). LAB may possibly reduce diarrhea in some ways including competition with pathogens for nutrients and space in the intestines (Reid *et al*., 2003). For instance *L. casei* and *B. bifidum* effectively prevent or treat infantile diarrhea (Reid *et al*., 2003) by several ways:

1) Compete with pathogens for nutrients and space in the intestines.

2) Some metabolism by-products such as acidophilin and bulgarican produced by *L. casei, L. acidophilus* and *L. bulgaricus* have a direct effect against inhibition of pathogens growth.

3) Enhance immune system which has effect against diarrhea, particularly through alleviation of intestinal inflammatory responses and intestinal immunoglobulin A (IgA) responses which cause create gut-stabilizing effect (Isolauri *et al*., 2001; Reid *et al*., 2003).

7) Improving immune function and preventing infections

Lactic acid bacteria are assumed to have some valuable effects to enhance immune function. These include the improvement of immune function by increasing the number of IgA producing plasma cell, increasing or educating phagocytosis other than increasing the proportion of T lymphocytes and natural killer cell (Reid *et al.*, 2003). They may protect against pathogen and to prevent or treat infections such as postoperative infections (Broussard *et al.,* 2004), respiratory infections (Hatakka *et al.*, 2001), and the growth of *Helicobacter pylori,* a bacterial pathogen responsible for type B gastritis and peptic ulcers.

8) Anti-inflammatory effects

Probiotics have been shown to modulate inflammatory and hypersensitivity reactions. They can affect the intestinal flora and may have beneficial effects in inflammatory bowel disease (IBD), which includes ulcerative colitis, Crohn's disease and pouchitis (Reid *et al.,* 2003). Clinical studies suggest that they can prevent reoccurrences of IBD in adults (Reid *et al.,* 2003), as well as enhance milk allergies and decrease the risk of atopic eczema in children (Kirjavainen *et al.*, 2003).

2.3 Fermentation

Fermentation is an anaerobic process of degradation of organic nutrients through biological reactions which involve no oxidative phosphorylation to maintain the production of ATP by glycolysis (Klein *et al*., 2006). During fermentation pyruvate is break down to several different compounds. The primary purposes of

fermentation by microorganisms are the furnishing of energy for their metabolism and growth (Klein *et al*., 2006). The nutrients in milk i.e. carbohydrates, amino acids, lipids, vitamins and minerals are required by LAB for growth and in the process will undergo biochemical changes through the activities of microorganisms. An important feature of this LAB fermentation of milk is the release of some amino acids during simulated gastrointestinal digestion as shown by the modification of protein elution profiles obtained after digestion with trypsin (Wattiaux and Howard, 2000; Hernandez-Ledesma, 2004). Proteolysis during fermentation may lead to the formation of novel peptides during gastrointestinal digestion.

The changes in milk components after fermentation include:

a) Increased formation of organic acids (sucinic acid, fumaric and others), lactic acid, galactose, glucose and polysaccharide whereas lactose content is decreased (Shah, 2000; Ostlie *et al*., 2003).

b) Peptides and free amino acids increase whereas proteins decrease after fermentation (Möller *et al*., 2008).

c) Urea decreases and ammonia increases after fermentation.

d) Volatile and long chain free fatty acids increase whereas fat decreases

e) Vitamins such as folic acid and others increase and vitamins such as B12, C and Biotin decrease.

However, fermentation has slight effect on the mineral content of milk and consequently the total mineral content is unchanged in the yogurt (Amellal-Chibane and Benamara, 2011).

2.3.1 Lactic acid fermentation

Fermentation can be divided into two types on the basis of formation of the end products i.e homolactic fermentation and heterolactic fermentation. Homolactic

fermentation is the production of lactic acid from pyruvate; alcoholic fermentation is the conversion of pyruvate into ethanol and carbon dioxide and heterolactic fermentation is the production of lactic acid as well as other acids and alcohols.

a) Homolactic fermentation

It is the simplest pathway, homo-fermentative LAB alter the existing energy source (sugar) into lactic acid via pyruvate to yield energy and to equilibrate the redox balance (Leroy and De Vuyst, 2004) and no gas is produced. Homolactic fermentation is responsible for soured milk in the production of many dairy products. It is a characteristic of *Lactobacillus, Streptococcus* and *Bacillus.* The fermentation of 1 mole of glucose yields two moles of lactic acid.

b) Heterolactic fermentation

Heterolactic fermentation will produce ethanol, CO_2, and lactic acid. It is a characteristic of *Leuconostoc* and *Lactobacillus*. The fermentation of 1 mole of glucose yields 1 mole each of lactic acid, ethanol and carbon dioxide.

During fermentation of milk the main role of LAB is to utilize lactose as a substrate and alter it into lactic acid. Lactose, taken up as the free sugar by the LAB is cleaved by internal enzyme *β*-galactosidase to glucose and galactose. Both glucose and galactase are metabolized concurrently via the glycolytic and *D*-tagatose 6-phosphate pathways respectively (Miallau *et al*., 2007). In addition galactose can also be further metabolized by enzymes of the Leloir pathway (Miallau *et al*., 2007).

2.4 Rheological and physical properties of yogurt

The fermentation of milk during yogurt making causes irreversible changes in the properties of milk. Heat treatment a normal process in yogurt making also results in considerable thermal denaturation of the whey proteins and subsequent partial fixation on the casein micelles that lead to the formation of stronger gels (Sendra *et*

al., 2010). Physical characteristics of yogurt such as absence of whey separation and apparent viscosity are essential facets of the quality and overall sensory consumer satisfaction of yogurt (Lee and Lucey, 2010). To achieve this, the design of flow processes, quality control, storage stability, sensory assessments of consistency and mechanical processing are important in predicting the rheological properties of fermented dairy products (Kealy, 2006).

Yogurt exhibits time-dependent shear thinning behaviour and thus it is not a true thixotropic material since structural breakdown owing to shear is not completely reversible once the shear stops (Lee and Lucey, 2010). The rheological characteristics of yogurt are controlled by milk composition, dry matter content, temperature and time of milk heat pre-treatment, type and quantity of starter culture employed to inoculate the milk, fermentation temperature and storage conditions of the final product (Remeuf *et al*., 2003; Sodini *et al*., 2005; Girard and Schaffer-Lequart, 2007; Renan *et al*., 2009). The textural properties of yogurt may be improved by adding some alternative materials including gelatine (Gonçalvez *et al*., 2005), pectin (Matia-Merino *et al*., 2004), k-carrageenan (Sohrabvandi *et al*., 2013), inulin (Guven *et al*., 2005; Ozer *et al*., 2005; Guzel-Seydim *et al*., 2005) and dietary fibres (Fagan *et al*., 2006).

2.5 Proteolysis of milk protein

Lactic acid bacteria are fastidious microorganisms with regard to nutritional requirements (Guarner *et al*., 2005; Lee *et al*., 2001). They have limited biosynthetic ability hence the requirement for an exogenous source of amino acids (such as isoleucine, leucine, valine, histidine and methionine) or peptides for optimum growth (Vermeirssen *et al*., 2002; Donkor *et al*., 2005; Papadimitriou *et al*., 2007). Since milk is deficient in such low-molecular components the growth of the starter bacteria

depends on their proteolytic systems to hydrolyze caseins (Ong and Shah, 2008). The amino acids released by the bacteria and accumulated in the milk affect the nutritional potential and biological value of the fermented product. Amino acids may not be directly contributory to the flavour and aroma of fermented milk. However, they act as precursors for a number of reactions that produce carbonyl compounds (Considine *et al*., 2000). The spectrum and level of free amino acids in fermented milk depend on several variables such as type of milk, composition of the starter, method of preparation and storage conditions. Caseins are the main source of amino acids ensuring 98% of the growth (Matsuura *et al*., 2005; Salami *et al*., 2011). The contribution of caseins to the provision of essential amino acids depends on the type of proteinase (Salami *et al*., 2011). Proteinase is capable of initiating the degradation of casein to oligopeptides which are transported into the bacteria and afterwards degraded through a complex sequence of intracellular peptidases (Salami *et al*., 2011). The amino acid necessity and production activity in mixed cultures can be modified using selected strains of lactobacilli (Lee *et al*., 2001) capable of intracellular splitting of oligopeptides or of attacking peptides and proteins in the nutrient medium by means of the proteolytic enzyme systems synthesized (Lee *et al*., 2001).

In the mixed yogurt culture *L. bulgaricus* has higher proteolytic activity than *S. thermophilus* and thus the free amino acids produced by *L. bulgaricus* are also used by *S. thermophilus* (Gobbetti *et al*., 2002; Pescuma *et al*., 2011). The total amino acid content in yogurt reflects the balance between proteolysis and assimilation by bacteria (Gobbetti *et al*., 2002). The pathway of peptide hydrolysis in yogurt bacteria ensures the release of amino acids respectively and the growth relation between *S. thermophilus* and *L. bulgaricus* (Shihata and Shah, 2000; Robinson and Tamime, 2002; Pescuma *et al*., 2011). Proteolysis in fermented milk is mainly related to yogurt

cultures which explain the high level of proteolysis in fresh biokefir after storage compared to other fermented milk (Gobbetti *et al*., 2002). The pathway of casein catabolism through yogurt organisms can be altered via endopeptidase activity as described for strains of *S. thermophilus* and *Lactococcus lactis ssp*. lactis, and aminopeptidase as described for *L. bulgaricus* and *Lactobacillus helveticus* (Gobbetti *et al*., 2002).

2.5.1 Proteolytic agents in yogurt

During yogurt fermentation proteolysis in yogurt is catalysed by enzymes from:

(1) Coagulant (e.g. chymosin, pepsin, microbial or plant acid proteinases).

(2) Milk (plasmin and perhaps cathepsin D and other somatic cell proteinases).

(3) The starter or non-starter culture.

(4) Secondary cultures (e.g. *P. camemberti, P. roqueforti*, *Propionibacterium sp., B. linens* and other coryneforms).

(5) Exogenous proteinases or peptidases, or both, which are produced during yogurt fermentation.

The initial hydrolysis of caseins during yogurt fermentation is occurred by the coagulant and to a minor range by plasmin which caused the creation of large- (water-insoluble) and intermediate-sized (water-soluble) peptides that are released afterward by the coagulant and enzymes from the starter and non-starter microflora of the yogurt (Donkor *et al*., 2005; Papadimitriou *et al*., 2007). The extracellular cell envelope-associated proteinase of *Lactococccus* (lactocepin, endopeptidase lactocepin) contributes to the formation of small peptides in yogurt. This occurred possibly by the hydrolysis of larger peptides produced from αs1-casein by chymosin or from β-casein by plasmin (Fox *et al*., 2000; Harte *et al*., 2003). The peptidases (which are intracellular) released from lysed cells are responsible for the breakdown

of short peptides and the liberation of free amino acids (Gobbetti *et al*., 2002; Korhonen and Pihlanto, 2003). The resulted products of proteolysis are free amino acids. Thus, their quantity in yogurt at any phase of fermentation is the net result of the released amino acids from casein, their degradation to catabolic products and maybe some synthesis by the yogurt microflora (Considine *et al*., 2000). Therefore proteolysis can vary significantly between variety e.g. coagulants which are completely denatured by fermentation temperature used in yogurt manufacture.

2.5.2 Functionality of bioactive peptides

The physiologically active components of proteins in the food are being widely recognized. The physiological action of natural proteins present in raw food materials can exert directly or indirectly upon enzymatic hydrolysis *in vitro* or *in vivo*. Dietary proteins deliver a good source of naturally active peptides (Korhonen and Pihlanto, 2006). These peptides are inactive within the structure of the native protein but can be liberated via hydrolysis with digestive enzymes and/or proteolytic activity of microorganisms or plants. It is currently well-known that biologically active peptides are generating from food proteins during fermentation with LAB and gastrointestinal digestion. The production and properties of bioactive peptides have been reported in several studies (Fujita *et al.*, 2003; Fitzgerald and Murray, 2006; Korhonen and Pilanto, 2006; Papadimitriou *et al*., 2007; Shimizu and Son, 2007; Korhonen, 2009).

Bioactive peptides have been defined as particular protein fragments that have physiological effect on human body and may eventually influence health (Kitts and Weiler, 2003). Oral administration of bioactive peptides has significant effect on functions of human body systems such as cardiovascular, digestive, immune and

nervous systems depending on the amino acid sequence of these peptides. Therefore, the potential of different dietary peptide sequences to stimulate human health by lowering the risk of chronic diseases or improving immune system has been widely studied (Papadimitriou *et al*., 2007; Shimizu and Son, 2007; Korhonen, 2009). Several known peptide sequences showed therapeutic properties such as anti-microbial, anti-oxidative, anti-thrombotic, anti-hypertensive and immunomodulatory activities (Fitzgerald and Murray, 2006; Figure 2.2). These activities are relied on the amino acid composition and sequence of these peptides. The length of bioactive peptides is generally 2–20 amino acid residues and some peptides can exert multi-functional properties (Hartmann and Meisel, 2007). Nowadays, the most important source of bioactive peptides is milk proteins because these peptides have been identified and isolated from milk protein hydrolysates and fermented dairy products (Korhonen and Pilanto, 2006; Papadimitriou *et al*., 2007; Shimizu and Son, 2007; Korhonen, 2009; Salami *et al*., 2011).

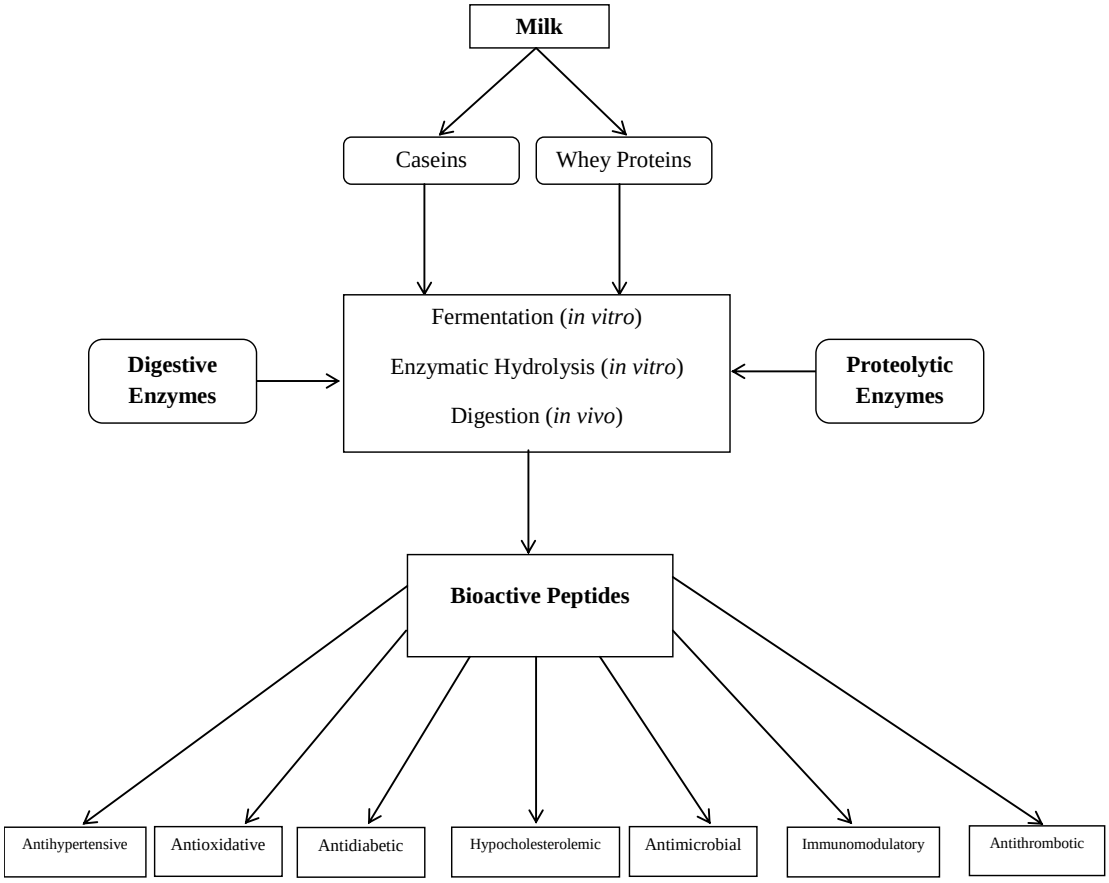


Figure 2.2 Potential means of formation of biologically active peptides from major milk proteins.

2.6 Hypertension

Hypertension is the most common cardiovascular diseases. It is a universal problem of epidemic proportions, that affects 10% – 20% in the adult population and 40% - 50% in people aged 50 or older (Karakurt and Kasikci, 2012). It is one of the serious chronic health problems associated with several diseases such as arteriosclerosis, stroke, myocardial infarction and end-stage renal disease. So, the role of the rennin–angiotensin system (RAS) in cardiovascular physiology is well studied and exploited pharmacologically (see Figure 2.3).

The angiotensin converting enzyme (ACE), a component of RAS catalyzes the formation of the strong pressor agent angiotensin II from angiotensin I help to control

high blood pressure (Unger, 2002; Coates, 2003). ACE inhibitors are competitive substrates for ACE. The primary structural control this inhibitory response is the C-terminal tripeptides sequence. These peptides may interact with subsites s_1, $s_{1'}$ and $s_{2'}$ at the active site of ACE (Figure 2.4). Substrates and inhibitors containing hydrophobic amino acid residues in the three C-terminal positions are preferable for ACE (Ramchandran and Shah, 2009). For example, aliphatic, basic and aromatic residues are binding in the penultimate positions, whereas aromatic, proline and aliphatic residues are binding in the ultimate positions. The positive charge of arginine or the ε-amino group of lysine at the C-terminus has been shown to play role of several ACE- peptides (Vermeirssen *et al*., 2003). Several ACE inhibitors such as captopril, enalapril, lisinopril and temocapril are known for the management of hypertension. All of these drugs produced side effects thus, justifying the search for natural ACE inhibitors for safe and economical use (Coates, 2003; Kang *et al.*, 2003).

Several compounds from plants have been recognized to possess *in vitro* ACE inhibitory activity including hydrolysable tannins, phenylpropanes, proanthocyanidins, flavonoids, xanthones, fatty acids, terpenoids, alkaloids oligosaccharides and peptide amino acids (Park *et al*., 2003; Ramesar *et al*., 2008). Other resources of anti- ACE peptides have been identified from plant proteins (water-soluble extracts of broccoli, mushroom, garlic, buckwheat and wine), protein hydrolysates of soybean, mung beans, sunflower, rice, corn, wheat, buckwheat and spinach (Guang and Phillips, 2009) as well as from animal (chicken muscle, sardine and tuna muscle).

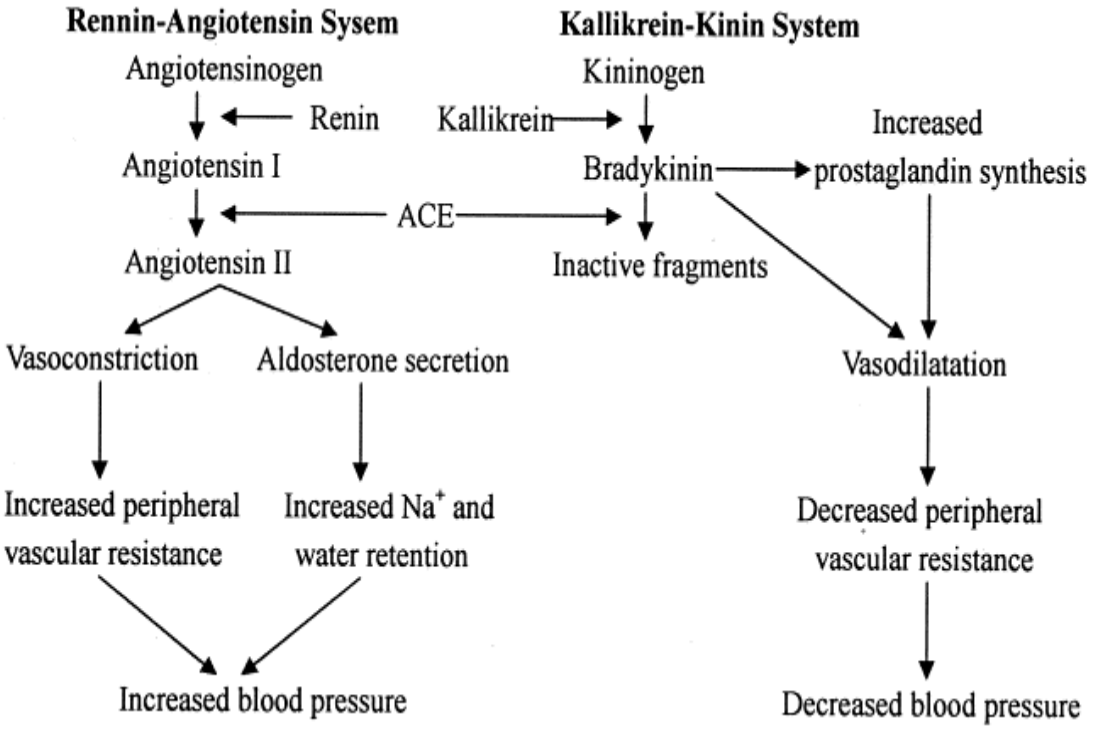


Figure 2.3 Regulation of blood pressure: role of angiontensin-I-converting enzyme in rennin- angiotensin system and Kallikrein-Kinin system, adapted from Li *et al*. (2004).

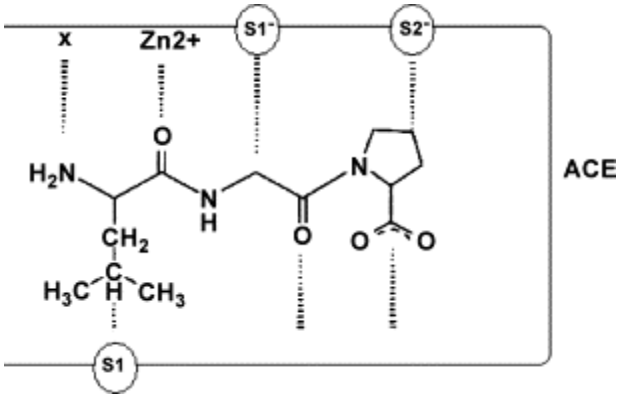


Figure 2.4 Active site of ACE showing the three subsites for interaction (Source: Hong et al., 2008).

2.6.1 Milk-protein-derived peptides with antihypertensive effects

Recently the ingesting of yogurt has increased because of the fact that this dairy product fulfills several of human nutritional requirements. It is a ready to eat food moderately low in fat and fulfil the requirements of human nutrition. Recently, the use of functional foods has been increased because of increasing consciousness among people of the linkage between food and health (Fitzgerald and Murray, 2006).

Yogurt considered to be functional food particularly when it contains probiotic bacteria. It is also offer additional benefits related to the bioactive peptides that are generated during manufacturing and storage. Presently, an excessive attention has been focused on bioactive-peptides that can reduce the blood pressure in hypertensive people (Fitzgerald and Murray, 2006).

The action of these peptides is relying on the inhibition of angiotensin-I converting enzyme (ACE, E.C. 3.4.15.1). However, the activity of these peptides could include various complex mechanisms that may increase the therapeutic properties of yogurt followed by further benefits for consumer health (Fuglsang *et al*., 2003; Ijäs *et al*., 2004; Vermeirssen *et al*., 2004). ACE is an enzyme that has an important role in the rennin-angiotensin system by controls the arterial blood pressure and the balance of water and salt in the body. An elevation in blood pressure is occurred when the enzyme catalyzes the hydrolysis of angiotensin I to angiotensin II that act as strong agent (vasoconstrictor) with the aid of vasodilative action resulted of the degradation of bradykinin (Coates, 2003). The proteolytic activity of LAB during milk fermentation and/or the action of pure proteinases on milk proteins leaded to produce numerous of peptides with anti-hypertensive properties (Gobbetti *et al*., 2002; Tauzin *et al*., 2002; Hernandez-Ledesma *et al*., 2004). Several peptides have been shown anti-hypertensive action on spontaneously hypertensive rats and on small groups of human volunteers (Vermeirssen *et al*., 2005; Fitzgerald and Murray, 2006). In addition, several ACE inhibitory peptides have been isolated from the enzymatic hydrolysis of fermentation milk with lactic acid bacteria (Gobbetti *et al*., 2002; Papadimitriou *et al*., 2007; Nejati *et al*., 2013) or chemical synthesis of peptides according to milk protein sequences (Miller *et al*., 2007).

The degradation of milk proteins with proteinases from *L. helveticus* produced peptides with ACE-inhibiting activity had a significant antihypertensive effect in spontaneously hypertensive rats (Tuomilehto *et al*., 2004). The same effect was observed with fermented milk containing *L. helveticus* (Nakamura *et al*., 1995). Two tripeptides valyl-prolyl-proline (Val-Pro-Pro; VPP) and isoleucyl-prolyl-proline (Ile-Pro-Pro; IPP) were identified as the bioactive peptides which were responsible for this effect (Nakamura *et al*., 1995). A liquid chromatography–mass spectroscopy (LC–MS) method with Ala-Pro-Pro as an internal standard was used for the quantitative determination of these two peptides in casein hydrolysates (Matsuura *et al*., 2005). In several short- and long-term human studies where VPP and IPP containing fermented milk products were ingested a blood-pressure lowering effect was observed (Seppo *et al*., 2002; Seppo *et al*., 2003; Tuomilehto *et al*., 2004; Bütikofer *et al*., 2008).

2.6.2 Production of fermented dairy products with ACE inhibitory peptides

In dairy products, the production of ACE inhibitory and anti-hypertensive peptides *in situ* aroused a lot of interest from scientists since this provides further therapeutic properties to fermented dairy products. During milk fermentation and production of yogurt a excessive amount of peptides are liberated from milk proteins as a result of the action of plasmin (indigenous milk enzyme) and proteolytic activity of starter and non-starter LAB.

Ferment milk with highly proteolytic species of LAB is widely used to increase the amount of bioactive peptides in fermented dairy products. Thus, selecting the right strains or mixture of strains with highly proteolytic activity and lysis tendency is big challenge to in this approach. Bacterial species ought to not be excessively proteolytic to spoil the product by other peptides such as bitter peptides

but yet to provide a high proteolysis of bioactive peptides such as ACE-inhibitory peptides. Since the concentration of ACE-inhibitory peptides appears to rely on a balance between their formation and degradation into inactive peptides and amino acids subject to storage periods and conditions (Ryhänen *et al*., 2001; Gobetti *et al*., 2004).

2.7 Diabetes

Diabetes is a condition whereby there is an elevation of blood glucose. Insulin produced by the pancreas is an important hormone needed by ours body because it enables glucose to be transported into the cells (Figure 2.5). Under diabetic condition the cells may not respond properly to insulin or the body does not produce enough insulin, or both. This situation will cause glucose accumulation in the blood that leads to so many complications (Li *et al*., 2007).

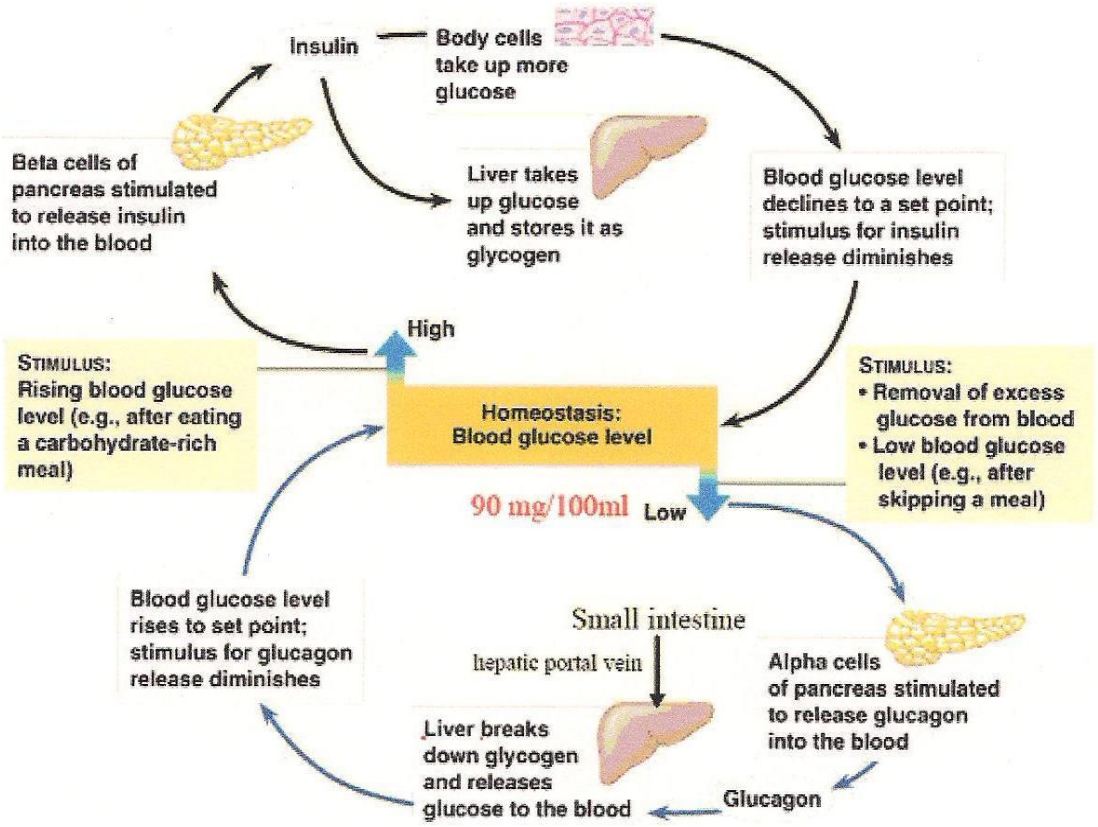


Figure 2.5 The role of the pancreas in glucose homeostasis (Cheng and Fantus, 2005).
There are two types of diabetes:

Type 1: This type of diabetes results from the failure of the body to produce insulin (Khardori and Pauza, 2003). This type also known formerly as insulin-dependent diabetes mellitus (IDDM) which is childhood diseases common in developed European countries and some newly prosperous countries (Khardori and Pauza, 2003). The pathogenesis of type 1 diabetes is summarized in (Figure 2.6).

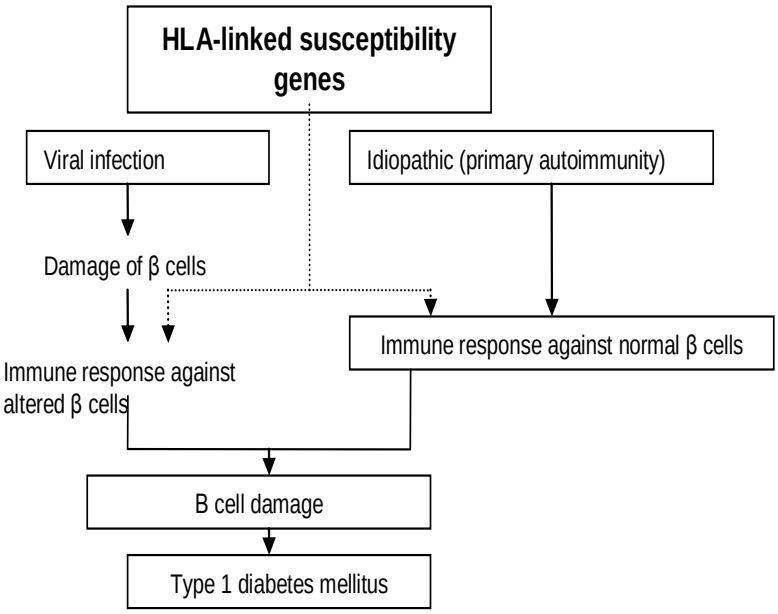


Figure 2.6 Pathogenesis of type 1 diabetes mellitus (Kumar*et al*.,1992).

Type 2: This kind of diabetes results from insulin resistance, a condition whereby cells failed to respond to insulin stimulation (Bazzano *et al.*, 2005). This type, also known as non-insulin dependent diabetes mellitus (NIDDM), develops in middle or later life and the complication attributed to type 2 diabetes include insulin resistance, hyperinsulinemia, impaired insulin secretion, reduced insulin mediated glucose uptake and utilization (Tiwari and Rao, 2002). The pathogenesis of insulin resistance and type 2 diabetes is summarized below (Figure 2.7). The increase in the levels of blood glucose resulting in hyperglycemia in type 2 diabetes patients occurs because of hydrolysis of starch by pancreatic α-amylase enzymes and absorption of glucose by intestinal α-glucosidases (Figure 2.8). Thus, an effective strategy in the management of type 2 diabetes is the strong inhibition of α-glucosidases and mild inhibition of α-amylase (Krentz and Bailey, 2005).

Alpha-glucosidase inhibitors, such as acarbose and miglitol are used for type 2 diabetes management (Tarling *et al*., 2008). These inhibitors are helping to maintain

levels of blood sugar within the normal range by slowing down the absorption of glucose from food to blood system. These drugs are most beneficial for persons who have been newly diagnosed with type 2 diabetes and who have blood sugar levels higher than normal but not considered above criteria for full-blown diabetes. They are as well suitable for persons taking sulfonylurea medication or metformin to use it as supplements for keeping blood sugar levels within the normal range (Tarling *et al*., 2008).

Amylase inhibitors are well-known as starch blockers since they have materials that can inhibit the absorbtion of dietary starch by the body. Starch is "complex carbohydrates that can not be absorbed until broken down by the digestive enzyme amylase and other secondary enzymes" (Krentz and Bailey, 2005). Highly concentrated versions of amylase inhibitors were reported to be able to reduce carbohydrate absorption in humans (Krentz and Bailey, 2005). Phenolics compounds may also play a mediator role for amylase inhibition and for that reason have potential to control type 2 diabetes (McCue and Shetty, 2004). Several natural α- amylase and α-glucosidase inhibitors isolated from plants possess mild inhibitory activity toward α-amylase and strong inhibitory activity toward α-glucosidase which potentially known as effective treatment for postprandial hyperglycemia with minimal side effects (Kwon *et al*., 2006).

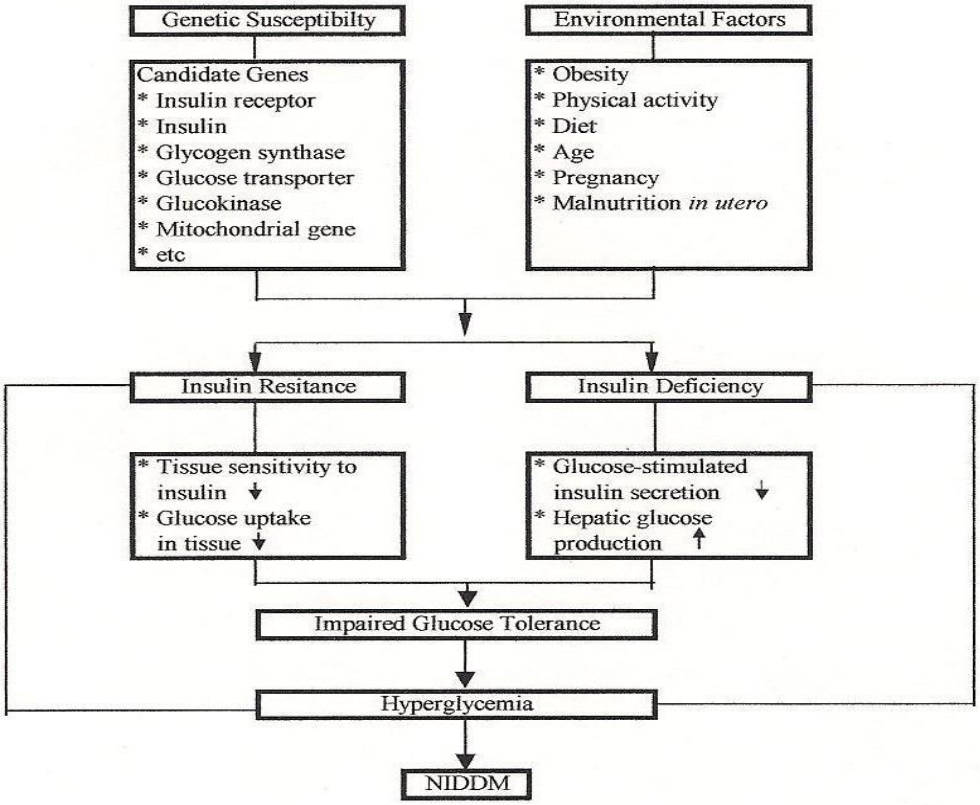


Figure 2.7 Progressive pathogenesis of type 2 diabetes mellitus (DeFronzo, 2004).

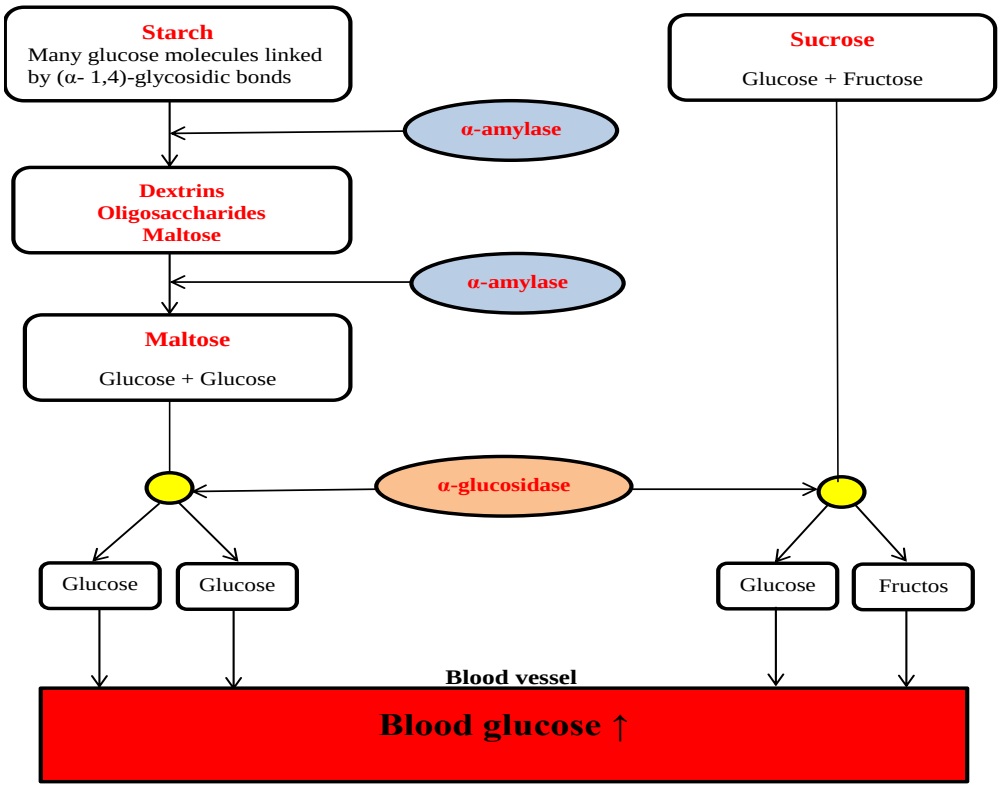


Figure 2.8 Digestion of Carbohydrate (starch and sucrose) in the body.

2.7.1 α-Amylase inhibitor enzyme

A number of protein inhibitors of α-amylases are present in microorganisms, higher plants and animals produce to regulate the activity of these enzymes (Gupta *et al*., 2003; Tangphatsornruang *et al*., 2005; Pytelková *et al*., 2009). The action of these inhibitors happens by direct blocking of the active site of the enzyme at various positions. In animals, α-amylase inhibitors decrease the glucose level after a meal by slow down the digestion of starch to simple sugars (Wild *et al*., 2004). This is particularly importance in people with type 2 diabetes. Plants also use α-amylase inhibitors as a protection strategy called anti-feedants (Grover *et al*., 2002; Simão *et al*., 2012). These inhibitors delay the digestive action of α-amylases and proteinases in the insect gut. Thus, α -amylase inhibitors have potential in several domain including crop protection and the management of diabetes.

Starch molecules hydrolyse by amylases to produce various complexes including dextrins and increasingly smaller polymers composed of glucose (Tarling *et al*., 2008). Amylases can be divided into two classifications, endoamylases and exoamylases (Gupta *et al*., 2003; Reddy *et al*., 2003). Endoamylases catalyse hydrolysis at random way in the central of the starch molecule. This action leads to the creation of linear and branched oligosaccharides of different chain lengths. Exoamylases hydrolyze from the non-reducing end, successively causing in short end products (Gupta *et al*., 2003; Reddy *et al*., 2003). The enzyme is normally produced and secreted in salivary glands (salivary α-amylase or AMY1) and pancreas (pancreatic α-amylase or AMY2A).

The human pancreatic α-amylase (HPA) is responsible for cutting large malto-oligosaccharides to smaller oligosaccharides which act as substrates for intestinal α-glucosidases. The digestion procedure is essential for glucose to be easily absorbed

through the blood system. In addition, control of blood glucose levels can be observed by controlling HPA activity. Indeed, HPA activity has been correlated to postprandial hypoglycemia (Tarling *et al*., 2008) and α-amylase inhibitors have been effectively known for diseases treatment specially where control of blood glucose level is needed such as diabetes or obesity (Krentz and Bailey, 2005).

α-Amylases are usually analyzed using soluble starch or modified starch as the substrate. α-Amylase catalyzes the hydrolysis of α-1, 4-glycosidic linkages in starch to produce glucose, dextrins and limit dextrins (Krentz and Bailey, 2005). Another substrate for specific α-amylases determination is nitrophenyl derivatives of maltosaccharides. The method measures the release of free ρ-nitrophenyl groups. The use of nitrophenyl-maltosaccharides in conjunction with a specific yeast α-glucosidase can be applied but these substrates are rapidly breakdown by glucoamylases commonly available in the culture broths. The employ of non-reducing end blocked ρ-nitrophenyl maltoheptoside has also been clarified. The blocking group (4, 6-O-benzylidene) inhibits the hydrolyzis of the substrate by the exo-acting enzymes that specific for α-amylase (Gupta *et al*., 2003).

One of the functions of α-amylase is delaying the absorption of glucose through the inhibition of the carbohydrate-hydrolyzing enzymes in the digestive tract. Inhibitors of these enzymes retard carbohydrate digestion and extend period of carbohydrate digestion. This action leads to lower the rate of glucose absorption and thus reducing rapid and sustained the postprandial plasma glucose increase (Rhabasa-Lhoret and Chiasson, 2004).

2.7.2 α-Glucosidase inhibitor enzyme

α-Glucosidase inhibitors are used for diabetes mellitus type 2 that preventing the digestion of disaccharides into smaller sugars available for the intestinal absorption. The inhibition of this enzyme is recognised to be beneficial for controlling diabetes by delaying the absorption of glucose liberated from starch (Wild *et al*., 2004). This enzyme is widely distributed in microorganisms, plants, and animal tissues. Three types of α-glucosidase inhibitors exist which are polyhydroxylated N-substituted heterocyclic compounds, polyhydroxylated cycloalkenes and oligomers of pseudosugars. Most inhibit α-glucosidases by simulating the pyranosyl moiety of the α-glucosidase. There are α-glucosidase inhibitors such as acarbose and voglibose produced from microorganisms and nojirimycin and 1-deoxynojirimycin from plants (Kim *et al*., 2004).

The α-glucosidase enzyme is sited in the brush border of the small intestine and is essential for the degradation of carbohydrates to monosaccharides that can absorb easily. It prevents the absorption of ingested carbohydrates, reducing the postprandial glucose and insulin peaks (Adolfo Andrade-Cetto *et al*., 2007). α-Glucosidase inhibitor works by decreasing the quantity of glucose absorb by the intestine. This inhibits the abnormal rise in blood sugar levels that occurs for diabetic people after meals. This inhibition decrease glucose absorption, thereby reducing alimentary hyperglycemia and hyperinsulinemia (Goji *et al*., 2009).

2.8 Gastrointestinal tract (GI)

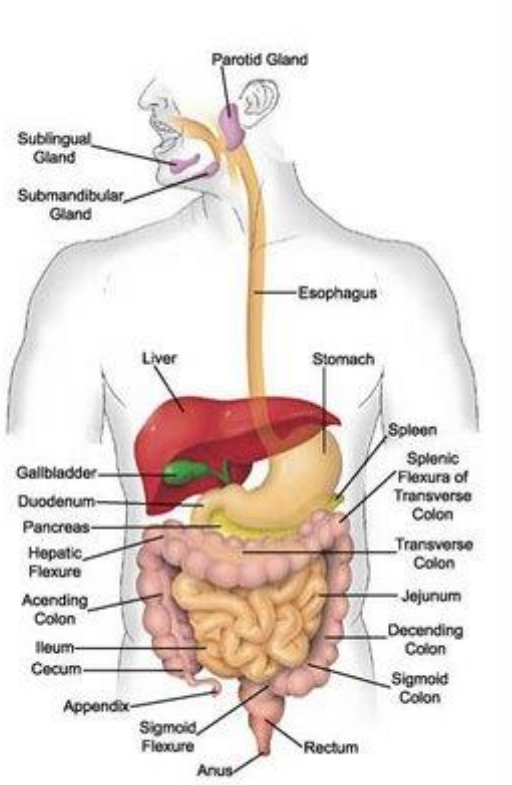


Figure 2.9 Human digestive system

Human digestive system contains a multipart series of organs and glands (Figure 2.9) which digest food via physical and chemical means. An adult human has approximately 5 meters of upper and lower GI tracts. Most of the digestive organs are tube-like such as stomach and intestine, and this GI tract releases hormone such as gastrin, secretin, cholecystokinin and ghrelin to help the regulation of the digestion process (Shetzline and Liddle, 2002).

The process of digestion starts in the mouth. The food had been eaten is broken down by the process of chewing and also chemical action of salivary enzymes where this enzymes are formed by the saliva and break down starch into smaller molecules. The process will then proceed to the esophagus on the way to the stomach. Stomach is a large sack-like organ that sank the food in a very strong acid called

gastric acid. The volume of stomach can be as low as 50 ml when empty and up to 4 liter when full and the pH inside stomach could be as low as pH 1.5 (Shetzline and Liddle, 2002) or as high as pH 6 or above after the digestion (Shetzline and Liddle, 2002). This partly digested food mixed with the acid is called chime. The food will subsequently enter the duodenum, which is the first part of small intestine. There are 3 regions that make up the small intestine, which are duodenum, jejunum and ileum (cilla *et. al*., 2009). The food will pass through the jejunum and then ileum which is the final part of small intestine. In this small intestine, the ingested food will be mixed with bile (that produced in the liver and stored in gall bladder, function as emulsification of lipid), pancreatic enzymes, and others digestive enzymes produced by the wall of small intestine which help in the broken down of food. The presence of villi and microvilli in the small intestine will increase the surface area for better absorption. The critical condition of small intestine is due to the presence of bile salts and also pancreatin (cilla *et. al*., 2009). In the large intestine, most water and electrolytes (such as sodium) will be reabsorbed into the blood. Many microbes like *Bacteroides*, *Lactobacillus acidophilus*, *Escherichia coli*, and *Klebsiella* which are present in large intestine support the digestion process. At the end of the digestion process, the water content of the undigested materials in the large intestine is reabsorbed and the solid waste is kept in the rectum until it is excreted through the anus (Shetzline and Liddle, 2002).

The structural design of food-based delivery systems has been increased in the past few years in order to encapsulate, protect and release bioactive components believed to benefit people health (McClements *et al*., 2009). These delivery systems may be depends on release of bioactive components at a particular location in the human gastrointestinal (GI) tract under environmental trigger (pH, ionic strength or

enzyme activity; Hur *et al*., 2011). Simulate the complex physicochemical and physiological actions that occur in the human GI tract are important to testing the efficacy of designed delivery systems models. Animals or humans i*n vivo* method is provide the most accurate results, but they are time consuming and expensive (Wattiaux and Howard, 2000; Vosloo, 2005). Thus, *in vitro* digestion models provide a useful alternative for rapidly screening food ingredients (Coles *et al*., 2005).

2.9 Medicinal plants

Traditional medicinal plants has become more important by provide valuable therapeutic agents. The use of herbal to treat various metabolic diseases such as diabetes, adiposity and cardiovascular complications are readily welcomed due to the limitations of chemo-therapeutic agents in addition to the side effects and high rates of secondary failure (Grover *et al*., 2002; Guang *et al*., 2009; Gumienna *et al*., 2009; Nileeka Balasuriya and Vasantha Rupasinghe, 2011). Plant kingdom offers endless list of natural effective oral anti-diabetic and anti-hypertensive agents with slight or no side effects. Several medicinal herb extracts already showed significant hypoglycemic or hypolipidemic properties (Mentreddy, 2007; Pytelková *et al*., 2009; Nileeka Balasuriya and Vasantha Rupasinghe, 2011). The present study has focused on two types of plant medicinal (*Cinnamomum verum* and *Allium sativum*) because they possesses many healthful properties that are related to their bioactive compounds and they become recognized for their great value in the prevention of many diseases such as diabetes and hypertension (Vuksan and Sievenpiper, 2005). These herbs found to improve glucose metabolism not only by hypoglycemic effects but also by educating lipid metabolism, antioxidant activity and capillary function (Mentreddy, 2007).

2.9.1 Cinnamon (*Cinnamomum verum*)

Figure 2.10 *Cinnamomum verum* Bark

2.9.1.1 Botanical description

The genus *Cinnamomum* comprises over 250 aromatic evergreen trees and shrubs primarily located in Asia and Australia (Thang *et al*., 2008). Cinnamon or Ceylon cinnamon is a common name for the culinary herb *Cinnamomum verum* J. Presl (*Cinnamomum zeylanicum* Blume, *Laurus cinnamomum* L.; Figure 2.10) of the family Lauraceae (laurel family). Cinnamon tree is a large evergreen tree with young branches that are smooth and brown (Thang *et al*., 2008). The leaves are opposite, leathery, ovate to broadly, ovate with three and rarely five prominent veins. Young leaves are reddish but later turn dark green. Small pale yellow flowers are borne in axillary or terminal panicles. The fruit is a fleshy, ovoid drupe which contains one seed and turns dark purple or black when ripe (Thang *et al*., 2008).

2.9.1.2 Chemical composition

Volatile oils can be obtained by distillation from the bark, leaves and flowers or buds of *Cinnamomum* species. The chemical composition of these oils is monoterpenes, sesquiterpenes and related oxygen derivatives of these two types of compounds. The major monoterpene hydrocarbons in volatile components of cinnamon extracts are α-pinene, camphene and limonene (Miyazawa *et al*., 2001).

The main constituent of cinnamon bark oil is cinnamaldehyde (41.3%) whereas eugenol is the main constituent (e.g. about 81-85%) of cinnamon leaf oil (Gupta *et al*., 2008; Ranjan *et al*., 2012).The essential oils from leaves of *Cinnamomum* species accounts for about 0.5% dry weight. Analysis of a steam-distilled volatile oil from cinnamon fruit stalks yielded 27 compounds with cinnamyl acetate (36.59%) and caryophyllene (22.36%) being the major components (Jayaprakasha *et al*., 2003).

Analysis of the hydro-distilled volatile oil from the buds of *Cinnamomum verum* (*C. zeylanicum*) yielded terpene hydrocarbons (78%) and oxygenated terpenoids (9%) with the sesquiterpenes, α- bergamotene (27%) and α-copaene (23%) being the most common compounds (Jayaprakasha *et al*., 2002). Minor compounds included α-humulene, α-muurolene and δ-cadinenes. The volatile oil of the buds contains more monoterpene and sesquiterpene compounds than oils from the flowers and fruits whereas the concentration of *trans*-cinnamyl acetate is much higher in the volatile oils from flowers and fruit than from the buds.

2.9.1.3 Therapeutic uses of cinnamon

Cinnamon displays insulin-enhancing activity *in vitro* (Broadhurst *et al*., 2000; Khan *et al*., 2003) and also insulin-like biological activity *in vitro* (Broadhurst *et al*., 2000). The aqueous cinnamon extracts (CEs) mimic some of the properties of insulin and enhence insulin action (Jarvill-Taylor *et al*., 2001; Anderson *et al*., 2004) possibly by preventing fructose feeding-induced decreases in insulin sensitivity by enhancing the insulin signaling pathway (Qin *et al*., 2004). Reduction in fasting blood sugar levels in pre-diabetic men and women with the metabolic syndrome (Ziegenfuss *et al*., 2006) as well as improvement in antioxidant status (Roussel *et al*., 2009), insulin sensitivity in women with polycystic ovary syndrome (Wang *et al*., 2007) and blood

glucose and lipids of patients with Type 2 diabetes (Khan *et al.,* 2003) are also other possible benefits of CE's.

Cinnamon or its components has also potential lipid lowering properties in persons with type 2 diabetes (Khan *et al.,* 2003) in fructose (Kannappan *et al.,* 2006) and cholesterol-fed animals (Lee *et al.,* 2003) and in streptozotocin (STZ)- induced diabetic rats (Subash *et al*., 2007). Studies in diabetic mice showed that cinnamon lowered blood glucose, total cholesterol and triglyceride levels while raising HDL cholesterol levels (Kim *et al*., 2006). The first clinical trial evaluating the effect of cinnamon (1, 3 and 6 g daily) in individuals with type 2 diabetes (Khan *et al.,* 2003) showed cinnamon powder ability to reduce mean fasting serum glucose (18 –29%), triglyceride (23– 30%), LDL cholesterol (7–27%) and total cholesterol (12–26%) levels with a 40 day period.

Cinnamon can boost the levels of proteins that important in insulin signaling, glucose transport and inflammatory response (Anderson *et al.,* 2004). The proanthocyanidin a type of polyphenol may have insulin-like properties (Anderson *et al.,* 2004) and these phenolic compounds lower blood glucose levels by enhancing glucose transport thus implicating valuable candidate for a new anti-diabetic drug (Kim *et al.,* 2006).

Cinnamon extracts have antibacterial activities effective in preventing the growth of different bacteria including *Staphylococcus aureus, E. coli, Enterobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa, Vibrio cholerae, Vibrio parahaemolyticus* and *Samonella typhymurium* and fungi including yeasts (four species of Candida, *C. albicans, C. tropicalis, C. glabrata,* and *C. krusei*) and molds (Ooi *et al.,* 2006). In addition, cinnamon may be useful as anti-inflammatory agents that are essential in inhibiting or mitigating arthritis as well as cardiovascular disease

(McCarty, 2006). Moreover, the correlation between inflammation and insulin function in Alzheimer's (causing some to refer to the neurodegenerative disease as "type 3 diabetes") suggests cinnamon's capability to block inflammation and improve insulin function may make it beneficial in combating that disease as well (McCarty, 2006).

2.9.2 Garlic (*Allium sativum*)

Figure 2.11 *Allium Sativum* (Garlic)

2.9.2.1 Botanical description

Allium sativum (Figure 2.11) grows in the wild in areas where it has become naturalized (Dini *et al*., 2011). Stinking rose, poor man's treacle, heal-all and garlic are a commons names for the culinary herb *Allium sativum*, *A. controversum* of the family Alliaceae. Garlic is a perennial herb with a globose bulb containing 5 - 15 cloves, protected by white or mauve-tinged skin (Dini *et al*., 2011). The plant has plane leaves and produces an umbel of green-white to pink flowers, with a deciduous spathe, that appear in summer.

2.9.2.2 Garlic preparations and their chemical compounds

Raw garlic homogenate is the main method of garlic preparation for concentrated research studies since it is the most common way of garlic consumption. Raw garlic homogenate is basically the same as water extract of garlic and it has

Allicin (allyl 2-propenethiosulfinate or diallyl thiosulfinate), the principal bioactive compound (Cutler and Wilson, 2004; Shukla and Kalra, 2007). This is because when garlic is cut or smashed the allinase enzyme available in garlic is stimulated and acts on alliin (present in intact garlic) to produce allicin. Other main sulfur containing compounds present in garlic homogenate are allyl methyl thiosulfonate, 1-propenyl allyl thiosulfonate and γ-Lglutamyl- S-alkyl-L-cysteine (Baghalian *et al*., 2005).

Abdullah *et al.*, (1988) and Augusti and Sheela, (1996) reported that fresh garlic contains water, carbohydrates (e.g. fructose), proteins, fiber and fat as well as 33 sulfur compounds, 17 amino acids, germanium (14 µg/100 g), calcium (50–90 µg/100 g), copper (0.02–0.03 µg/100 g), iron (2.8–3.9 µg/100 g), potassium (100–120 µg/100 g), magnesium (43–77 µg/100 g), chromium (0.3–0.5 mg/ 100 g), manganese (0.2–0.6 mg/100 g), boron (0.3– 0.6 mg/100 g), barium (0.2–1 mg/100 g), aluminum(0.5–1 mg/100 g), sodium (10–22 mg/100 g), phosphorous (390–460 mg/100 g), zinc (1.8–3.1 mg/100 g), selenium (15–35 µg/100 g), thiamine (0.25 mg/100 g), riboflavin (0.08 mg/100 g), vitamin C (5 mg/100 g), nicotinic acid (0.5 mg/100 g), retinal (15 µg/100 g) and energy (39–140 cal/100 g wet wt.). The main component of the volatile oil are sulfur compounds especially allicin, diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS) and ajoene. It also contains unique organosulfur compounds, which give its specific flavour and aroma and most of its effective biological activity (Block, 2009). Actually, above 90% of studies on garlic's active mechanism have focused on the sulfur compounds 85% of which contain of alliin and two main γ-glutamylcysteines. The total allicin produce has been determined as 2.5 mg/g of fresh crushed garlic or about 5–20 mg per clove. Further alteration of organosulfur compounds (OSCs) can happen after interplay with

free sulfhydryl groups, including those present in cysteine, glutathione or proteins (Block, 2009).

2.9.2.3 Therapeutic uses of garlic

Garlic often been used in the treatment of diabetes by increasing either the insulin secretion from pancreatic β-cells or its release from bound insulin (Patumraj *et al.,* 2000; Srinivasan, 2005). Allicin has been reported to have important hypoglycemic effect. This effect is assumed to be as a result of improved hepatic metabolism, increased release of insulin or insulin-sparing effect (Patumraj *et al.,* 2000; Srinivasan, 2005). The previous mechanism seems to be the main factor, as allicin and other sulfhydryl compounds in garlic interaction with insulin (also a disulfide protein) for insulin-inactivating compounds, which causes a rise in free insulin (Johnson *et al.,* 2006; Urios *et al*., 2007). The antioxidant action of S-allyl cysteine sulfoxide (isolated product from garlic) may also have positive influence in diabetes (Augusti and Sheela, 1996; Lee *et al*., 2009).

Garlic possesses important protective effect against heart disease and strokes through its capacity to influence the process of atherosclerosis (Sukandar *et al*., 2010). Foremost in garlic's ability to offer substantial protection toward heart disease and strokes is its aptitude to lower blood cholesterol and triglyceride levels (Harauma and Moriguchi, 2006). It also has a hypotensive activity assisting to lower blood pressure (Sukandar *et al*., 2010).

Garlic also acts as antioxidant which has ability to protect blood vessels from the deleterious effects of free radicals (Lin *et al*., 2008). Furthermore, this antioxidant action has been shown to reduce blood cholesterol leves and lower cholesterol deposits on the walls of blood vessels. This is lead to lower lipids in the blood (such as cholesterol and triglycerides) which important to heart-health. It is also known as

good cholesterol because help to decrease low-density lipoprotein (LDL) in the blood as well as alter the ratio of low-density lipoproteins in favor of high-density lipoproteins (HDL), which supports the liver process fat materials in the blood rather than deposited them in the tissue (Lin *et al*., 2008). It also improves the blood flow to the capillaries resulting in reduce blood pressure.

Garlic is one of the best useful foods for the digestive system. It practices a positive action on the lymph, helps in elimination of noxious waste substance in the body. It catalyzes peristaltic activity and the secretion of the digestive juices. Smashed cloves of garlic may be soaked in water or milk and drunk for all kinds of digestion disorders. It has an anti-septic activity and is a perfect choice for treating infectious disease and stomach and intestine inflammations. Garlic oil is absorbed into the alimentary tract and is removed partially through the urine (Bjarnsholt *et al*., 2005).

Garlic has been approved to have broad-spectrum antimicrobial action toward several species of bacteria, viruses, worms and fungi (Wilson and Adams, 2007). These discoveries go along with the historical use of garlic in the therapy of a range of infectious diseases. Study has reported that garlic juice and allicin prevented the growth of *Staphylococcus, Streptococcus, Bacillus, Brucella,* and *Vibrio* species at low concentrations (Bongiorno *et al*., 2008).

2.9.3 Phenolic phytochemicals

Phenolic compounds are widely present in fruits, vegetables and spices. These compounds may have potent antioxidants activity by applying antioxidative action as terminators of free radicals and chelating metals that have ability for catalyzing lipid peroxidation. They may act by donating a hydrogen atom to radicals which results in the formation relatively stable phenoxy radical intermediates making it more difficult for a new chain reaction to initiate (Sroska and Cisowski, 2003; Ranilla *et al*., 2010).

The efficiency of phenolic compounds may be associated with factors such as number of hydroxyl groups and the site of binding as well as the mutual position of hydroxyls in the aromatic ring (Sroska and Cisowski, 2003). Thus diets rich in vegetables, fruits and spices have been associated with a lowered incidence of degenerative diseases, diabetes (Brash and Havre, 2002) and hypertension diseases (Kris-Etherton *et al.*, 2002). Some phenolic substances may have applications in controlling pathogens in foods (Mandavia *et al*., 2000; Anson *et al*., 2009) and crops (Mandavia *et al.*, 2000) as well as possessing anti-inflammatory effects (Trouillas *et al*., 2003). Phenolics compounds occur primarily in conjugated form with one or more sugar residues linked to hydroxyl groups as well as with other compounds such as carboxylic, organic acids, amines and lipids (Sroska and Cisowski, 2003). Enzyme hydrolysis of these phenolic glycosides appears to be a usefull way to improve the number of free phenolics with nutraceutical and pharmacological properties (Zheng and Shetty, 2000). Examples of classification of dietary polyphenols with their pharmacological properties are given in Figure 2.12.

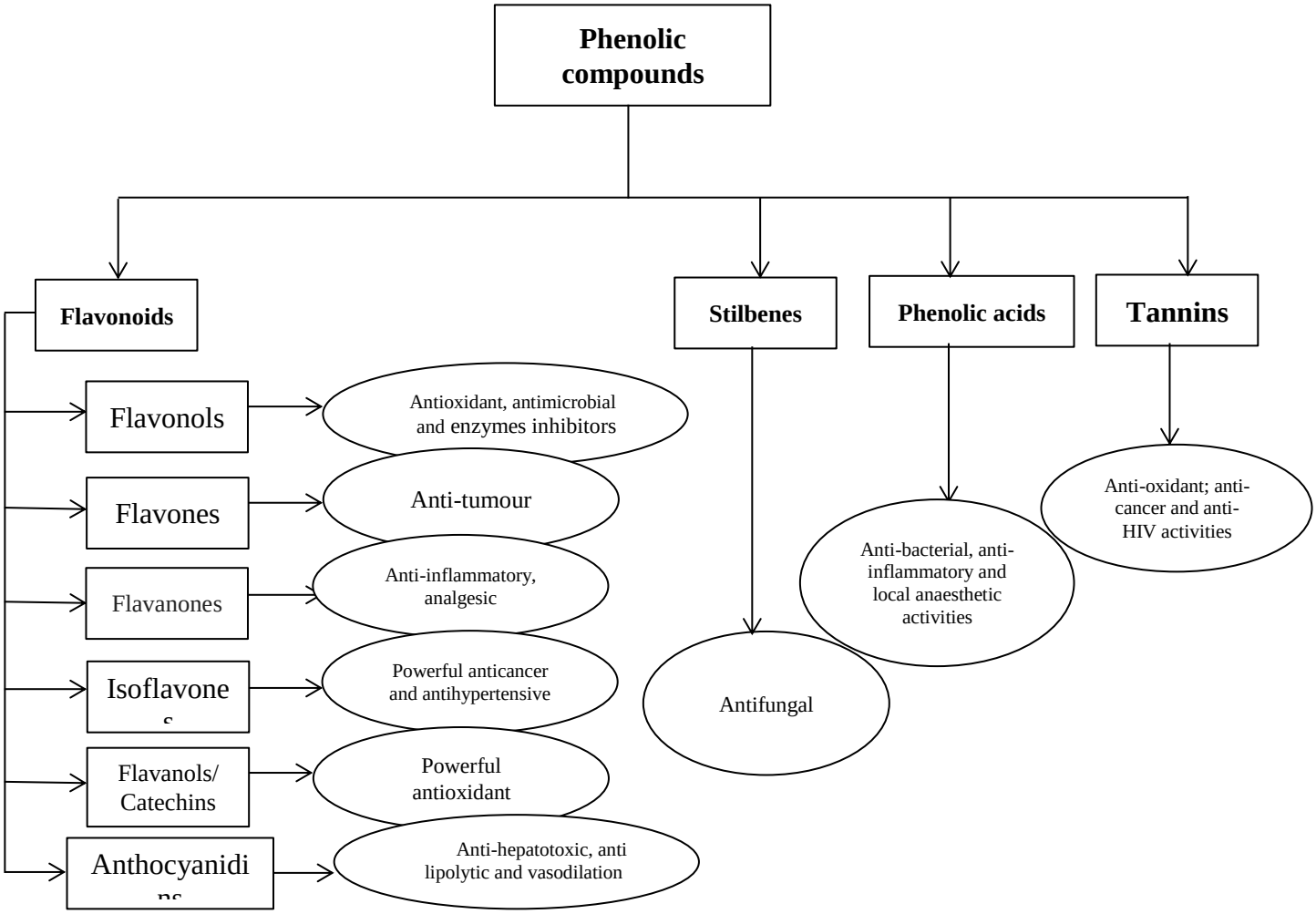


Figure 2.12 Classification of dietary polyphenols with their pharmacological properties.

2.9.4 Natural foods antioxidants and their health benefits

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) ae synthetic antioxidants which have been used as antioxidants in food because of its excellent solubility in food, heat stable and providing extended shelf life. There are limitations on applying of these compounds in food because of their carcinogenicity effects (Brash and Havre, 2002; Rahimi *et al*., 2005). There are other options to replace synthetic antioxidants by natural and safe sources of food antioxidant (Psaltopoulou *et al*., 2011) which include vegetables, fruits and plants in general.

Plants are increasingly used for the manufacture of raw ingredients or preparations including phytochemicals with major antioxidant activities and

therapeutic properties (Exarchou *et al*., 2002). Crude extracts rich in phenolics such as fruits, herbs, vegetables and cereals are widely used in the food manufacturing and processing since they increase the quality and nutritional value of food by delaying oxidative degradation of lipids. In fact many plants spices and herbs have protective effect related to the presence of antioxidant and antimicrobial compounds in their cell walls (Srinivasan, 2005; Wilson and Adams, 2007; Ranilla *et al*., 2010).

The role of food antioxidants in the maintenance of health and reduction of risks developing cancer, high blood pressure, diabetes and other diseases is increasingly apparent as reflected in the increase in consumer's preference for functional foods with specific health properties (Anonymous, 2002). Antioxidants compounds are known to delay or prevent the oxidation of lipids or other compounds by inhibiting the beginning or proliferation of oxidative chain reactions (Sroska and Cisowski, 2003). Thus the addition of antioxidants to food products specifically to lipids and lipid-containing foods can improve the food shelf life. The antioxidative effect is generally caused by phenolic components such as flavonoids (Chan *et al*., 2012), phenolic acids and phenolic diterpenes (Chan *et al*., 2012) which can absorb and neutralize free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Psaltopoulou *et al*., 2011; Chan *et al*., 2012). A lot of these phytochemicals have significant antioxidant activity such as effective neutralizing effects on free radicals that can control high blood pressure and diabetes (Hunter and Fletcher, 2002; Anonymous, 2002).

The exposure of living organisms to reactive oxygen species (ROS) is unavoidable in aerobic life since the generation of ATP from molecular oxygen demands electrons. ROS fall into two groups i.e. those that contain unpaired electrons (O_2^-, OH^-) or those that have the ability to extract electrons from other molecules

(H_2O_2, HOCl). These species may damage biomolecules directly or initiate chain reactions in which ROS are passed from one molecule to another resulting in extensive damage to cell structures such as membranes and proteins.

Breakdown or deficiency of these defenses against ROS can lead to damage which has been strongly associated with a wide variety of chronic diseases including Alzheimers, autoimmune disease, cancer, cardiovascular disease, diabetes, multiple sclerosis and arthritis (Trouillas *et al*., 2003; Rahimi *et al*., 2005; Shetty *et al*., 2008; Ranilla *et al*., 2010). In contrast, levels of ROS must not become too low given their important roles in the immune system. Therefore, there is a need for constant monitoring and regulation of the redox potential of the blood.

3.0 Effects of inclusion of *Allium sativum and Cinnamomum verum* in milk on the acidification, proteolysis and growth of lactic acid bacteria during fermentation

3.1 INTRODUCTION

Yogurt has long been known as a product with many desirable effects for health (Analie and Bennie, 2001). The excellent sensory properties and the health benefits of yogurt (Adolfsson *et al*., 2004) can be credited to the action of yogurt bacteria and their metabolites (Analie and Bennie, 2001). Lactic acid bacteria (LAB) particularly *Lactobacilli, Streptococci* and *Bifidobacteria* are the most important microorganisms associated with the health status of human gastrointestinal tract which justifies the reason for calling them friendly bacteria. They are dependent on carbohydrates such as lactose and glucose for their energy sources and yield lactic acid as a major end product. In yogurt production, changes in the milk substrate by LAB during fermentation are attributed to fermentation temperature, ingredients added during manufacturing, fermentative action of the inoculated starter cultures and the secretion of nutritional and chemical substances by the microorganisms (Analie and Bennie, 2001). Since conventional yogurt starter bacteria, *S. thermophilus* and *L. bulgaricus* are very sensitive to survive passage through the low pH in gastric tract (Shah, 2000), the combination of live strains of *L. acidophilus* and species of *Bifidobacterium* to yogurt bacteria (Analie and Bennie, 2001) have been widely used in yogurt manufacturing. A product is called yogurt if live bacteria are present in the final product. The viable number of probiotics in the final product was suggested to be at least 10^6–10^7 cfu/g to be accepted as the therapeutic minimum (Madureira *et al*., 2011).

Most yogurt are considered "ripe" somewhere in the pH range of 4.0-4.5, depending on how strong or mild a product is preferred (Hui *et al*., 2007). The fermentation is terminated at pH 4.5 because this is the preferred pH in commercial dairy products. The pH values lower than 4.0 are undesirable because *L. bulgaricus*

tends to produce excessive lactic acid, acetaldehyde and proteolytic by-products in this pH range (Stephaine *et al*., 2009). On the other hand, pH of about 4.5 can help maintain the yogurt throughout shelf life, maintain a mild flavor and a pleasant product appearance and eliminate the graininess that commonly develops during breaking and cooling of yogurt (Hui *et al*., 2007).

Allium sativum and Cinnamomum *verum* (also known as garlic and cinnamon) are medicinal plant which possesse many healthful properties. Their bioactive compounds have great therapeutical values towards treatment of diabetes and hypertension (Broadhurst *et al*., 2000; Jarvill-Taylor *et al*., 2001; Qin *et al.*, 2003; Harauma and Moriguchi, 2006). The inclusion of these two herbs by virtue of the rich phytochemicals is expected to affect microbial growth during fermentation of milk. Thus, the present study investigated the effect of *A. sativum* and C. *verum* water extracts on LAB during fermentation of three types of milk (cow, goat and camel milk) by determining the acidification process, microbial growth and their proteolytic activity during milk fermentation.

3.2 MATERIALS AND METHODS

3.2.1 Substrates and chemicals

The herbs used in the present study, *Cinnamomum verum* and *Allium sativum*, were purchased from local food store. The former was ground to powder form whereas the latter was obtained from commercial sources in powder form (McCormick®, 4.2 g equals 4 cloves of fresh garlic). Homogenized and pasteurized full cream cow milk (Dutch Lady, Malaysia) and camel milk (Al-Turath, Saudi Arabia) were purchased from supermarket. Camel milk was kept frozen (-20°C) for 2 weeks until required for yogurt making. Goat milk was purchased fresh from local goat farm. It was heated at 85°C for 30 min and kept refrigerated and used within 2

weeks. Commercially available direct vat set (DVS) starter culture powder used in yogurt preparation consist of a mixture of *Lactobacillus acidophilus* LA-5, *Bifidobacterium* Bb-12, *Lactobacillus casei* LC-01, *Streptococcus thermophilus* Th-4 and *L. delbrueckii ssp. bulgaricus* (Chris-Hansen, Denmark) in the ratio of 4:4:1:1:1. De Man Rogosa and Sharpe (MRS) agar, M17 agar, buffered peptone water, lithium chloride, sodium propionate, hydrochloric acid, Sodium hydroxide, phenolphthalein, O-phtaldialdehyde (OPA), sodium tetraborate, sodium dodecyl sulphate, β-mercaptoethanol and tryptone, were purchased from Sigma Chemical Company (St Louis, MO, USA).

3.2.2 Experimental design

The present study examined the effect of *A. sativum* or *C. verum* water extract on the probiotics and yogurt bacteria, acidification and proteolytic activity during fermentation of milk. Three groups of set bio-yogurt were prepared using cow, camel and goat milks with each group consisting of plain-yogurt and yogurt containing *A. sativum* or *C. verum* water extract. The milk or milk mixed with *A. sativum* or *C. verum* water extract were inoculated with the starter culture followed by incubation at 41°C until the pH reached 4.5. The parameters evaluated include pH changes, titratable acidity (lactic acid equivalent), bacterial cell counts and the extent of proteolysis.

3.2.3 Plant water extraction

A. sativum or *C. verum* powder was mixed with sterile dH_2O at the ratio of 1: 10 in a 250 ml bottle. The final concentration of both herbal extracts was 0.1g/ml. The mixture was left for 12 hours (Behrad *et al.*, 2009) in a water bath at 70°C (Julabo, Model Sw-21c or Haake Model SWD 20) followed by centrifugation (1000 rpm,

15minutes at 4^{o}C; Eppendoft 5804 R). The supernatant was removed and used as herbal water extract in the making of herbal-yogurt.

3.2.4 Yogurt manufacturing process

3.2.4.1 Preparation of starter culture

The starter culture was prepared according to the producer's recommendation for DVS culture used. Chilled pasteurized full cream cow milk or frozen camel milk (1 L each) was pre-heated to 41°C. A small volume of each milk (100ml) was placed into a sterilized beaker. The probiotic yogurt bacteria powder mix was added into each type of milk. The mixture was stirred thoroughly and then mixed evenly with the remainder of the respective milk followed by incubation for 12 hours at 41°C (Julabo, Model Sw-21c or Haake Model SWD 20). The yogurt formed were refrigerated (4°C) and used as starter cultures within 3 days in the making of yogurt. The changes in pH, titratable acidity (TA) and bacterial cell counts of starter culture on 1, 3 and 7 days of storage at 4°C were monitored (Table 3.1).

Table 3.1 Changes in pH, titratable acidity (TA) and bacterial cell counts of starter culture during 1, 3 and 7 days of refrigerated storage.

Day	Starter culture														
	pH			TA (%)			*Lactobacillus spp. Counts* ($x10^6$ cfu/ml)			*S. thermophilus* counts ($x10^9$ cfu/ml)			Probiotics counts ($x10^9$ cfu/ml)		
	Bio-WMY	Bio-LMY	Bio-GMY	Bio-WMY	Bio-LMY	Bio-GMY	Bio-WMY	Bio-LMY	Bio-GMY	Bio-WMY	Bio-LMY	Bio-GMY	Bio-WMY	Bio-LMY	Bio-GMY
1	4.3 ± 0.02	4.4 ± 0.03	4.03 ± 0.1	0.8 ± 0.05	0.7 ± 0.00	0.75 ± 0.1	4.9 ± 0.2	35.7 ± 4.9	5.4 ± 0.04	16.6 ± 4.4	4.8 ± 1.0	16.8 ± 0.1	15.1 ± 3.0	27.7 ± 4.2	13.9 ± 0.2
3	4.1 ± 0.02	4.2 ± 0.01	3.77 ± 0.1	0.9 ± 0.09	0.8 ± 0.00	1.17 ± 0.1	5.9 ± 0.9	26.4 ± 1.9	10.7 ± 0.1	7.6 ± 2.3	8.5 ± 0.7	9.04 ± 0.1	0.2 ± 0.1	21.8 ± 2.3	2.2 ± 0.1
7	3.9 ± 0.02	4.0 ± 0.01	3.43 ± 0.2	1.0 ± 0.05	0.9 ± 0.10	1.35 ± 0.1	3.9 ± 0.5	20.3 ± 0.3	4.8 ± 0.1	5.4 ± 1.1	3.1 ± 0.2	1.5 ± 0.1	0 ± 0.0	5.9 ± 1.4	0.03 ± 0.1

WMY = Cow milk yogurt, LMY = Camel milk yogurt and GMY = Goat milk yogurt. Values are presented as a mean (n = 3) ± standard error.

3.2.4.2 Yogurt preparation

Pasteurized full cream milk (1 L) was heated to 41^{o}C in a 3000 ml beaker. The milk was subsequently divided into 3 portions of 255 ml placed into 500 ml beaker. The first portion was used as a plain-yogurt (control) after the addition of 30 ml dH_2O and inoculation with 15g starter culture. The second and third portions were used to prepare *A. sativum*- and *C. verum*- yogurt by adding 30 ml of *A. sativum* or *C. verum* water extract (0.1g/ml) respectively and 15g of starter culture into each portion. Aliquots of 100 ml from each portion were placed into a disposable 150 ml plastic containers and these were held at 41°C (Julabo, Model Sw-21c or Haake Model SWD 20) until the required pH of 4.5 was reached. The fermentation was stopped at this pH by placing the containers immediately in ice bath for 1 hour followed by storage at

4°C (Figure 3.1). The changes in acidity, proteolysis and viability of LAB and probiotics of cow, camel and goat milks whether in the presence or absence of herbal-water extract were monitored by taking 10 ml samples for every 30 min for acidification measurement and every one hour for the other analysis until the pH reached 4.5.

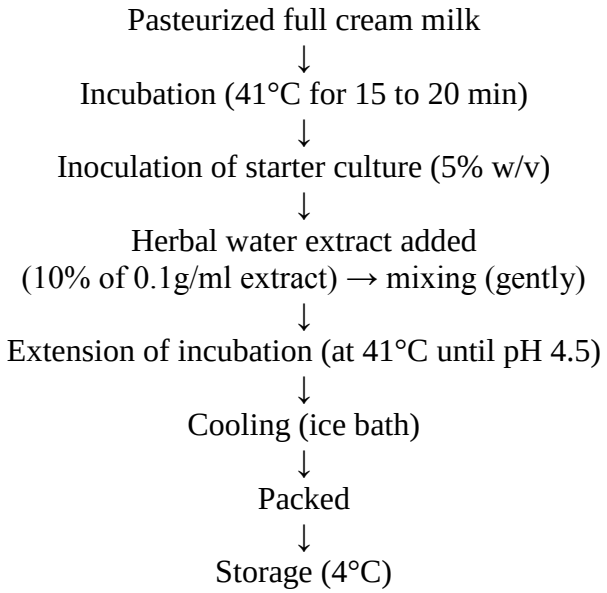


Figure 3.1 The flow diagram of traditional production of herbal-yogurt.

3.2.5 Measurement of pH and titratable acidity (TA)

The pH change was monitored by determining the free H^+ in yogurt in distilled water mixture (1:1) using a digital pH meter (Mettler Toledo 320). The pH meter was calibrated to pH 4.0 and 7.0 using standard solution.

Titratable acidity (TA; % lactic acid equivalent) was determined by titration using 0.1N NaOH. Yogurt sample (1ml) was transferred into an Erlenmeyer flask containing 9ml dH_2O, followed by the addition of a few drops of 0.1% phenolphthalein (Behrad *et al*., 2009). NaOH (0.1N) was added into the sample with continuous stirring until a definite pink colour lasting for 30 seconds was obtained.

The volume of NaOH required to neutralize the acid in yogurt was used to calculate the content of TA (Sadler and Murphy, 1998) by using the following formula

$$\text{TA (\% lactic acid)} = \frac{\text{d.f. X } V_{NaOH} \text{ X 0.009g x 0.1}}{\text{W (g)}} \text{ x 100\%}$$

Dilution factor (d.f.) = 10

V_{NaOH} = Volume of NaOH used to neutralize the lactic acid

0.009= conversion factor, 1ml NaOH (0.01N) neutralizes 0.009g of lactic acid

0.1 = Normality of NaOH

W = weight of yogurt sample for titration

3.2.6 Determination of proteolytic activity

Proteolytic activities in yogurt were assessed during fermentation by measuring liberated free amino groups using the O- phthaldialdehyde (OPA) method.

3.2.6.1 Sample preparation (yogurt water extract)

The yogurt water extract was prepared according to Martini, *et al*. (1987). Yogurt sample (10 g) and 2.5 ml dH_2O were homogenized with a homogenizer (Polytron PT2100) at the maximum setting for 10 seconds. The pH was then adjusted to 4.0 with 1M HCl. Coagulation of protein was allowed to occur at 45°C for 10 minutes prior to centrifugation (10000rpm, 10 minutes at 4^oC) to separate the supernatant from precipitated proteins. The supernatant was removed and this was neutralized at pH 7.0 using NaOH (0.5M). The supernatant was centrifuged again (10000 rpm, 10 minutes at 4^oC) to remove residual proteins. The yogurt water extract was either kept on ice and used within 12 hours of preparation or stored at -20ºC freezer until required for analysis.

3.2.6.2 O-phthaldialdehyde (OPA) assay

A rapid, sensitive and convenient o-phthaldialdehyde (OPA) based spectrophotometric method was determined according to Church, *et al.* (1983). The OPA solution was made by combining the following reagents: 25ml of 100mM sodium tetraborate; 2.5ml of 20% (w/w) sodium dodecyl sulphate (SDS); 40mg of OPA and 100µl of β-mercaptoethanol. The volume was made up to 50ml by adding dH_2O. This reagent was freshly prepared and used within 2 hours. Since OPA reagent is light-sensitive, it was protected from light source during preparation and running of the assay. A small aliquot of standard solution or yogurt- water extract (30 µl) was added directly into 1.0ml of OPA reagent in a 1.5ml cuvette. The mixture was mixed briefly by inversion and left at room temperature for 2 minutes. The absorbance was read at 340nm (Spectrophotometer, Shimadzu UV Mini 1240).

For standard curve samples tryptone solution of known concentrations (0.25-1.50 µg/ml; Figure 3.2) were also prepared. Linear regression of free amino groups concentration versus absorbance measurements allows the calculation of unknown free amino groups concentration. Typical equation of the standard curve used for calculating free amino groups is as follows:

$$\text{Free amino groups concentration (µg/g)} = \frac{A_{340} - 0.03}{0.1041}$$

where A_{340} was the spectrophotometric absorbance at 340 nm.

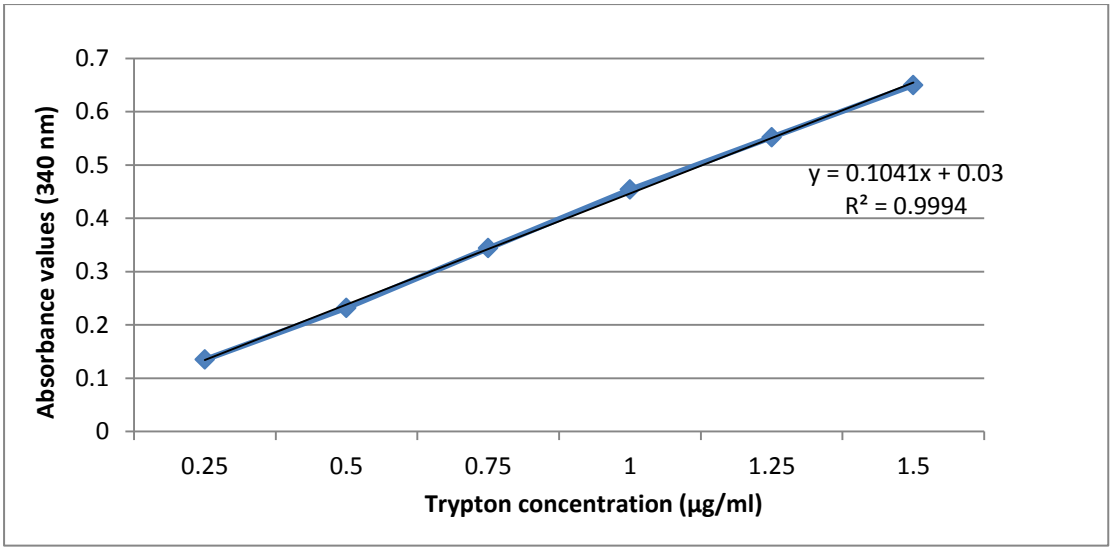


Figure 3.2 Typical calibration curve for free amino groups concentration.

3.2.7 Microbial viable cell count (VCC) in yogurt

3.2.7.1 Sample preparation

Yogurt samples (1ml) were mixed with 9ml of 0.15% sterile buffered peptone water (20 g/L dH_2O). The mixture was thoroughly stirred and serial decimal dilutions were prepared by using buffered peptone water.

3.2.7.2 Enumeration of *Lactobacillus* spp

Lactobacillus spp was enumerated as described by Kailasapathy *et al*. (2007). Diluted yogurt (1ml) was mixed with 15ml of autoclaved melted MRS media (62 g/L dH_2O, 45˚C) and the mixture was then placed onto the petri dishes. The mixture was stirred thoroughly by gently tilting and swirling the dishes. The dishes were then left at room temperature for 30 min to allow the media to solidify. The plates were sealed with parafilm and incubated (37˚C, 48 hours) in an inverted position. Viable microbial count (*Lactobacillus spp*) was calculated (Sivakumar and Kalaiarasu, 2010) as follows:

$$\text{CFU*/ml} = \frac{\text{Number of colonies formed X dilution factor of sample}}{\text{1 ml of sample}} \quad (3.1)$$

*CFU: Colony forming unit

3.2.7.3 Enumeration of *Streptococcus thermophilus*

S. thermophilus was enumerated as described by Rybka and Kailasaphaty (1995). Diluted yogurt (0.1ml) was inoculated into petri dish, which contained solidified M17 media (48.25 g/ 950 ml dH_2O with 50 ml of 10% w/v lactose solution). The sample was spreaded evenly on the surface using a sterile glass hockey-shaped spreader. The plates were placed in an inverted position in incubator (37˚C) for 48 hours. Viable microbial count was calculated (Sivakumar and Kalaiarasu, 2010) as follows:

$$\text{CFU*/ml} = \frac{\text{Number of colonies formed X dilution factor of sample}}{\text{0.1 ml of sample}}$$

*CFU: Colony forming unit

3.2.7.4 Enumeration of probiotic bacteria (*Bifidobacterium bifidum*)

The probiotic cultures count of *Bifidobacterium bifidum* were enumerated using MRS-LP agar. The formulation of MRS-LP was prepared according to Vinderola *et al*. (2000) where 0.2% (w/v) of lithium chloride (solid-powder) and 0.3% (w/v) of sodium propionate (solid-powder) were added to the MRS media (62 g/ 930 L dH_2O, 45˚C). Diluted yogurt (1ml) was mixed with 15ml of autoclaved melted MRS-LP media (see Section 3.2.6.2). The probiotic cultures were anaerobically incubated (GasPak System-OXOID) at 37^{o}C for 72 hours. The viable *B. bifidum* counts were calculated using equation 3.1 (see Section 3.2.6.2).

3.2.8 Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA, SPSS 14.0), followed by Duncan's post hoc test for mean comparison. All results presented are means of three independent replicates. The criterion for statistical significance was $p<0.05$. In addition, the standard curve used to calculate free amino groups was plotted by using linear regression method of fitting the plotted points from the absorbance's of the standard solution versus the concentrations which performed using Microsoft® Excel XP.

3.3 RESULTS

3.3.1 Acidity trend during milk fermentation

The pH (4.8±0.09) of *A. sativum* water extract was significantly ($p<0.05$) lower than *C. verum* water extract (6.3±0.02) but the TA values (0.3%) in both extracts were equals (Table 3.2) were similar. Figures 3.3, 3.4 and 3.5 show the changes in pH of cow, goat, and camel milk respectively in the presence and absence of *A. sativum* or *C. verum* water extract during fermentation at 41°C. it is well known that microbial fermentation process of milk includs 1) lag phase (slow accumulation of acid due to slow bacteria growth), 2) logarithmic phase (rapid pH decrease due to fast bacteria growth), and 3) stationary phase (slowdown of acidification rate due to acid inhibition of bacteria growth). Cow milk did not show lag phase compared to other milks in the presence and absence of *A. sativum* or *C. verum* water extract (Figure 3.3). Both goat and camel milk samples started to show observable pH reduction after one hour of incubation (Figures 3.4 and 3.5). The presence of *A. sativum* or *C. verum* in the three types of milk had no significant effect on pH reduction as compared to respective controls. However, the addition of *A. sativum* in camel milk was observed to resulted

in shorter incubation (240 min) time as compared to plain milk (300 min, $p<0.05$; Figure 3.5).

The changes in titratable acidity (TA) in cow, goat and camel milks during fermentation are shown in Figures 3.6, 3.7 and 3.8 respectively. TA in cow milk at the start of incubation (0 hour) until the 1st hour of fermentation was 0.21% lactic acid equivalent (LAE). In the presence of *C. verum* TA was significantly ($p<0.05$) increased to 0.27% LAE; $p<0.05$ after one hour of fermentation (Figure 3.6). *A. sativum* + cow milk showed higher ($p<0.05$) TA after 4 hours of fermentation as compared to plain milk. At the end of fermentation (pH 4.5) TA in *A. sativum*-yogurt (0.78% LAE) was higher ($p<0.05$) than that in *C. verum*-yogurt (0.54% LAE).

In goat milk at the beginning of incubation TA was 0.24% LAE (Figure 3.7). The TA value was not significantly changed in the presence of *A. sativum* or *C. verum* (0.27% and 0.29% LAE respectively). Goat milk + *A. sativum* or *C. verum* water extract showed higher TA ($p<0.05$) than plain milk between 90 min to 150 min of fermentation. At the end of fermentation, the TA value of yogurt in the presence of *A. sativum* or *C. verum* were closers (1.02% LAE) to that in plain-yogurt (0.99% LAE; Figure 3.7).

TA in camel milk was (0.30% LAE) at the start of fermentation but it was lower than that of *A. sativum* or *C. verum* + milk. The TA in *A. sativum*- or *C. verum*-camel milk increased to similar extent compared with plain- yogurt during fermentation (Figure 3.8).

Table 3.2. Changes in pH and titratable acidity (TA) as lactic acid equivalent %) in *A. sativum* and *C. verum* water extracts.

Sample	pH	TA%
AS-water extract	4.8±0.09	0.3±0.1
CV-water extract	6.3±0.02*	0.3±0.1

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts = 0.1g/ml. Results are shown as a mean (n = 3) ± standard error. *p < 0.05

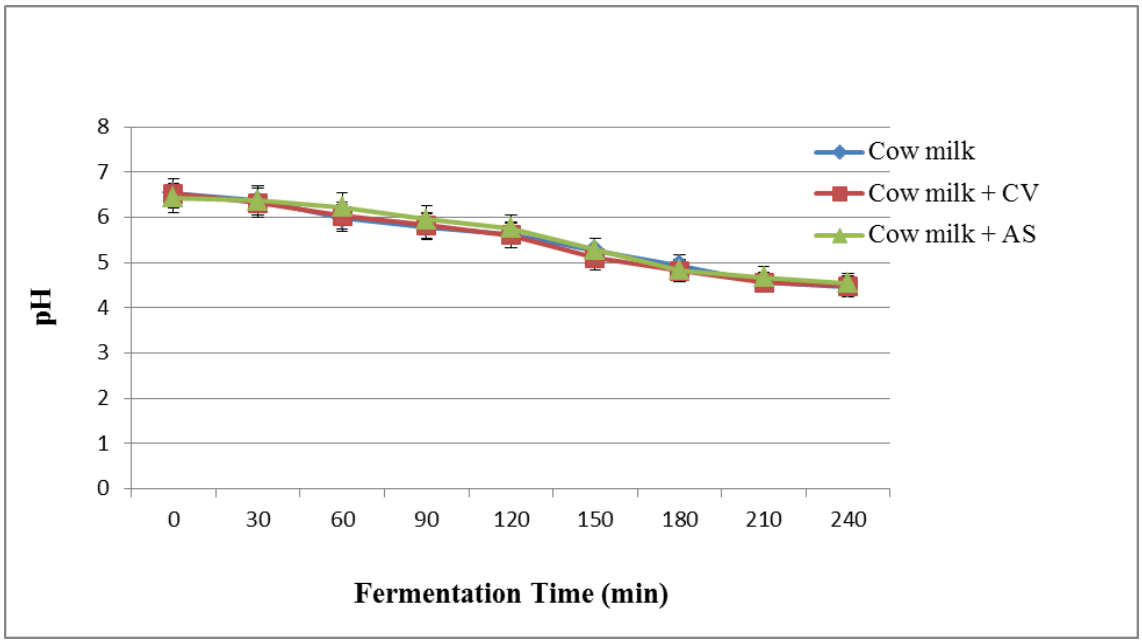


Figure 3.3 Changes in pH of cow milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

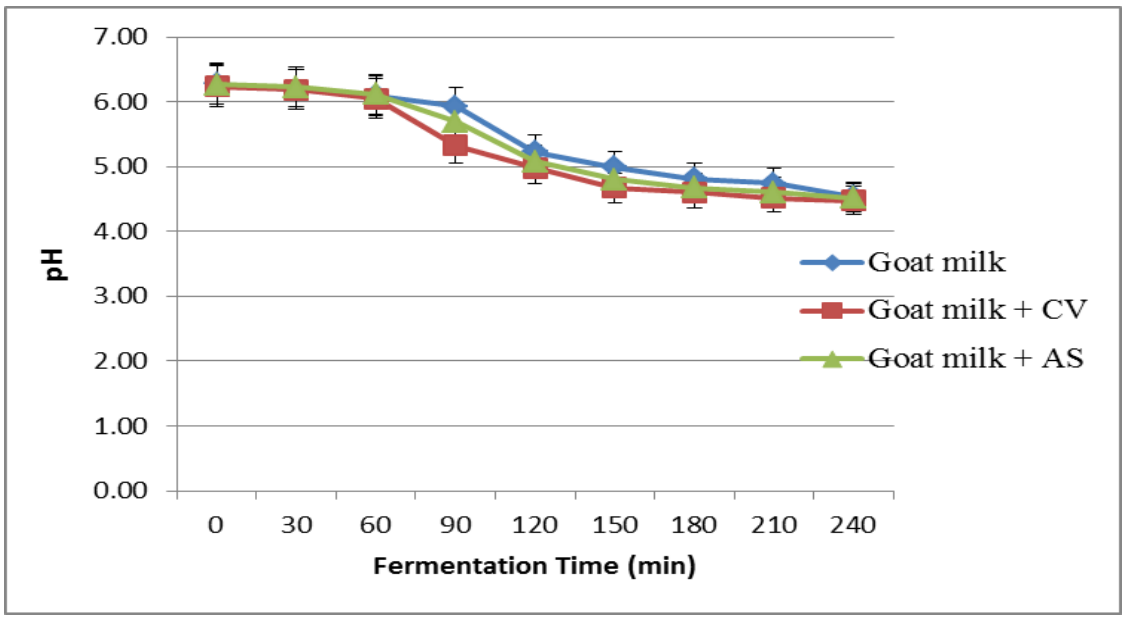


Figure 3.4 Changes in pH of goat milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

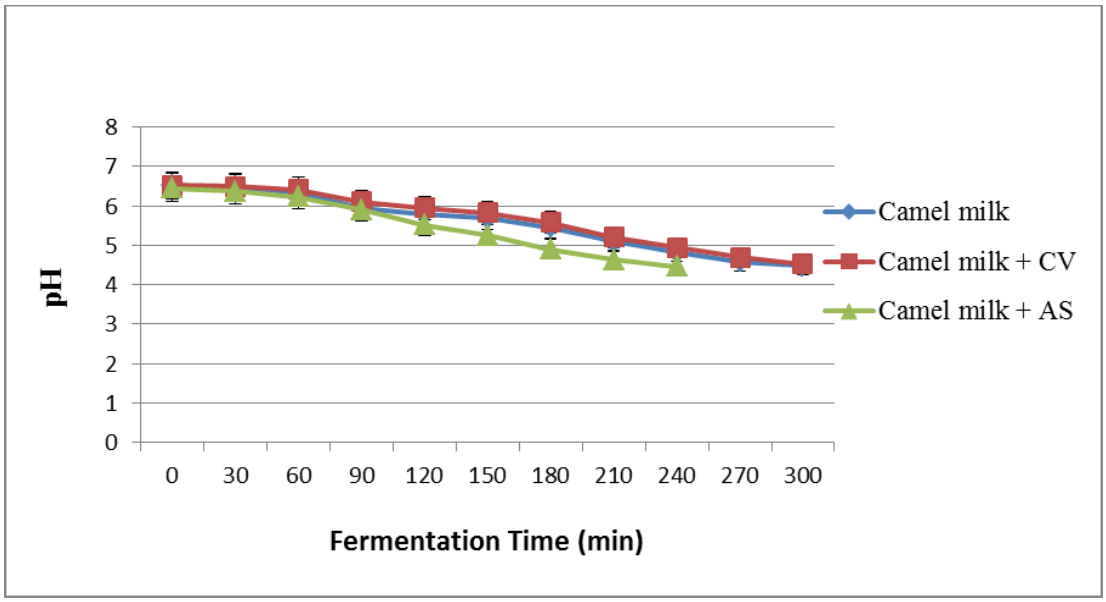


Figure 3.5 Changes in pH of camel milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

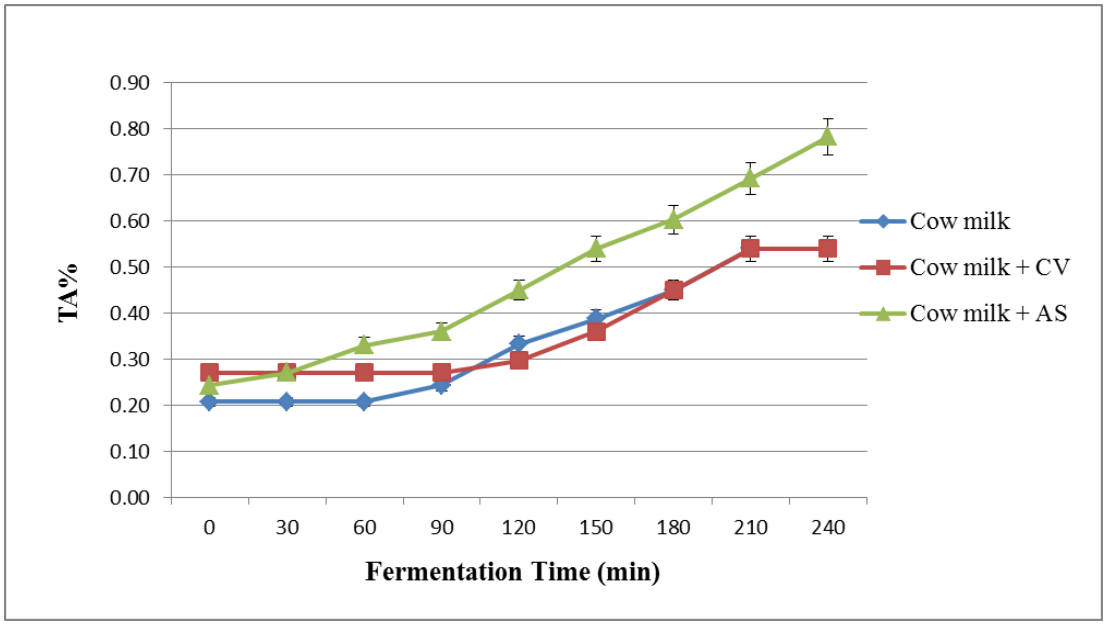


Figure 3.6 Changes in titratable acidity (TA; lactic acid equivalent %) of cow milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

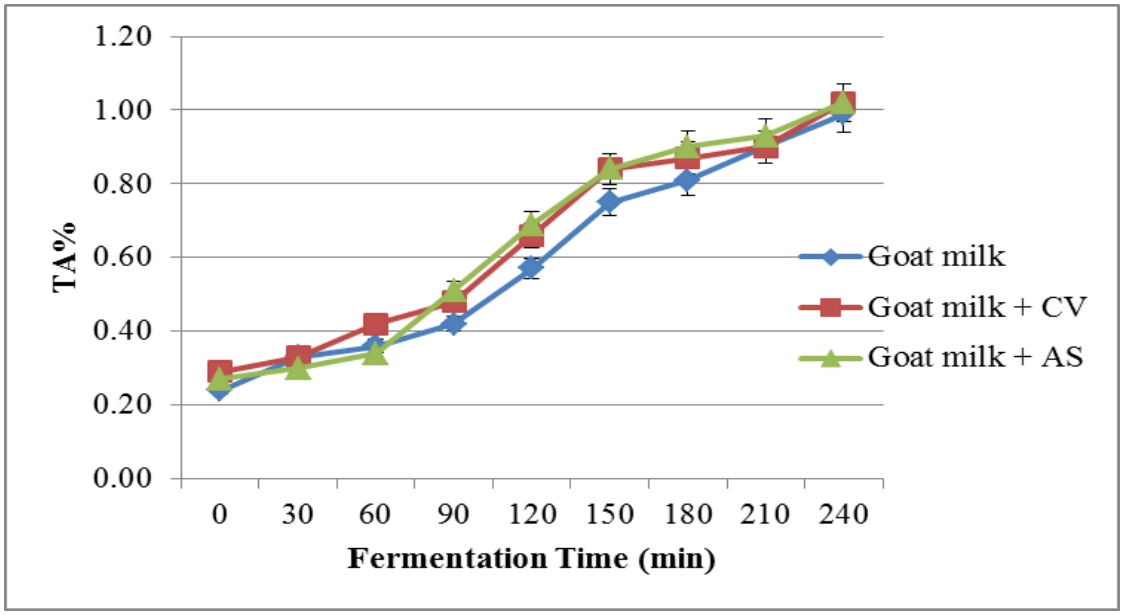


Figure 3.7 Changes in titratable acidity (TA; lactic acid equivalent %) of goat milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

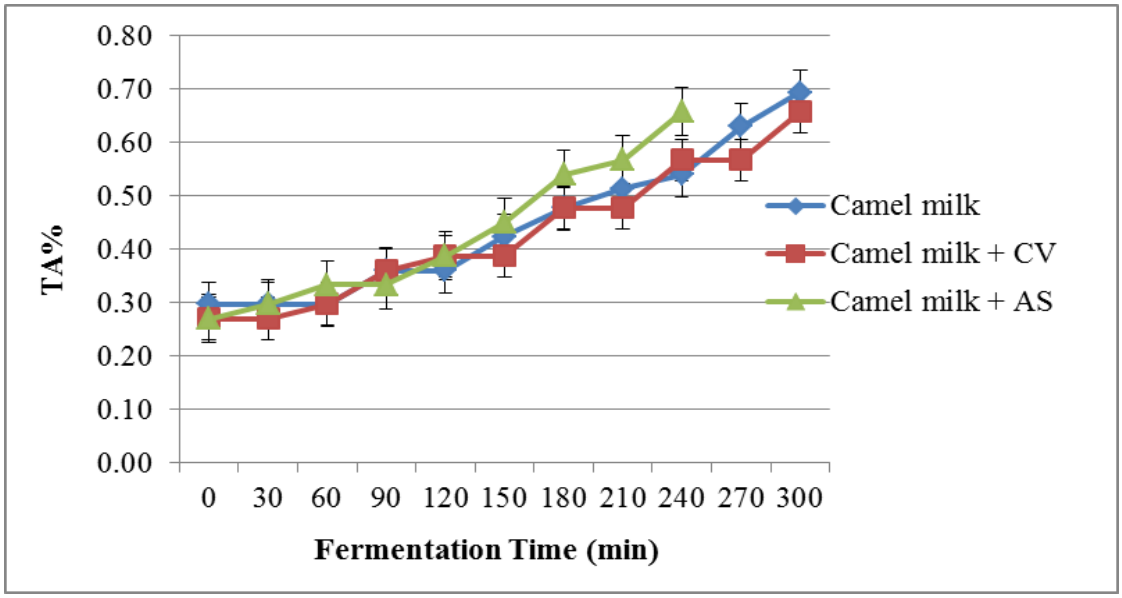


Figure 3.8 Changes in titratable acidity (TA; lactic acid equivalent %) of camel milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

3.3.2 Free amino group in hebal extracts and yogurt

A) Concentration of free amino groups in herbal extracts

The free amino groups concentration of *A. sativum* water extract (22.4±0.1 µg/g) were significantly higher than that of *C. verum* extract (10.2±0.1 µg/g; Table 3.3).

Table 3.3 Total free amino groups (µg/g) in *A. sativum* and *C. verum* water extracts.

Sample	Concentration (µg/g)
AS	22.4±0.1*
CV	10.2±0.1

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts = 0.1g/ml. Results are shown as a mean (n = 3) ± standard error. *p < 0.05

B) Proteolytic activity in cow milk yogurt during fermentation

The initial free amino groups value in of cow milk mixture with *C. verum* water extract (19.21±0.1µg/g) showed no significant difference compared to milk alone (12.81±0.1µg/g; Figure 3.9). The free amino groups value in milk + *C. verum*

significantly ($p<0.05$) increased to 172.90±0.03 µg/g after 4 hours of fermentation whereas that of milk alone reached to about 80.10±0.02 µg/g. The presence of *A. sativum* water extract in milk increased the free amino groups to 166.51±0.1 µg/g (0 hour). Fermentation of milk in the presence of *A. sativum* increased free amino groups to 262.57±0.1 µg/g after 4 hours of incubation (Figure 3.9). By the end of fermentation the highest increase of proteolytic activity was noticed in *C. verum*-yogurt (9 folds) followed by plain-yogurt (6.3 folds) and *A. sativum*-yogurt (1.6 folds).

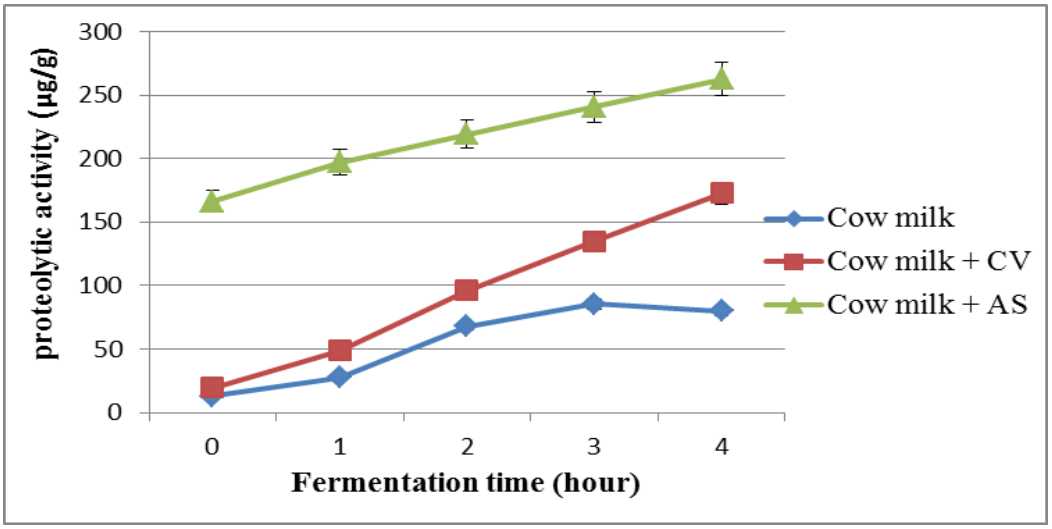


Figure 3.9 Changes in proteolytic activity (µg/g) of cow milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

C) Proteolytic activity in goat milk yogurt during fermentation

The initial free amino groups value in goat milk before fermentation was 213.26±0.05 µg/g (Figure 3.10). Free amino groups value was higher ($p<0.05$) in the mixture of milk with *A. sativum* water extract (285.87±0.06 µg/g; 0 hour) but not in milk + *C. verum* (235.83±0.05 µg/g) compared to milk alone at the beginning of

fermentation. Proteolytic activity was significantly increased in the 2nd hour of fermentation for plain milk (252.09±0.05 µg/g) and milk + *C. verum* (295.1±0.04 µg/g). The proteolytic activity in milk + *A. sativum* decreased after the 1st hour (227.5±0.05 µg/g) followed by increased towards initial value during the next 3 hours. Differences in free amino groups were significant between control and both milk treatments at the 3rd and 4th hours of fermentation (Figure 3.10).

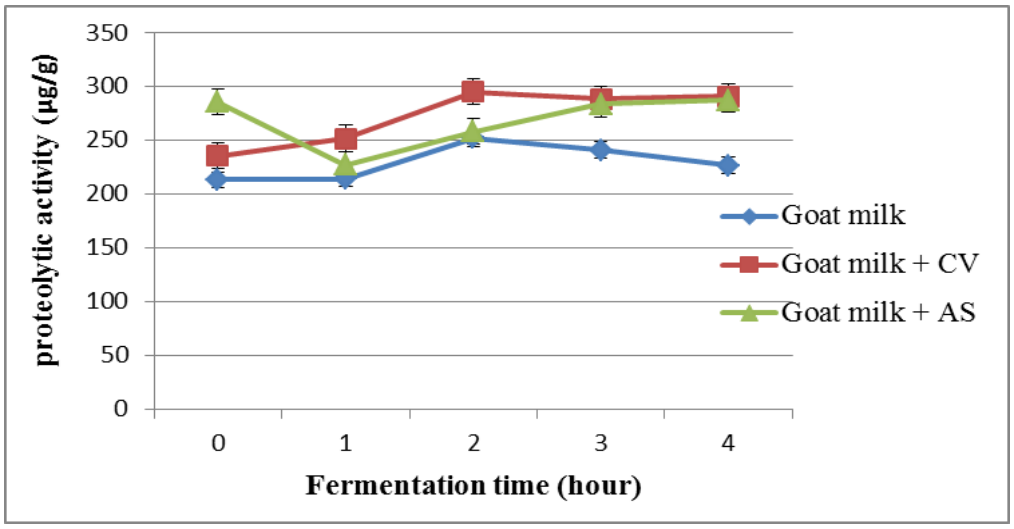


Figure 3.10 Changes in proteolytic activity (µg/g) of goat milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

D) Proteolytic activity in camel milk yogurt during fermentation

The initial free amino groups value in camel milk was 268.97± 0.03 µg/g (Figure 3.11). This value was increased to 294.59± 0.02 µg/g ($p>0.05$) in the presence of *C. verum*. The addition of *A. sativum* extract into milk increased free amino groups to 425.87± 0.1 µg/g ($p<0.05$) at 0 hour. Free amino groups values during 4 hours fermentation were higher in milk + *A. sativum* (432.17 µg/g - 470.66 µg/g) than milk alone (274.23 µg/g - 352.49 µg/g). Free amino groups in milk + *C. verum* during 5 hours fermentation range of 307.15 µg/g - 397.07 µg/g. The increase of proteolytic

activity in yogurt as a result of fermentation both in presence and absence of *A. sativum* or *C. verum* water extract ranged from 1.1 folds to 1.4 folds (Figure 3.11).

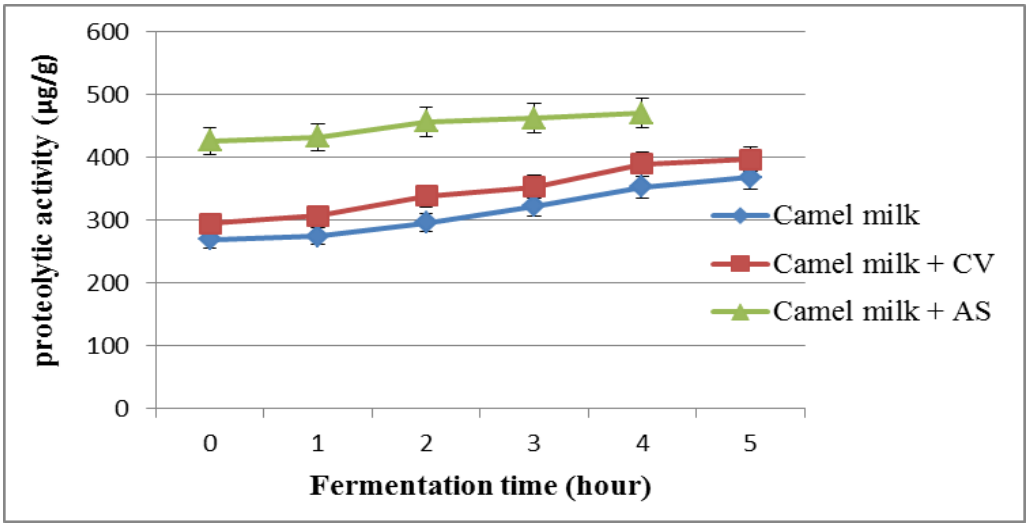


Figure 3.11 Changes in proteolytic activity (µg/g) in camel milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C) for milk + *A. sativum* and 5 hours fermentation for plain milk and milk + *C. verum*. Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

3.3.3 The growth of LAB during milk fermentation

3.3.3.1 Viable cell count (VCC) of *S. thermophilus*

A) In cow milk yogurt

VCC of *S. thermophilus* in the mixture of milk with *A. sativum* or *C. verum* water extract was not different (0.73 x 10^8cfu/ml and 0.66 x 10^8cfu/ml respectively) as compared to that of milk alone (0.62 x 10^8cfu/ml; Figure 3.12). The presence of herbal extracts did not affect the growth of *S. thermophilus* during the first hour of fermentation. However, the VCC of *S. thermophilus* increased ($p<0.05$) to 2.03 x 10^8cfu/ml, 2.66 x 10^8cfu/ml and 2.31 x 10^8cfu/ml for milk, milk + *A. sativum* and milk + *C. verum* respectively after the next 3 hours of fermentation (Figure 3.12). The VCC of *S. thermophilus* was higher in milk + *C. verum* (2.70 x 10^8cfu/ml; $p<0.05$)

and milk + *A. sativum* (2.60 x 10^8cfu/ml; p>0.05) than plain yogurt (2.40 x 10^8cfu/ml) at the end of fermentation. The increase in VCC of *S. thermophilus* during the fermentation was the highest in *C. verum*-yogurt (4.1 folds) followed by plain-yogurt (3.8 folds) and *A. sativum*-yogurt (3.6 folds).

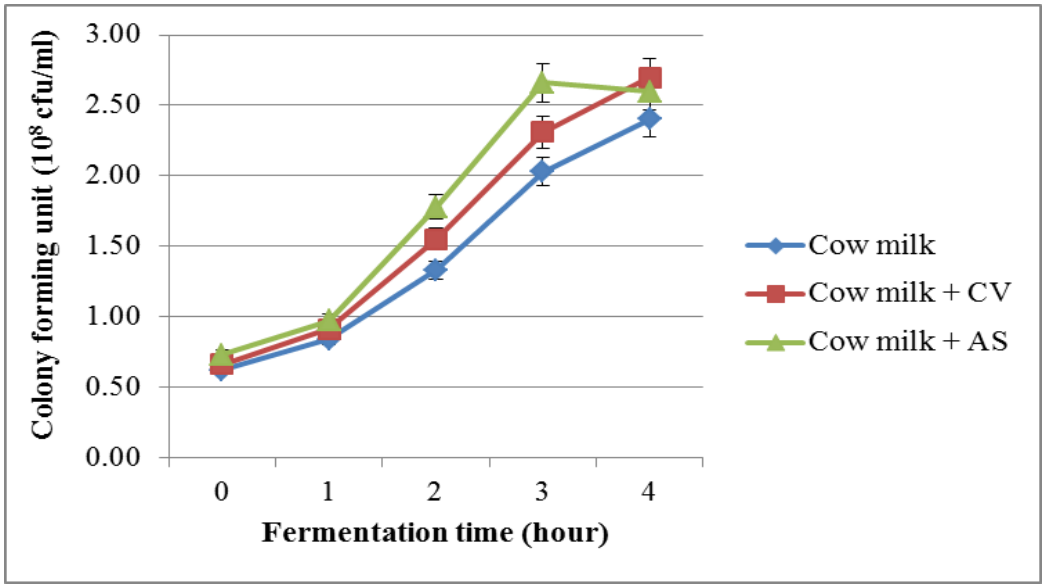


Figure 3.12 Changes in viable cell count (VCC) of *S. thermophilus* (10^8cfu/ml) in cow milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

B) In goat milk yogurt

The initial VCC of *S. thermophilus* in goat milk (0.94 x 10^8cfu/ml) were not affected by the presence of *A. sativum* or *C. verum* water extract in milk (1.02 x 10^8cfu/ml and 0.98 x 10^8cfu/ml respectively; Figure 3.13). Fermentation of milk for one hour had no significant effect on *S. thermophilus* growth in all treatments. However, the effects of addition of *A. sativum* or *C. verum* extract in milk on *S. thermophilus* VCC was significant after the 2nd hour to the end of fermentation (1.81 x 10^8cfu/ml - 2.87 x 10^8cfu/ml and 1.7 x 10^8cfu/ml - 2.92 x 10^8cfu/ml respectively)

compared to milk (1.45 x 10^8cfu/ml - 2.43 x 10^8cfu/ml). The increase in VCC of *S. thermophilus* was the highest in *C. verum*-yogurt (3 folds) followed by *A. sativum*-yogurt (2.8 folds) and plain-yogurt (2.6 folds) by the end of the 4 hours of fermentation (Figure 3.13).

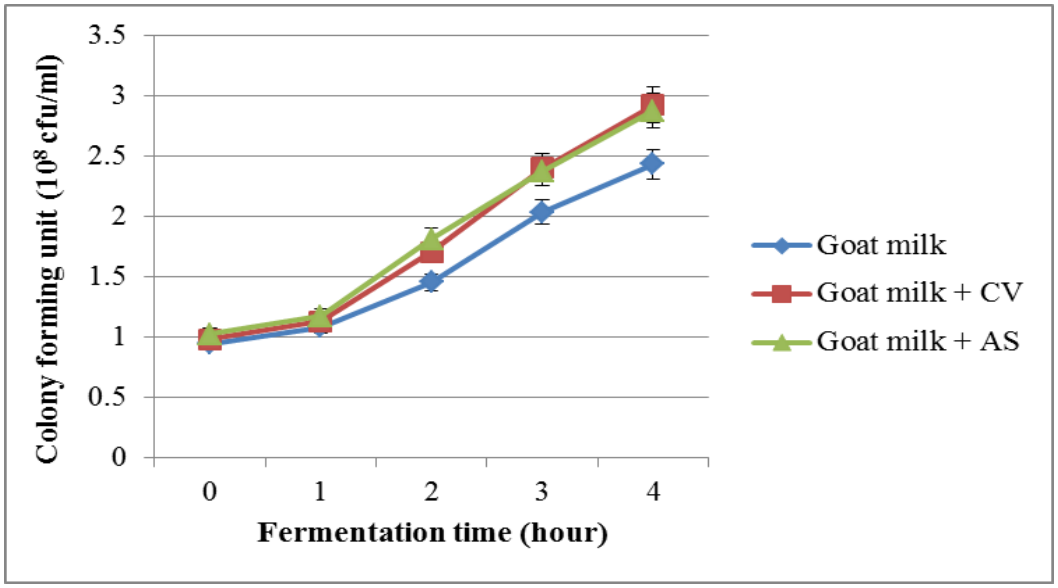


Figure 3.13 Changes in viable cell count (VCC) of *S. thermophilus* (10^8cfu/ml) in goat milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

C) In camel milk yogurt

The initial *S. thermophilus* cell counts of camel milk in the presence of *A. sativum* or *C. verum* water extract (1.31 x 10^8cfu/ml and 1.34 x 10^8cfu/ml respectively) were not different ($p>0.05$) from milk alone (1.14 x 10^8cfu/ml; Figure 3.14). The VCC of *S. thermophilus* did not change much ($p>0.05$) after 1 hour of fermentation but increased ($p<0.05$) after the 2nd hour of fermentation (Figure 3.14) for all treated samples. The highest *S. thermophilus* VCC was seen at the end of fermentation with 3.1 x 10^8cfu/ml for both plain- and *A. sativum*- yogurt and 3.6 x

10^8cfu/ml for *C. verum*-yogurt. The total increase in VCC was almost the same in plain- and *A. sativum*- yogurt (2.7 folds) whereas *C. verum*-yogurt had 2.3 folds higher by the end of fermentation.

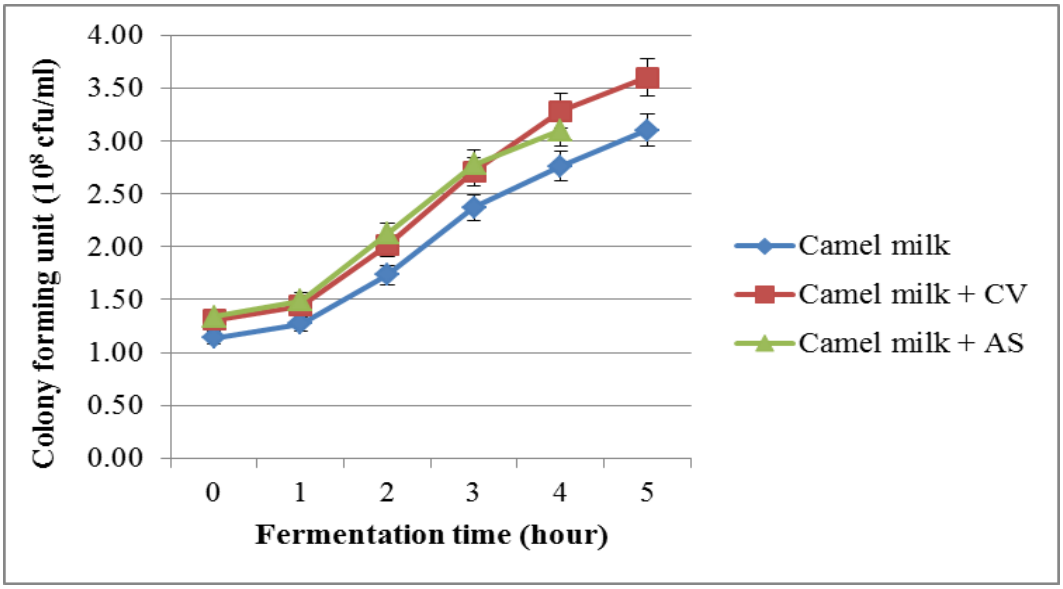


Figure 3.14 Changes in viable cell count (VCC) of *S. thermophilus* (10^8cfu/ml) in camel milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C) for milk + *A. sativum* and 5 hours fermentation for plain milk and milk + *C. verum*. Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

3.3.3.2 Viable cell count of *Lactobacillus* spp

A) In cow milk yogurt

The initial VCC of *Lactobacillus* spp was higher ($p<0.05$) in the mixture of milk with *A. sativum* or *C. verum* water extract (1.78 x10^6 cfu/ml and 1.65 x10^6 cfu/ml respectively) than in milk alone (1.19 x10^6 cfu/ml; Figure 3.15). *Lactobacillus* spp grow at similar extent in milk and milk + *A. sativum* during the first two hours of fermentation. VCC of *Lactobacillus* spp for the next two hours of fermentation reduced for plain- and *A. sativum*- yogurt (Figure 3.15). *Lactobacillus* spp VCC in milk + *C. verum* increased ($p<0.05$) after two hours of fermentation (2.10 x10^6 cfu/ml)

but remained the same for the next two hours of fermentation. The VCC at the end of the incubation (4 hours) was the highest in *C. verum*- yogurt (2.14 x10^6 cfu/ml) followed by *A. sativum*- yogurt (1.70 x10^6 cfu/ml) and plain- yogurt (1.39 x10^6 cfu/ml).

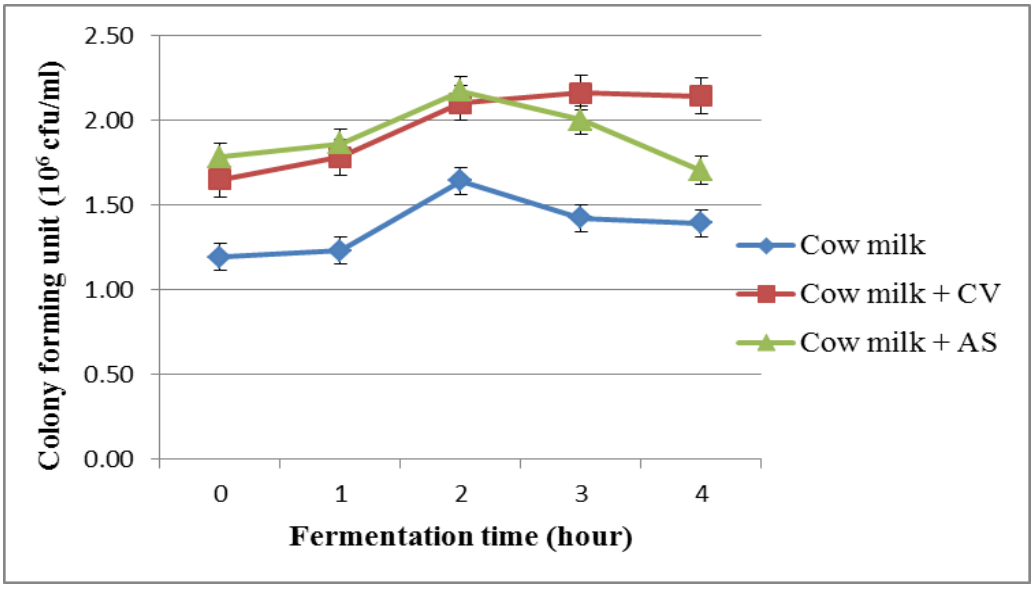


Figure 3.15 Changes in viable cell count (VCC) of *Lactobacillus* spp. (10^6cfu/ml) in cow milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

B) In goat milk yogurt

The initial VCC of *Lactobacillus* spp in goat milk was 1.44 x10^6 cfu/ml (Figure 3.16). This value was increased to 2.12 x10^6 cfu/ml and 1.72 x10^6 cfu/ml ($p<0.05$) in the presence of *A. sativum* and *C. verum* water extracts respectively. The VCC of *Lactobacillus* spp increased significantly ($p<0.05$) in all treatments from the 2nd hour of incubation onwards (Figure 3.16) and was about 2 folds higher in all types of yogurt by the end of fermentation.

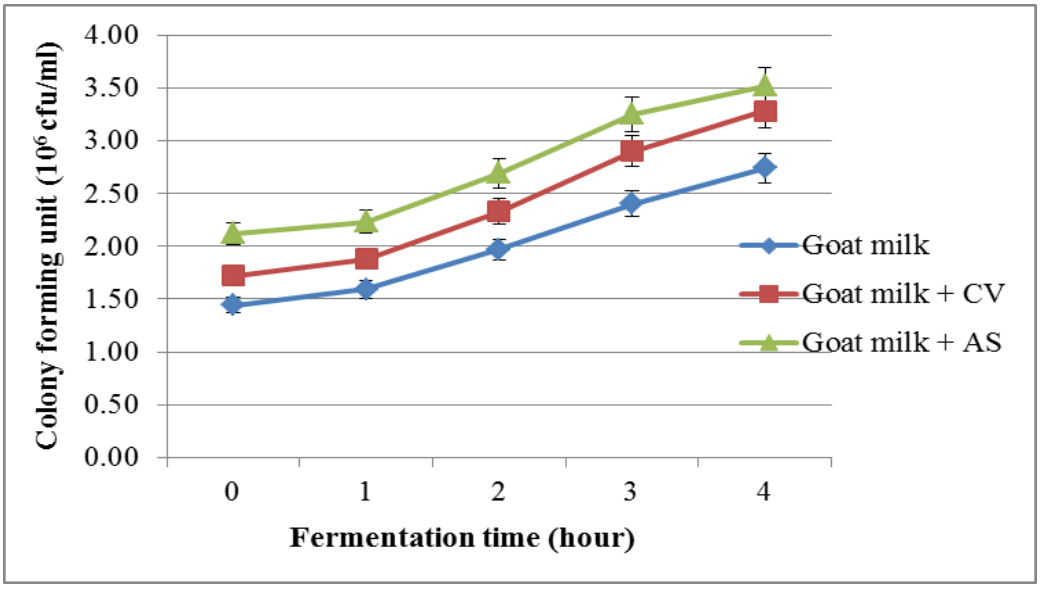


Figure 3.16 Changes in viable cell count (VCC) of *Lactobacillus* spp. (10^6cfu/ml) in goat milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

C) In camel milk yogurt

The initial VCC of *Lactobacillus* spp in camel milk was 3.05 x10^6 cfu/ml. The addition of *C. verum* extract into milk did not affect the initial VCC (3.29 x10^6 cfu/ml, Figure 3.17). However, milk + *A. sativum* showed higher ($p<0.05$) initial VCC of *Lactobacillus* spp (5.68 x10^6 cfu/ml) than milk alone. Linear growth of *Lactobacillus* spp occurred after the first hours of incubation with the fastest growth shown by *A. sativum*- yogurt followed by *C. verum*- and plain- yogurt (Figure 3.17). *A. sativum*- yogurt had in the highest ($p<0.05$) VCC of *Lactobacillus* spp (26.87 x10^6 cfu/ml) at the 4th hour of incubation compared to *C. verum*- yogurt (17.99 x10^6 cfu/ml) and plain- yogurt (13.79 x10^6 cfu/ml).

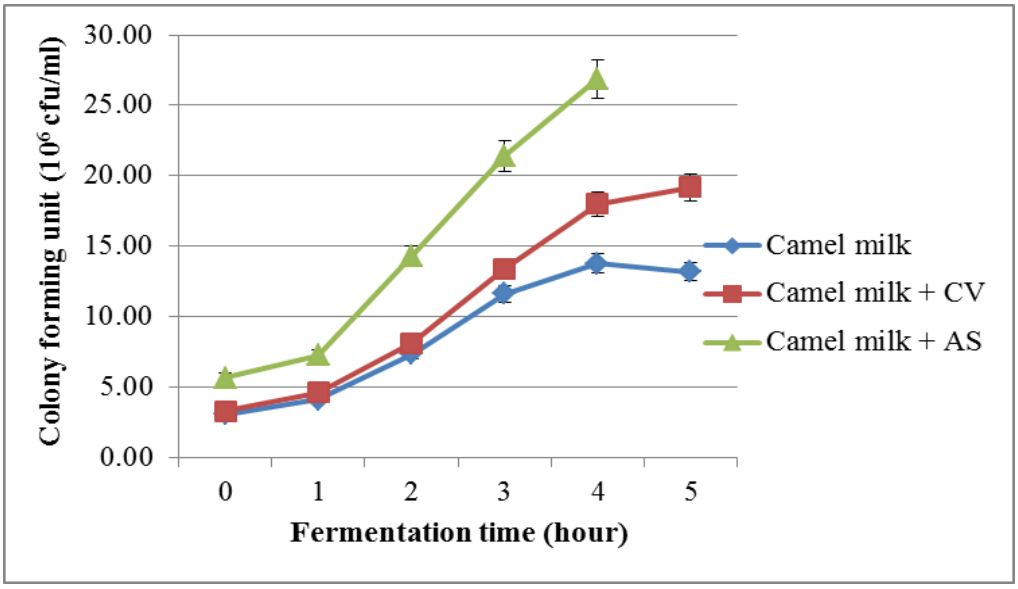


Figure 3.17 Changes in viable cell count (VCC) of *Lactobacillus* spp. (10^6cfu/ml) in camel milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C) for milk + *A. sativum* and 5 hours fermentation for plain milk and milk + *C. verum*. Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

3.3.3.3 Viable cell count of probiotic (*B. bifidum*)

A) In cow milk yogurt

The *B. bifidum* VCC in cow milk at the start of fermentation was 1.7 x 10^8cfu/ml (Figure 3.18). No difference in *B. bifidum* VCC was observed in milk + *A. sativum* or milk + *C. verum* (5.7 x 10^8 cfu/ml and 3.8 x 10^8 cfu/ml respectively). The VCC of *B. bifidum* increased ($p<0.05$) by the 1st hour of fermentation and reached the maximum VCC by the 3rd hour (72.2 x 10^8 cfu/ml, 100.2 x 10^8 cfu/ml and 21.3 x 10^8 cfu/ml for *C. verum* -, *A.* sativum- and plain- yogurt respectively). However, the VCC of *B. bifidum* reduced in *A. sativum*-yogurt to 81.0 x 10^8 cfu/ml; $p<0.05$ and in *C. verum*-yogurt to 65.9 x 10^8 cfu/ml; $p>0.05$ by the fourth hour of fermentation (Figure 3.18).

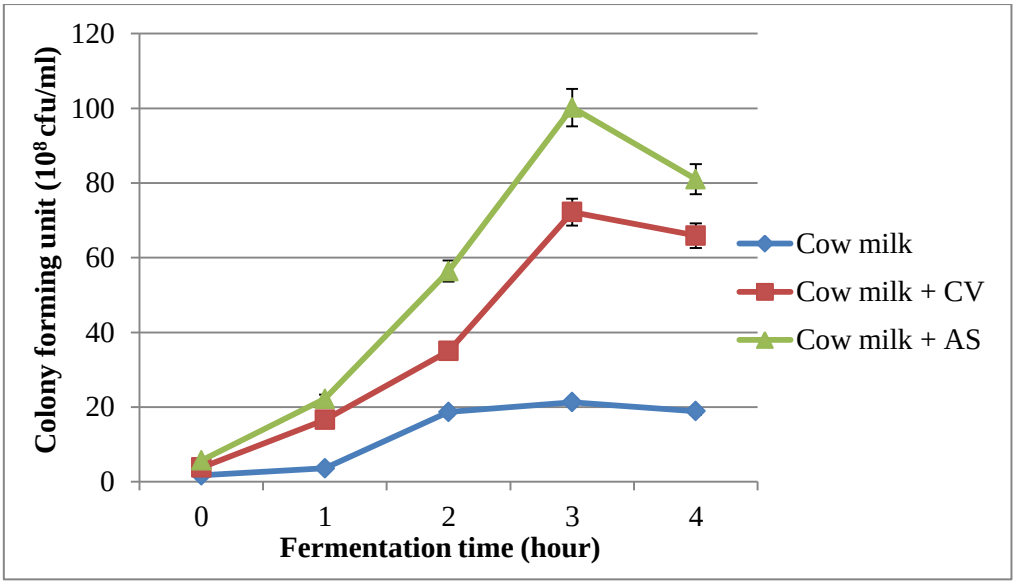


Figure 3.18 Changes in viable cell count (VCC) of *B. bifidum* (10^8cfu/ml) in cow milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

B) In goat milk yogurt

The initial *B. bifidum* VCC in goat milk + *A. sativum* or *C. verum* (12.0 x 10^8 cfu/ml and 13.5 x 10^8 cfu/ml respectively) was higher than in milk alone (8.8 x 10^8 cfu/ml; Figure 3.19). *B. bifidum* VCC showed no significant increase ($p>0.05$) after the 1st hour of incubation in all treated milks. Incubation of milk to three hours enhanced ($p<0.05$) the growth of *B. bifidum* in milk + *A. sativum* (98.4 x 10^8 cfu/ml) and milk + *C. verum* (113.0 x 10^8 cfu/ml) compared to milk alone (46.8 x 10^8 cfu/ml). No further increase in *B. bifidum* VCC after the 4th hour of fermentation (43.0 x 10^8 cfu/ml and 91.5 x 10^8 cfu/ml for plain- and *A. sativum*- yogurt respectively). The VCC in *C. verum*-yogurt increased to 134.3 x 10^8 cfu/ml at the fourth hour of fermentation (Figure 3.19). The highest growth of *B. bifidum* was shown in *C. verum*-yogurt (10 folds) followed by *A. sativum*-yogurt (7.6 folds) and plain-yogurt (4.8 folds) by the end of fermentation.

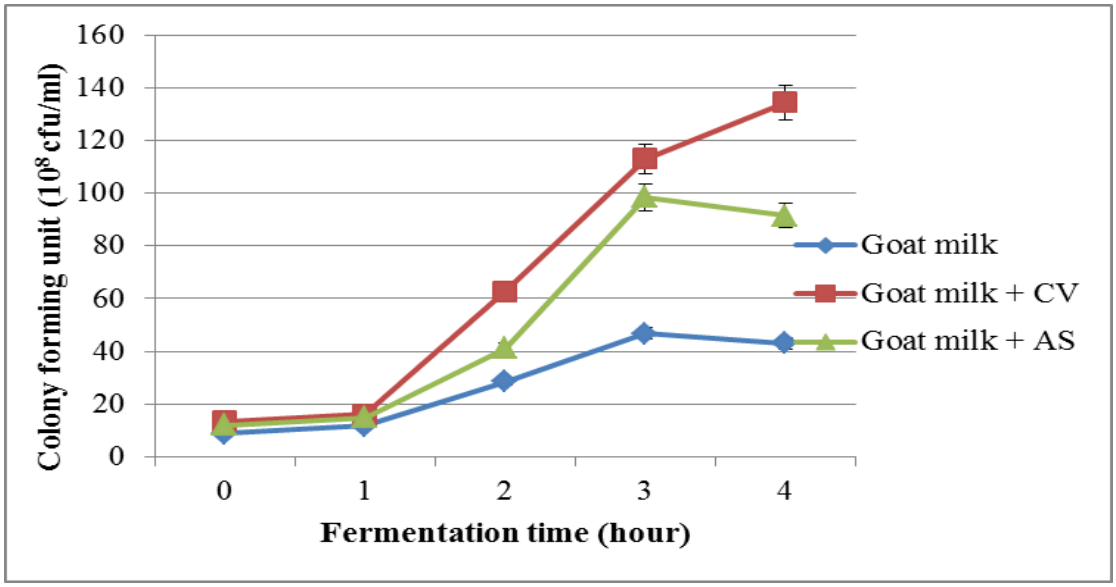


Figure 3.19 Changes in viable cell count (VCC) of *B. bifidum* (10^8cfu/ml) in goat milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

C) In camel milk yogurt

The *B. bifidum* VCC in the mixture of milk with *A. sativum* (~17.0 x 10^8cfu/ml) and *C. verum* (~18.0 x 10^8cfu/ml) was not significantly different from milk alone (~11.0 x 10^8cfu/ml) at zero hour of fermentation and even after 1st hour of fermentation (Figure 3.20). The highest *B. bifidum* VCC in plain-yogurt was seen on the 2nd hour of fermentation (51.1 x 10^8 cfu/ml) followed by a gradual reduction to 20.0 x 10^8 cfu/ml ($p<0.05$) by the 5th hour of fermentation. However, the VCC of *B. bifidum* in yogurt increased ($p<0.05$) in the presence of *A. sativum* or *C. verum* water extract (196.1 x 10^8 cfu/ml and 255.5 x 10^8 cfu/ml respectively) by the end of fermentation (Figure 3.20). *C. verum*- yogurt showed the highest increase in *B. bifidum* VCC (14 folds) throughout the fermentation followed by *A. sativum*- yogurt (11 folds) and plain- yogurt (2 folds).

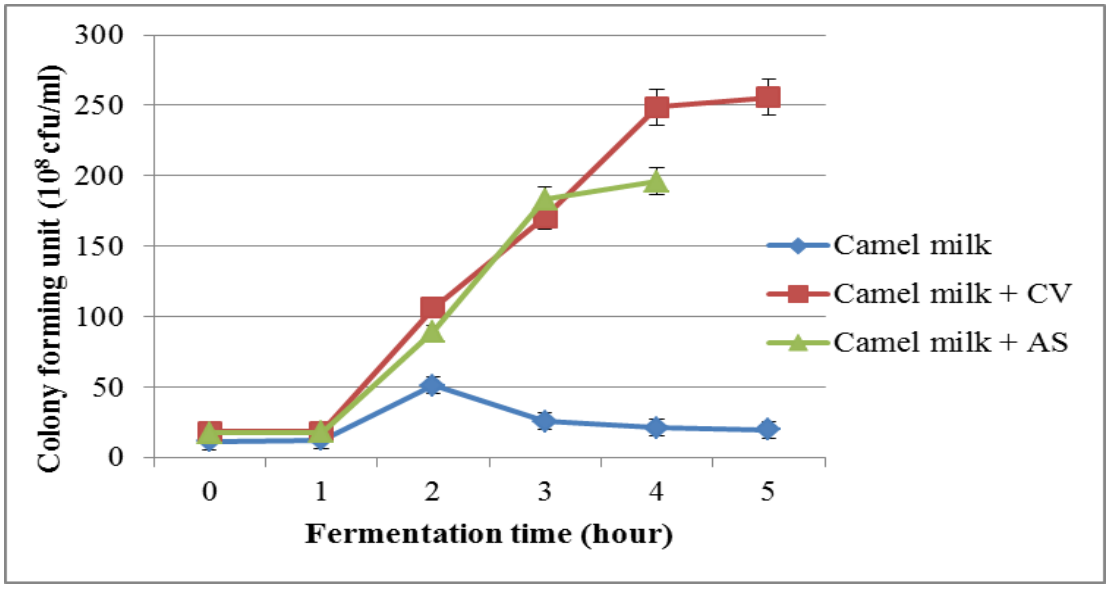


Figure 3.20 Changes in viable cell count (VCC) of *B. bifidum* (10^8 cfu/ml) in camel milk in the presence and absence of *A. sativum* (AS) or *C. verum* (CV) water extract during 4 hours fermentation (41°C) for milk + *A. sativum* and 5 hours fermentation for plain milk and milk + *C. verum*. Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to control at the same incubation period.

3.4 DISCUSSION

3.4.1 Changes in pH and titratable acidity

Acidification is a very essential process for food preservation via fermentation because the accumulation of acid (production of bacteria metabolic activities) reduces the pH and thus prevents the growth of spoilage bacteria (Novak and Lubiere, 2000). The pH reduction can be used as indicator of different growth rates of LAB and probiotics (Adolfsson *et al*., 2004). Thus the measurement of pH and titratable acidity offer some insights into the viability and growth of starter culture. In the case of yogurt, the starter culture added into milk utilize carbon sources from lactose, glucose and to a smaller extent galactose as substrates for energy production and in the process produce lactic acid and other organic acids as metabolic by-products (Novak and Lubiere, 2000). The present study showed variations in the rate of H^+ produced during the fermentation of cow, camel and goat milks.

Susceptibility of LAB to environmental stresses in logarithmic phase as compared to those in stationary phase has been previously reported in cow- (Sefa-Dedeh *et al.,* 2001; Al-Kadamany *et al.,* 2003; Medoua *et al.,* 2008), camel- (Lore *et al.,* 2005; Sulieman *et al.,* 2007; Abdelgadir *et al.,* 2008; Hassan *et al.,* 2008; Rahman *et al.,* 2009) and goat- (Park, 2007; Minervini *et al.,* 2009; Eissa *et al.,* 2010) milk fermentation systems. The actual shape of fermentation curve is strictly dependent on several factors including the milk base, the presence of the microorganisms and their associated enzymes in milk, starter culture, type and concentration of supplemented ingredients, milk heat treatment, and incubation temperature (Soukoulis, 2007). In the present study, pH curve in camel and goat milk fermentation showed longer lag phase than cow milk and this could be attributed to the higher antibacterial factors naturally present in camel and goat milk than in cow milk (Attia *et al.,* 2001; Pavlović *et al.,* 2006). The inclusion of *A. sativum* or *C. verum* water extract in three types of milk did not significantly influence pH reduction during fermentation. However, camel milk + *A. sativum* reached pH 4.5 after 4 hours, 1 hour less in fermentation time as compared to milk alone. This could be a consequence of higher ($p<0.05$) *Lactobacillus* spp. cell counts (Figure 3.17) which produced relatively more lactic acid than plain milk during fermentation. On the other hand, the increase of fermentation time (5 hours) for camel milk despite higher ($p<0.05$) *Lactobacillus* spp. cell counts than those in cow and goat milk (4 hours) could be explained by high buffering capacity in camel milk (Yagil and Van., 2000) compared to cow and goat milk.

An increase in titratable acidity (% lactic acid equivalent) as a result of accumulation of organic acids during fermentation is related to the metabolic activity of starter culture (Medoua *et al.,* 2008). Key microbial metabolic by-products

contributing to this acidification include acetic acid, citric acid, butyric acid, acetaldehyde, formic acid and lactic acid (Billard *et al.,* 2007). The present study showed higher titratable acidity (TA) in the mixture of cow milk with *A. sativum* than in the cow milk alone during fermentation. This could attribute to the higher growth of *Lactobacillus* spp (Figure 3.15) which resulted in increase in lactic acid production.

3.4.2 Proteolytic activity

Lactic acid bacteria are unable to synthesize essential amino acids and thus it is necessary for them to be capable of breaking down and efficiently utilize protein available from their surroundings (Ramachandran and Shah 2009). The proteolytic system of dairy LAB consists of exocellular proteinases, membrane-bound aminopeptidases, intracellular exopeptidases and proteinases (Shihata and Shah, 2000; Gobbetti *et al*., 2002). The proteolytic activity of these enzymes yield polypeptides of various sizes, each with free amino groups that can be determined quantitatively using OPA method. Thus the free amino groups in the yogurt indirectly reflect the proteolytic activity of LAB in different types of milk under the influence of additives such as *A. sativum* or *C. verum* water extracts. In the present study, the effectiveness of LAB to degrade milk proteins among the three types of milk in presence of herbal extracts occurred at the following order: cow milk + herbal extract > camel milk + herbal extract > goat milk + herbal extract. The proteolytic activity was higher in the presence of *A. sativum* and *C. verum* water extracts in cow milk than that in goat and camel milk during fermentation. This could occur as a result of lower antibacterial activity in cow milk (Abolghait *et al*., 2011) than other treated milks (Agrawal *et al*., 2005; Pavlović *et al*., 2006). In addition, yogurt bacteria probably could get access to readily available peptides/amino acids in goat and camel milks (Haenlein, 2004; Al-Alawi and Laleye, 2011).

Higher free amino groups in *A. sativum*- (22.4±0.1 µg/ml) than *C. verum*- (10.2±0.1 µg/ml) water extract (Table 3.3) could explain the higher free amino groups in *A. sativum*-yogurt than in *C. verum*-yogurt.

3.4.3 Viability of yogurt starter culture

The addition of probiotics to yogurt is a natural way of enhancing the functionality of yogurt (Ramchandran and Shah, 2009). There are two patterns of growth which can be observed in yogurt, one for *Lactobacillus* spp. and the other for *S. thermophilus*. *S. thermophilus* tend to grow faster during earlier fermentation (phase 1) due to liberation of amino acids from casein in milk. This is attributed to the accumulation of fermentation products such as lactic and acetic acids which stimulate the growth of *Lactobacillus* spp. (Robinson *et al*., 2002). The present study showed no significant differences in *S. thermophilus* VCC in milk alone and milk + *A. sativum* or *C. verum* water extract during the 1st hour of fermentation. This could be related to lag phase of the bacterial growth cycle which adapt themselves to growth conditions. However, the inclusion of *A. sativum* or *C. verum* water extract in cow, camel and goat milks enhanced ($p<0.05$) the growth of *S. thermophilus* from the 2nd hour to the end of fermentation as compared to milk alone. Since the present study showed that proteolytic activity increased only during herbal cow milk mixture fermentation (Figure 3.9), it can be suggested that the herbal extracts could have provided essential growth factors possibly in form of peptides and amino acids to improve the growth of starter culture in the milk (Ramachandran and Shah, 2009). The highest growth of *S. thermophilus* seen in cow milk among other treated milks in the presence of *A. sativum* or *C. verum* water extract could be attributes to lack antibacterial properties present in cow milk (Abolghait *et al*., 2011).

Lactobacillus spp. growth improved in the presence of *A. sativum* or *C. verum* water extract in milk compared to in the absence even during the 1st phase of fermentation for cow and goat milks as well as for camel milk + *A. sativum*. Again, this could be associated with some essential growth factors released from herbal extracts. The reason for significant reduction of *Lactobacillus* spp. VCC in *A. sativum*-cow milk yogurt at the end of fermentation could be attributed to the increase in waste products (production of hydrogen peroxide) or metabolite formation via bacteria metabolic activity (Shah, 2000; Vinderola and Reinheimer, 2003; Mishra and Prasad, 2005; Madureira *et al*., 2011) caused inhibition of *Lactobacillus* spp. growth. The growth of *Lactobacillus* spp. was the highest in camel milk + herbal extracts followed by goat herbal extracts and cow milk herbal extracts. This could be related to high peptides and amino acids contents naturally available in milk as a result of protoelytic activity of indigenous bacteria, in addition to camel and goat milk proteins being much easier to be broken down by the proteolytic activity of bacteria than cow milk (Haenlein, 2004; Agrawal *et al.*, 2005; El-Said *et al*., 2010).

Vinderola *et al*. (2000) reported that *Bifidobacteria* are vital bacteria and can grow efficiently even at 4°C. In the present study, higher VCC of *B. bifidum* was noticed in the presence of *A. sativum* or *C. verum* water extract in milk, thus could be explained that these two herbs may provide essential growth factors for bacterial growth during fermentation. Faster growth of *B. bifidum* ($p<0.05$) occurred in the mixture of cow milk with *A. sativum* or *C. verum* water extract than in goat or camel milk mixture with these two herbal extracts. Again this is possibly related to the low antibacterial activity in cow milk (Abolghait *et al*., 2011) compared to goat and camel milk (Agrawal *et al*., 2005; Pavlović *et al*., 2006).

3.5 CONCLUSIONS

The titratable acidity in the mixture of cow milk with *A. sativum* significantly increased due to low pH of *A. sativum* water extracts during fermentation whereas other treated milk with herbal extracts had slight effects as compared to respective milk alone. The addition of *A. sativum* or *C. verum* increased proteolytic activity in cow milk more than goat and camel milk during fermentation. The increase of *S. thermophilus* or *B. bifidum* VCC was the highest in the presence of herbal extracts in cow milk. On the other hand, increase of *Lactobacillus* spp. VCC was the highest in camel milk during fermentation. *A. sativum* and *C. verum* water extracts could have provide essential growth factors for LAB growth during the fermentation of milk.

4.0 Influence of *Allium sativum* or *Cinnamomum verum* water extract on post-acidification and proximate composition of yogurt made from cow, camel and goat milk during refrigerated storage

4.1 INTRODUCTION

Cow milk represents 85% of the milk consumed in the world with goat and sheep milk constituting a much smaller proportion of 10%. Other dairy animals (buffalo, yak, mare, and camel) despite being scarce have their own unique roles in the survival of mankind. Regarding to preference, cow milk ranked first followed by camel, sheep and goat milk (Eyassu, 2007). Camel milk fulfills the nutritional requirements of minor population in harsh and arid environment. It is different from other ruminant milk as it does not form coagulum in acidic environment (Shamsia, 2009). This lack of coagulum formation allows the camel milk to pass rapidly through stomach together with the specific insulin like protein/insulin and remains available for absorption in intestine (Agrawal *et al*., 2005). This is in contrast to the digestion of cow's milk which normally form a solid precipitate (curd) attributed to the high degree of phosphorylation of the caseins (Jumah *et al*., 2001). From nutrition point of view, camel milk has low cholesterol content and its fat consists of mainly polyunsaturated fatty acids that are completely homogenized and gives the milk a smooth white appearance (Yagil and Van, 2000). Camel's milk lactose is present in concentrations of 4.8%, but surprisingly in comparison to cow's milk lactose, this milk sugar is easily metabolized by persons suffering from lactose intolerance (Hanna, 2001). A possible explanation to this is that camel milk produces less casomorphin which would provoke less intestinal motility and thus would cause lactose to become more exposed to the action of lactase (Cardoso *et al*., 2010). Camel milk contains low amount of β-lactoglobulin (Merin *et al*., 2001; Al-Alawi and Laleye, 2011) and β-casein (Al-Alawi and Laleye, 2011). The presence of these two protein components in cow milk is responsible for allergies and due to this camel milk has little or no allergies effects (El-Agamya *et al*., 2009). On the other hand, goat milk has its own special nutritional properties that make it attractive to consumers

(Haenlein, 2004). It has fat rich in medium-chain triglycerides (MCT) made up of fatty acids and it easier to be digested because of forms smaller casein micelles and fat globules (Park *et al*., 2007). Besides, fermented goat milk products are ideal for the persons allergic to cow milk (Haenlein, 2004). Goats' milk also contains free taurine, one of the final metabolic products of sulphur-containing amino acids (Park *et al*., 2007) which play important roles in several biological functions including modulator of growth (Minervini *et al*., 2009) and of neuronal activity (Jiang *et al*., 2004), conjugation of bile salts (Chesney and Hedberg, 2010), regulation of osteoblast metabolism (Menzies, 2002), protection of cells against various types of injury and prevention of cardiovascular damage (Warskulat *et al*., 2007) and treatment of fatty liver of children (Pugh, 2002; Menzies, 2002). In comparison to cow milk, regular consumption of goat milk significantly improves the body weight, mineralization of skeleton, increased blood serum level of vitamin and hemoglobin (Bano *et al*., 2011).

Dairy products are continually being developed to increase the flavors and health benefits in order to appeal customers' satisfaction and enhance sales. Yogurt is a fermented dairy product obtained by lactic acid fermentation of milk by the action of yogurt starter bacteria and is a popular product throughout the world. Yogurt products in the market place are available with different functions targeted to different consumer groups. For example, fat free yogurt for people with cardiovascular problems, lactose free yogurt for lactose intolerant people and folic acid enriched yogurt for initial stages of pregnancy which help to prevent neural tube defects (Mozzi *et al*., 2003; Boeneke *et al*., 2007; Peng *et al*., 2009). Medicinal plants rich in natural antioxidants and phenolics are increasingly being used in food manufacturing because they provide valuable nutritional and therapeutic properties and retard oxidative degradation of lipids (Rahimi *et al*., 2005; Ranilla *et al*., 2010; Psaltopoulou *et al*., 2011). In addition, the quality and

nutritional values of foods regarded as functional such as herbal-yogurt may also be improved (Behrad *et al*., 2009; Shori and Baba, 2011; Amirdivani and Baba, 2011).

Allium sativum and *Cinnamomum verum* are medicinal plants rich in natural compounds with therapeutic proprieties (Ziegenfuss *et al*., 2006; Marta *et al*., 2007). Hence the presence of these plants during fermentation of milk could add unique values to the yogurt, either directly or indirectly via altered yogurt bacteria growth and metabolism. In addition, the differences in the chemical composition among cow, camel and goat milks could lead to different behaviour of the milk during fermentation and refrigerated storage which subsequently affect the quality of yogurt. Therefore, the present study was aimed to evaluate post- acidification and proximate composition of yogurt made from different milk species (cow, camel and goat milk) in the presence and absence of *A. sativum* and *C. verum* water extracts during 21 days of refrigerated storage.

4.2 MATERIALS AND METHODS

4.2.1 Substrates and chemicals

Sodium hydroxide pellets (NaOH), phenolphthalein, dinitrosalicylic acid (DNS), phenol reagent, sodium sulphite, potassium sodium tartrate, lactose, sodium carbonate, sulphuric acid, glucose, chloroform, methanol, potassium chloride and anhydrous sodium sulphate were purchased from Sigma Chemical Company (St. Louis, MO USA).

4.2.2 Experimental design

This study examined the physicochemical changes of set yogurt upon the inclusion of *A. sativum* or *C. verum* water extract. Three groups of set bio-yogurt were prepared using cow, camel and goat milks. The milk inoculated with the starter culture was held at 41°C and incubation was terminated at pH 4.5. Three batches of

each set yogurt were prepared including *A.* sativum-yogurt, *C.* verum-yogurt and plain-yogurt which was used as control. The parameters that were evaluated in each yogurt included pH changes, titratable acidity (lactic acid equivalent), total solids, moisture content, ash, fat content, solids-not-fat, lactose and carbohydrate contents during 21 days of storage at 4^{o}C.

4.2.3 Plant water extraction procedure

The plant was water extracted according to the method described in Section 3.2.3.

4.2.4 Yogurt manufacturing process

4.2.4.1 Starter culture and yogurt preparation

The preparation of starter culture was carried out using the method described in Section 3.2.4.1. Three groups of bio-yogurt were made using cow, camel and goat milks both in the presence and absence of *A. sativum* or *C. verum* water extract (see Section 3.2.4.2).

4.2.5 Measurement of pH and titratable acidity (TA)

The pH and titratable acidity changes were monitored as described in Section 3.2.5.

4.2.6 Determination of lactose

The lactose concentration was determined using dinitrosalicylic acid (DNS) method as described by Miller (1972) and Adeniran *et al*., (2008). DNS reagent was freshly prepared by dissolving 1g DNS and 0.2 ml of phenol in a total volume of 100 ml of 1% NaOH containing sodium sulphite (0.5 g). Water extract of yogurt (3 ml) containing lactose was appropriately diluted with dH_2O and 3 ml of DNS reagent were added. The mixture was heated for 15 min in a boiling water bath. One millilitre

of Rochelle salt solution (4% w/v; potassium sodium tartrate) was added when the contents of the tubes were still warm. The resulting mixture was then cooled down to room temperature (25°C) prior to the absorbance of the mixture was measured at 575 nm (Spectrophotometer, Shimadzu UV Mini 1240). The absorbance was converted to lactose concentration using a standard curve (Figure 4.1) that applied to an absorbance range corresponding to 0 to 60 µg/ml of lactose stock solution (100 mg/100ml dH_2O). This standard curve was poltted when each assay was carried out. Typical equation of the standard curve is as follows:

$$\text{Lactose concentration (µg/ml)} = \frac{A_{575} + 0.0018}{0.002} \qquad (4.1)$$

where A_{575} was the spectrophotometric absorbance reading at 575 nm. Linear regression of equation (4.1) allowed calculation of lactose concentration from absorbance measurements.

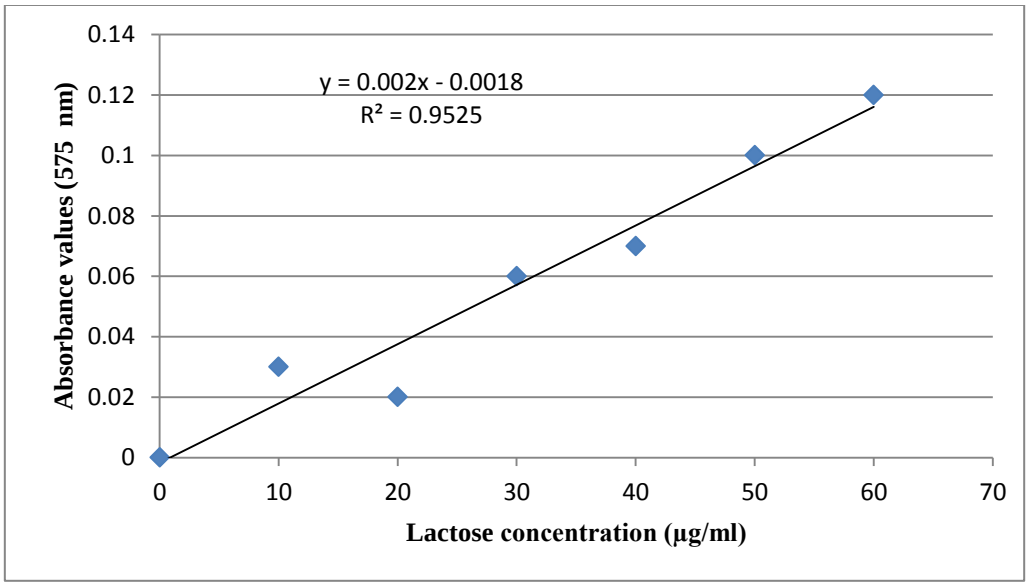


Figure 4.1 Typical calibration curve for total lactose concentration.

4.2.7 Determination of total carbohydrate

Total carbohydrate determination was adapted from the phenol–sulphuric acid method as described by Krishnaveni (1984). One gram of yogurt sample was diluted with 10 ml of dH_2O. The mixture was kept in a boiling water bath for three hours prior to cooling to room temperature. Solid sodium carbonate (5%) was then added to neutralize the solution. The mixture was then centrifuged (6000 rpm, 10 minutes) and the supernatant was harvested. One millilitre of the supernatant was mixed with 1 ml phenol solution (5% w/v) followed by the addition of 5 ml concentrated sulphuric acid (96% w/v). The mixture was left at room temperature for 30 min prior to measure the absorbance reading at 490 nm (Shimadzu spectrophotometer, UV Mini 1240). The total amount of carbohydrate was determined based on a standard calibration curve prepared using glucose solution of known concentrations (0-100 µg/ml; Figure 4.2) which was run each time assay was carried out. Typical linear equation of the standard curve was as follows:

$$\text{Total carbohydrate concentration (µg/ml)} = \frac{A_{490} - 0.0202}{0.0137} \qquad (4.2)$$

where A_{490} was the spectrophotometric absorbance reading at 490 nm. The linear regression of equation (4.2) allowed calculation of total carbohydrate concentration in unknown solutions.

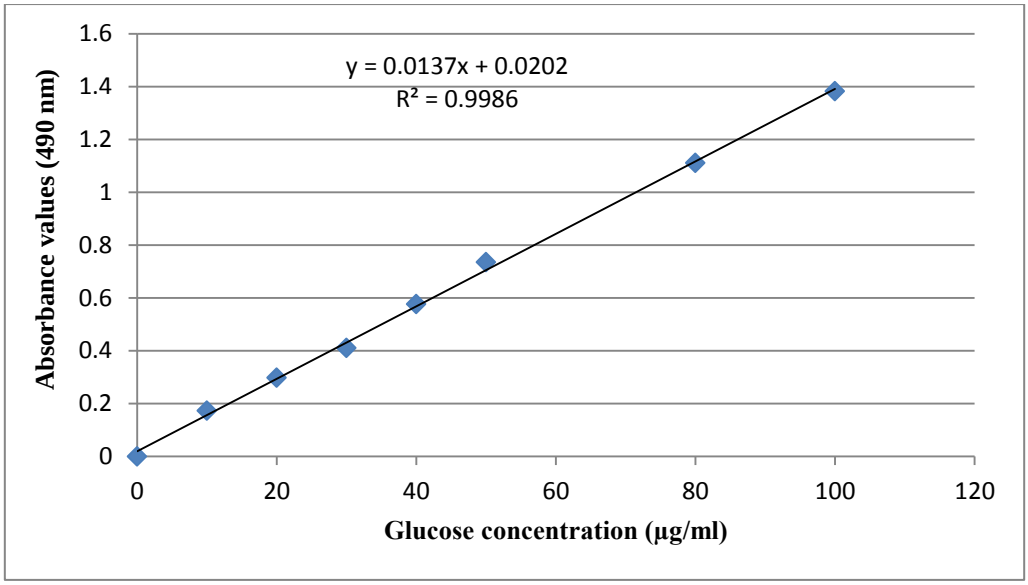


Figure 4.2 Typical calibration curve for total carbohydrate concentration.

4.2.8 Total solid and moisture content

Total solid (TS) measurement in yogurt was adapted from Hooi *et al*., (2004). Approximately 10 g of yogurt sample was placed in pre-dried dish of known weight (Adventure Ohaus) and kept in an oven at 100°C (Memmert) for 5 hours. The sample was then cooled in the desiccator containing cobalt (II) chloride anhydrous for 15 minutes prior to re-weighing. The sample was again reheated in the oven for another 1h, cooled and re-weighed. This was repeated until the dried sample showed a constant weight. The total solids and moisture contents were calculated as follows:

$$\textbf{\%Total solids} = \frac{\text{weight of dried sample plus dish - weight of dish}}{\text{Weight of sample}} \times 100 \qquad (4.3)$$

$$\textbf{\%Moisture} = 100 - \left[\frac{\text{weight of dried sample plus dish – weight of dish}}{\text{Weight of sample}}\right] \times 100$$

4.2.9 Determination of ash content

The ash content in each yogurt sample was determined according to AOAC (1995). Yogurt sample (10g) was ashed at 550°C (Memmert) for 24 h. The ash content is expressed as the inorganic residue left as a percentage of the total weight of yogurt incinerated which calculated by using equation in (4.3).

4.2.10 Determination of fat content

The fat in yogurt samples was extracted by solvent extraction method as described by El-Sohaimy and Hafez, (2010). Known weight of yogurt sample (~10 g) was added gradually to 20 ml chloroform/methanol (2:1 v/v) followed by vigorous shaking. The mixture was stirred further for 2 hours using an electromagnetic stirrer. The mixture was then filtered through Whatman No. 1 and the filter paper was rewashed with fresh solvent. The filtrate was collected and three drops of 1% phenolphthalein indicator (w/v in methanol alcohol) were added to visualize the appearance of interface between the extracting and the aqueous layer of the mixture. Fifty millitters of potassium chloride 0.88% (w/v) was added and the mixture was shaken. The aqueous layer (upper) was removed by aspiration and the washing procedure was repeated. The extract was then passed into a pre-weighed round bottom flask through a 2.5-cm thick layer of anhydrous sodium sulphate placed on Whatman No. 1 filter paper in a funnel followed by rinsing with 20 ml 2:1 (v/v) chloroform/methanol. The solvent was rotary evaporated (40°C) and the extract was then placed in a desiccator overnight and the extracted fat weight determined by difference. Extracted fat was calculated as follows:

Weight of fat extracted = (weight of container + extracted fat) − (weight of container)

$$\text{Fat content (\%)} = \frac{\text{weight of fat extracted (g)}}{\text{weight of original sample (g)}} \times 100$$

4.2.11 Determination of solids-not-fat

Solids-not-fat (%) were calculated as the arithmetic difference between the values of the total solids and the fat content (AOAC, 1995).

4.2.12 Statistical analysis

Statistical analyses of all data obtained were performed as described in Section 3.2.8. The lactose and glucose standard curves used to calculate concentration of lactose and carbohydrate respectively were plotted as described in Section 3.2.8 using Microsoft® Excel XP.

4.3 RESULTS

4.3.1 Changes in pH and titratable acidity (TA) during refrigerated storage

The adding of *A. sativum* or *C. verum* water extract to cow, camel and goat milks had no significant effect on pH reduction as compared to milk alone (Table 4.1). On the other hand, the mixture of *C. verum* or *A. sativum* water extract and cow milk had higher TA (0.27±0.03% lactic acid equivalent (LAE); $p<0.05$ and 0.24±0.05% LAE respectively) compared to milk alone (0.21±0.05 LAE; Table 4.1). The presence of these herbal extracts in camel milk had no increase in TA content significantly. The mixture of *C. verum* extract and goat milk increased TA (0.29±0.02% LAE; $p<0.05$) as compared to goat milk alone (0.24±0.01% LAE) whereas TA in *A. sativum* + goat milk was significantly unchanged (Table 4.1).

Figures 4.3, 4.4 and 4.5 show the effect of addition of *A. sativum* or *C. verum* water extract on the changes in pH during refrigerated storage of yogurt made from cow, camel and goat milks respectively. The pH of cow milk yogurt reduced further from 4.4 to 3.6 by day 21 of storage (Figure 4.3). However, camel and goat milk yogurt pH reduced by only about 0.3 and 0.5 units respectively during the same storage period (Figures 4.4 and 4.5 respectively). The presence of *A. sativum* or *C.*

verum water extract had no effect on the pH of cow-, camel- and goat- milk yogurt compared to respective controls.

TA increased during refrigerated storage of yogurt made from all three types of milk (Figures 4.6, 4.7 and 4.8). *A. sativum*-cow milk yogurt had higher TA ($p<0.05$) than plain- cow milk yogurt throughout the storage period whereas *C. verum*- yogurt was unchanged (Figure 4.6). The presence of *A. sativum* or *C. verum* in camel- or goat- milk yogurt did not affect TA during storage compared to their respective controls (Figures 4.7 and 4.8) except for *A. sativum*- goat milk yogurt (1.23% LAE) which was marginally higher than control (1.11% LAE) on the 14th day of storage (Figure 4.8).

Table 4.1 The pH and titratable acidity (%) in cow, camel and goat milk in presence and absence of *A. sativum* and *C. verum* water extracts.

Sample	pH	Titratable acidity (%)
Cow milk	6.53±0.1	0.21±0.05
AS+cow milk	6.43±0.07	0.24±0.05
CV+cow milk	6.52±0.1	0.27±0.03*
Camel milk	6.50±0.03	0.27±0.05
AS+camel milk	6.44±0.03	0.30±0.04
CV+camel milk	6.52±0.02	0.30±0.03
Goat milk	6.28±0.1	0.24±0.01
AS+goat milk	6.27±0.1	0.27±0.01
CV+goat milk	6.24±0.2	0.29±0.02*

AS= *A. sativum* and CV= *C. verum*. Cow, camel and goat milk presented as controls. Results are shown as mean (n = 3) ± standard error. *$p < 0.05$ as compared to control.

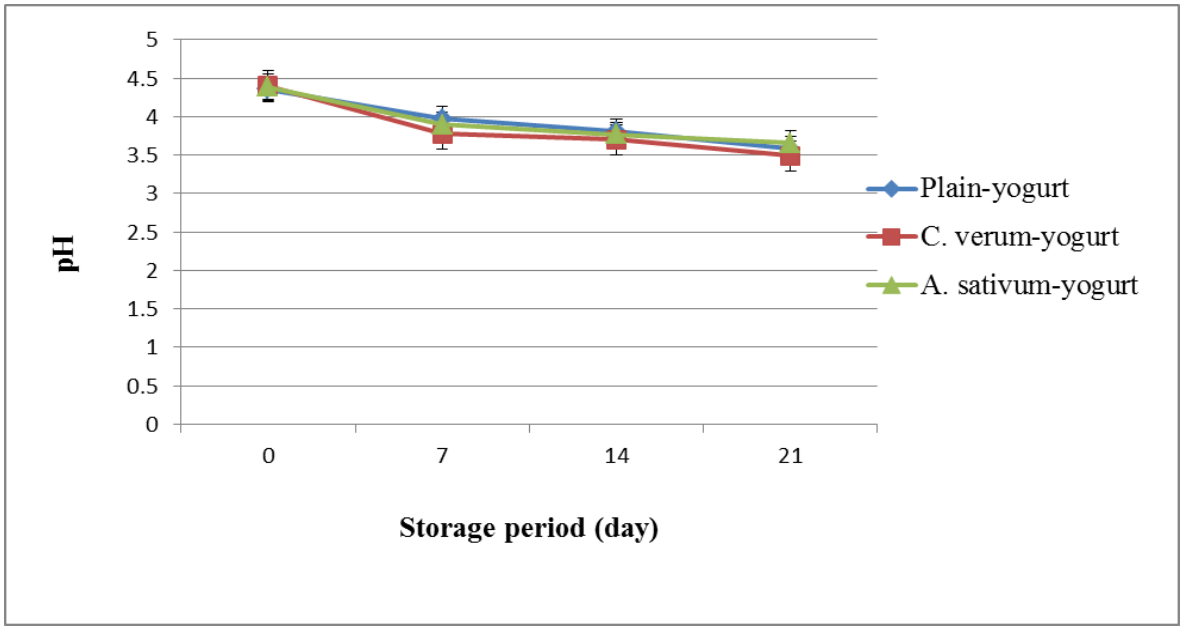


Figure 4.3 Changes of pH in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

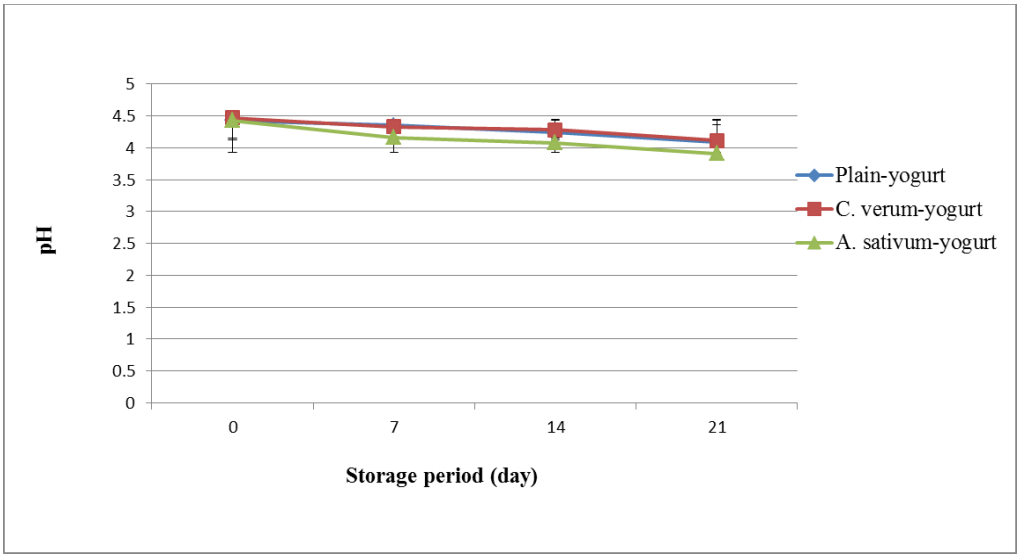


Figure 4.4 Changes in pH of camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

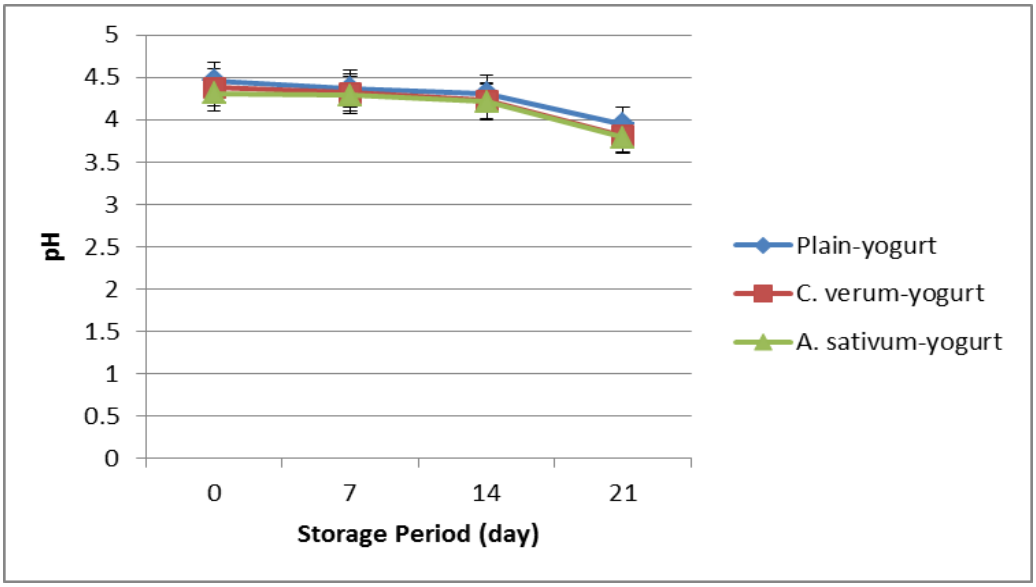


Figure 4.5 Changes in pH of goat milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

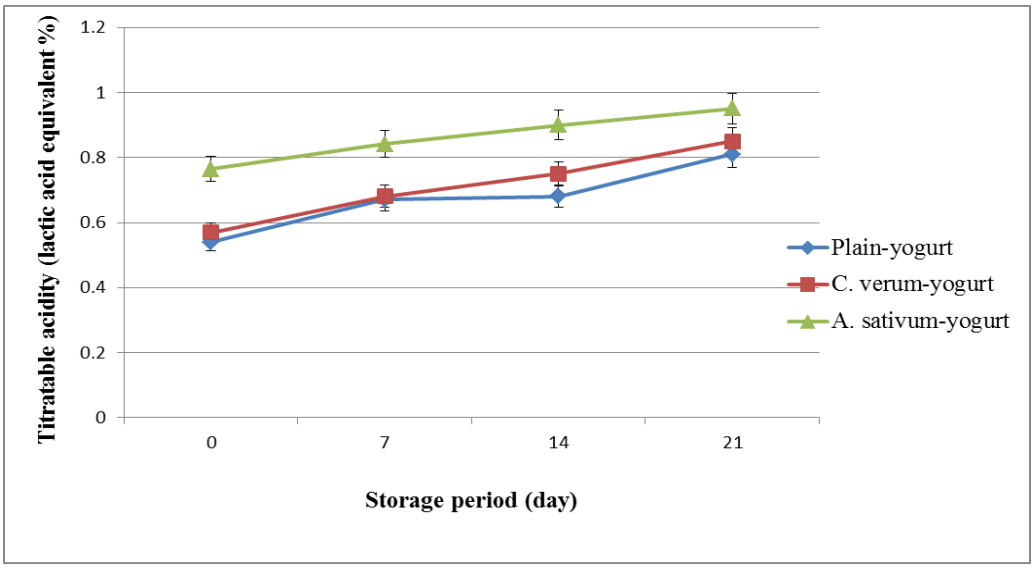


Figure 4.6 Changes of titratable acidity (lactic acid equivalent %) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

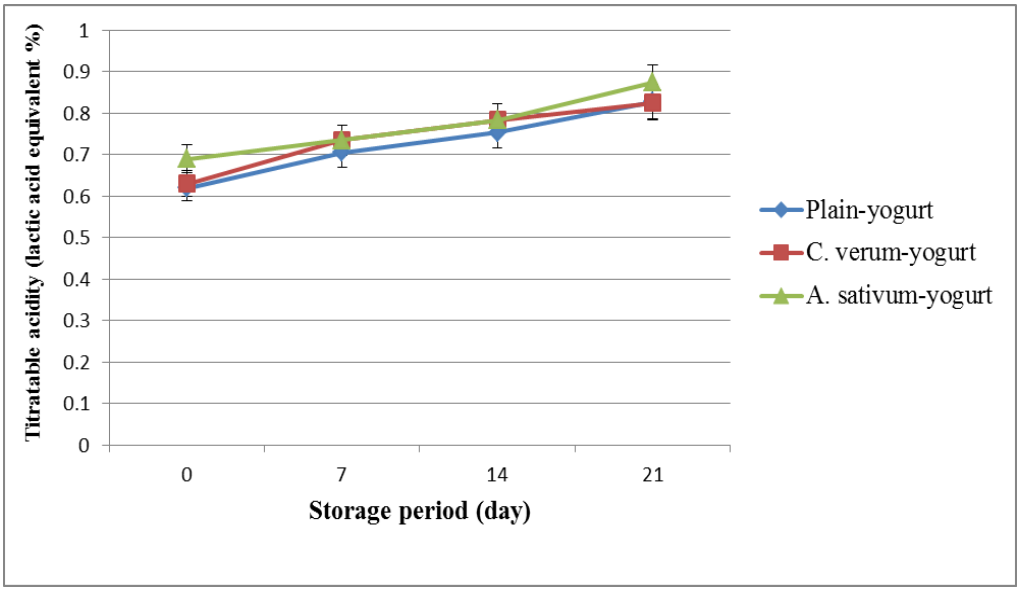


Figure 4.7 Changes of titratable acidity (lactic acid equivalent %) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

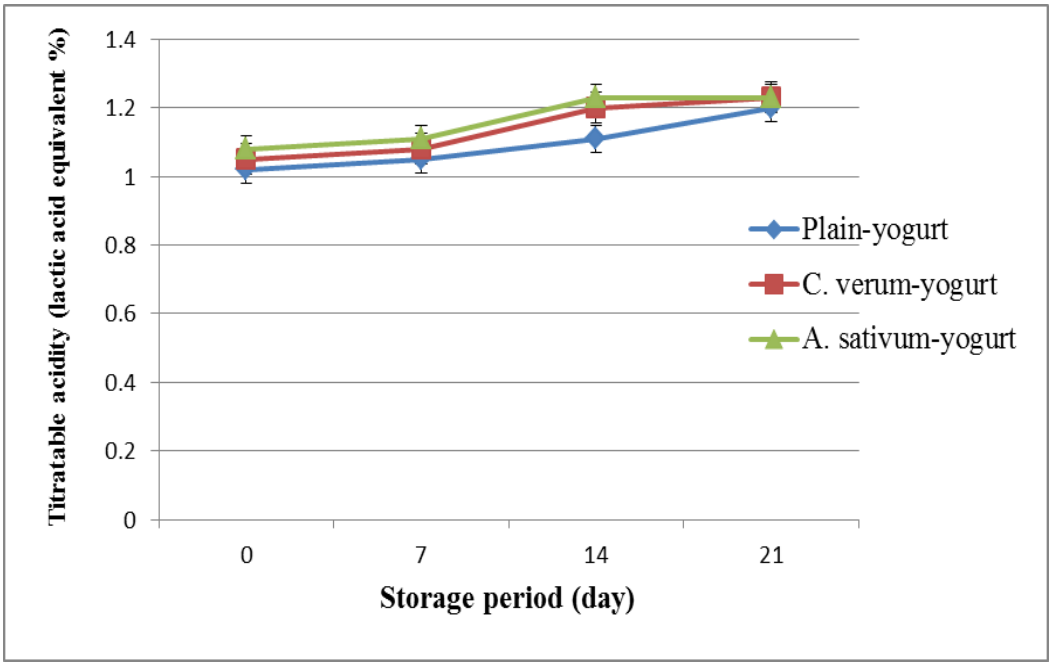


Figure 4.8 Changes of titratable acidity (lactic acid equivalent %) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

4.3.2 Total lactose content

Cow milk exhibited the highest ($p<0.05$) lactose content with an average of 4.42±0.1 g/100 g followed by goat milk 4.30±0.1 g/100 g and camel 4.14±0.2 g/100 g respectively (Table 4.2). The mixture of *A. sativum* or *C. verum* water extract with the three types of milk had no effect on lactose content. The lactose content of yogurt made from cow, camel and goat during 21 days of refrigerated storage are as shown in Figures 4.9, 4.10 and 4.11. The value of lactose content in plain- cow milk yogurt ranged from 4.10±0.1 to 3.45±0.1 (g/100 g) during 21 days of storage (Figure 4.9). *A. sativum* or *C. verum* increased lactose utilization during fermentation but had little effects on lactose sugar content with an average of 3.88- 3.39 g/100 g and 3.86- 3.31 g/100 g respectively during period of storage.

Similarly, the lactose sugar content of fresh *A. sativum*- and *C. verum*- yogurt made from camel milk was 3.53±0.1 g/100 g and 3.60±0.1 g/100 g respectively (Figure 4.10). However, refrigerated storage reduced lactose content ($p<0.05$) to 2.90±0.1 g/100 g and 2.96±0.1 g/100 g for *A. sativum*- and *C. verum*- yogurt respectively by 21 days of storage. Refrigerated storage of plain- yogurt reduced lactose sugar from 3.75±0.2 g/100 g to 3.29±0.1 g/100 g during the 21 days of storage.

Fresh *A. sativum*- and *C. verum*- yogurt made from goat milk contained lower lactose content (3.84±0.2 g/100 g and 3.85±0.1 g/100 g respectively) than control (3.99±0.1 g/100 g; $p>0.05$; Figure 4.11). Refrigerated storage of yogurt decreased ($p<0.05$) the lactose content gradually to the lowest values (3.26±0.1 g/100 g, 3.17±0.1 g/100 g and 3.20±0.2 g/100 g for plain-, *A. sativum*- and *C. verum*- yogurt respectively) by day 21 of storage.

Table 4.2 Lactose content (g/100 g) in cow, camel and goat milk in presence and absence of *A. sativum* and *C. verum* water extracts.

Sample	Lactose (g/100 g)
Cow milk	4.42±0.1
AS+cow milk	4.43±0.1
CV+cow milk	4.41±0.1
Camel milk	4.14±0.2
AS+camel milk	4.14±0.1
CV+camel milk	4.15±0.1
Goat milk	4.30±0.1
AS+goat milk	4.29±0.1
CV+goat milk	4.29±0.1

AS= *A. sativum* and CV= *C. verum*. Cow, camel and goat milk presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

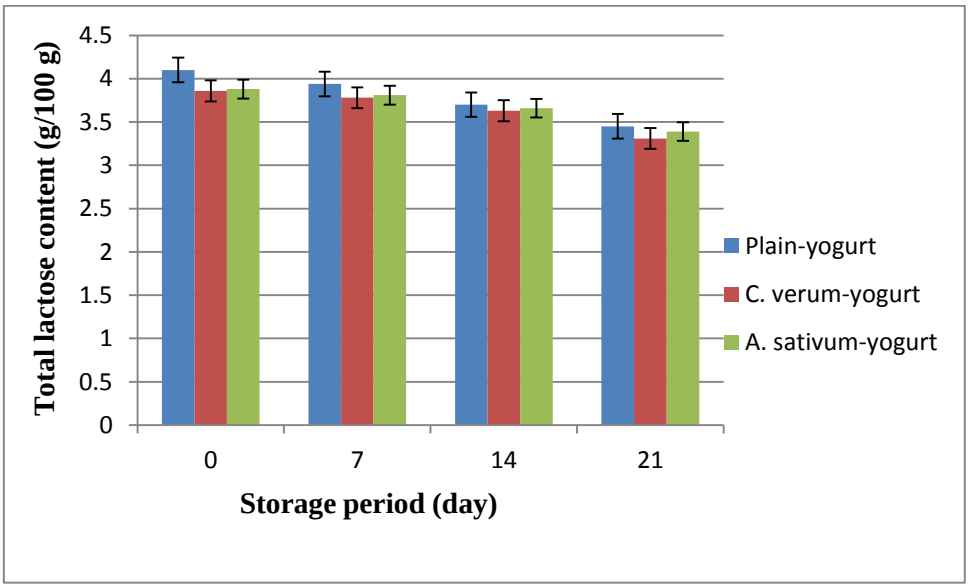


Figure 4.9 Changes of total lactose content (g/100 g) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

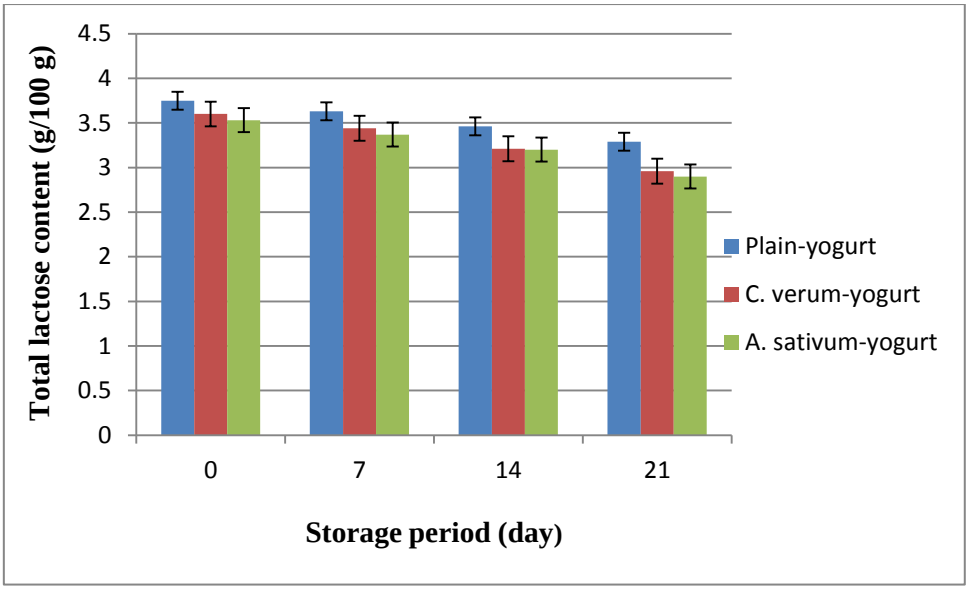


Figure 4.10 Changes of total lactose content (g/100 g) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

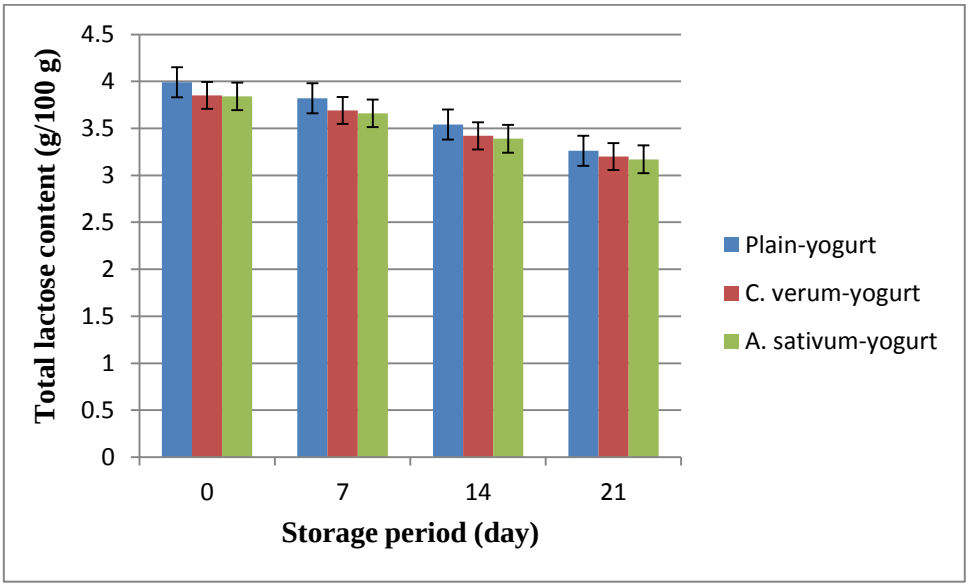


Figure 4.11 Changes of total lactose content (g/100 g) in goat milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

4.3.3 Total carbohydrate content

The presence of *A. sativum* and *C. verum* water extracts in all the three types of milk did not affect the carbohydrate content as compared to milk alone (Table 4.3). The carbohydrate content in fresh plain-cow milk yogurt was 9.7±0.2 g/100 g whereas the presence of *A. sativum* or *C. verum* water extract had no effect on the carbohydrate content (Figure 4.12). Refrigerated storage of yogurt to day 21 decreased the content of carbohydrate to 8.98±0.2, 9.26±0.2 and 8.62±0.2 g/100 g in plain-, *A. sativum*- and *C. verum*-yogurt respectively.

The carbohydrate content in fresh plain-yogurt made from camel milk was 10.32±0.04 g/100 g (Figure 4.13). This value was unchanged in the presence of *A. sativum* and *C. verum* (10.58±0.06 and 10.69±0.05 g/100 g respectively). However, refrigerated storage (21 days) decreased ($p<0.05$) the content of carbohydrate to 9.30±0.04, 9.51±0.05 and 9.58±0.4 g/100 g for plain-, *A. sativum*- and *C. verum*-yogurt respectively.

Fresh *A. sativum*- and *C. verum*-yogurt made from goat milk had similar carbohydrate contents (10.3 g/100 g) compared to control (9.9 g/100 g; Figure 4.14). Refrigerated storage (21 days) resulted in a small decrease in carbohydrate ($p>0.05$) to 9.12±0.2, 9.36±0.2 and 9.45±0.2 g/100 g for plain-, *A. sativum*- and *C. verum*-yogurt respectively.

Table 4.3 Carbohydrate content (g/100 g) in cow, camel and goat milk in presence and absence of *A. sativum* and *C. verum* water extracts.

Sample	Carbohydrate (g/100 g)
Cow milk	9.40±0.1
AS+cow milk	9.66±0.1
CV+cow milk	9.70±0.1
Camel milk	10.03±0.2
AS+camel milk	10.35±0.2
CV+camel milk	10.47±0.1
Goat milk	9.65±0.1
AS+goat milk	9.98±0.2
CV+goat milk	10.02±0.2

AS= *A. sativum* and CV= *C. verum*. Cow, camel and goat milk presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

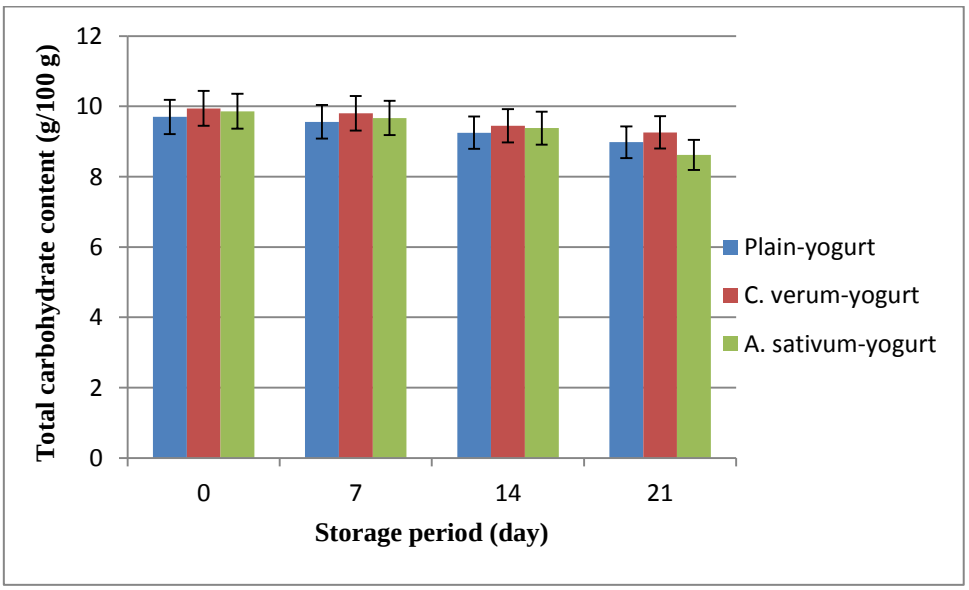


Figure 4.12 Changes of total carbohydrate content (g/100 g) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

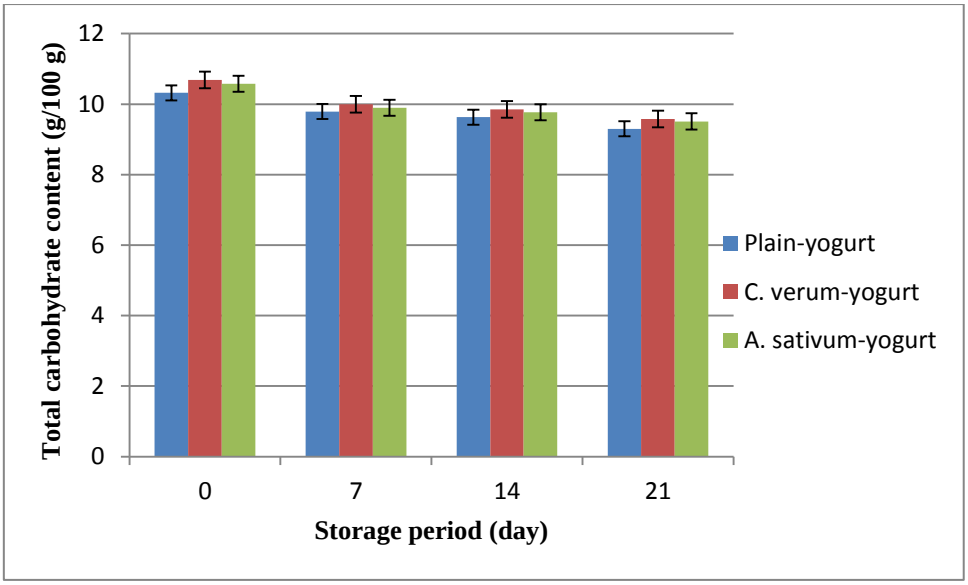


Figure 4.13 Changes of total carbohydrate content (g/100 g) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

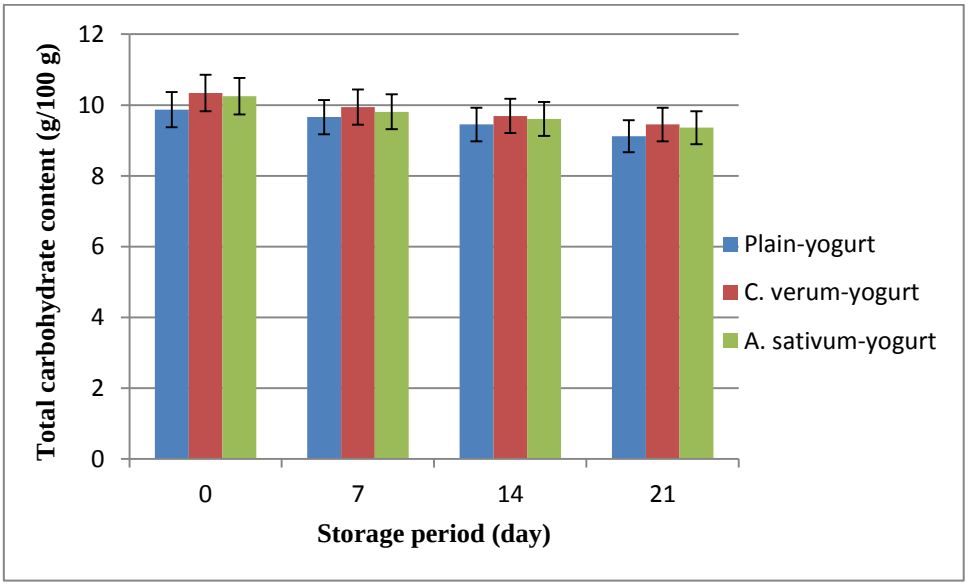


Figure 4.14 Changes of total carbohydrate content (g/100 g) in goat milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

4.3.4 Total solids (TS)

The presence of *A. sativum* and *C. verum* water extracts in cow, camel or goat milk did not also affect the TS as compared to their respective milk alone (Table 4.4). The TS of plain- cow milk yogurt ranged from 12.64±0.4% to 13.17±0.4% during 21 days of refrigerated storage (Figure 4.15). The presence of *C. verum* or *A. sativum* water extract in yogurt showed similar TS with an average of 12.90% - 13.40% during period of storage.

The TS of plain- camel milk yogurt ranged from 12.14±0.5% to 12.84±0.5% throughout 21 days of refrigerated storage (Figure 4.16). *A. sativum*- and *C. verum*-camel milk yogurt showed similar TS with an average range of 12.20% - 12.90% during the 21 days of storage.

TS in goat milk yogurt ranged between 12.71±0.2% and 13.22±0.2% during refrigerated storage to 21 days (Figure 4.17). The presence of *A. sativum* or *C. verum* water extract had little effects on TS during 21 days refrigerated storage with values ranging 13.20% - 13.90%.

Table 4.4 Total solids (%) in cow, camel and goat milk in presence and absence of *A. sativum* and *C. verum* water extracts.

Sample	Total solids (%)
Cow milk	12.51±0.01
AS+cow milk	12.72±0.01
CV+cow milk	12.74±0.01
Camel milk	12.08±0.01
AS+camel milk	12.17±0.01
CV+camel milk	12.15±0.02
Goat milk	12.55±0.01
AS+goat milk	13.02±0.01
CV+goat milk	12.98±0.01

AS= *A. sativum* and CV= *C. verum*. Cow, camel and goat milk presented as controls. Results are shown as mean (n = 3) ± standard error. *$p < 0.05$ as compared to control.

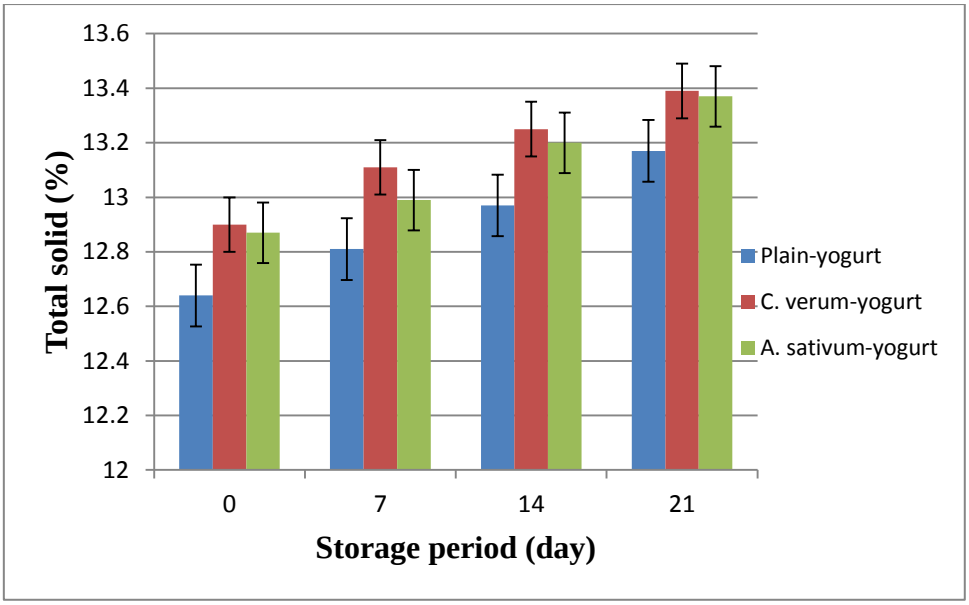


Figure 4.15 Changes of total solids (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

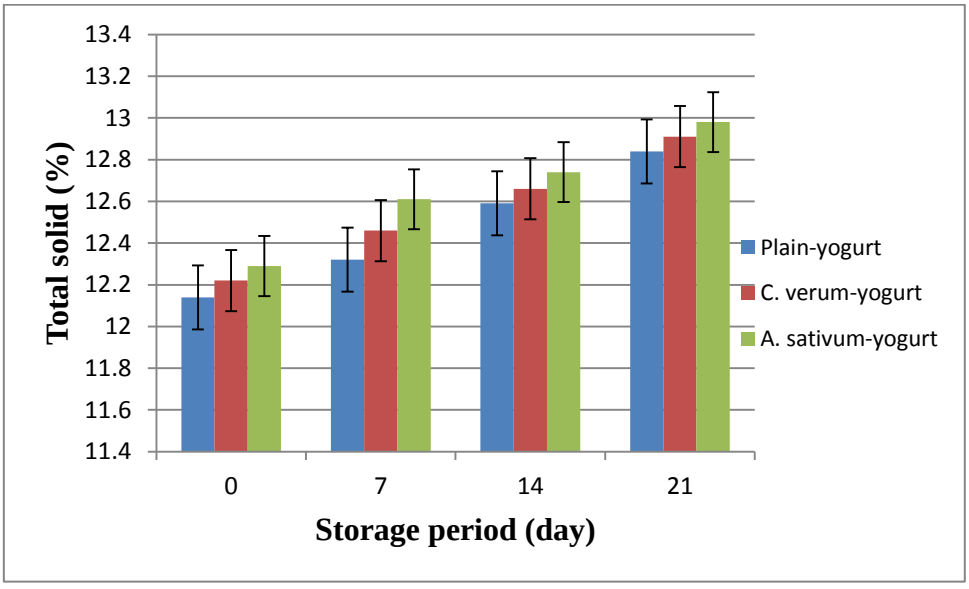


Figure 4.16 Changes of total solids (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

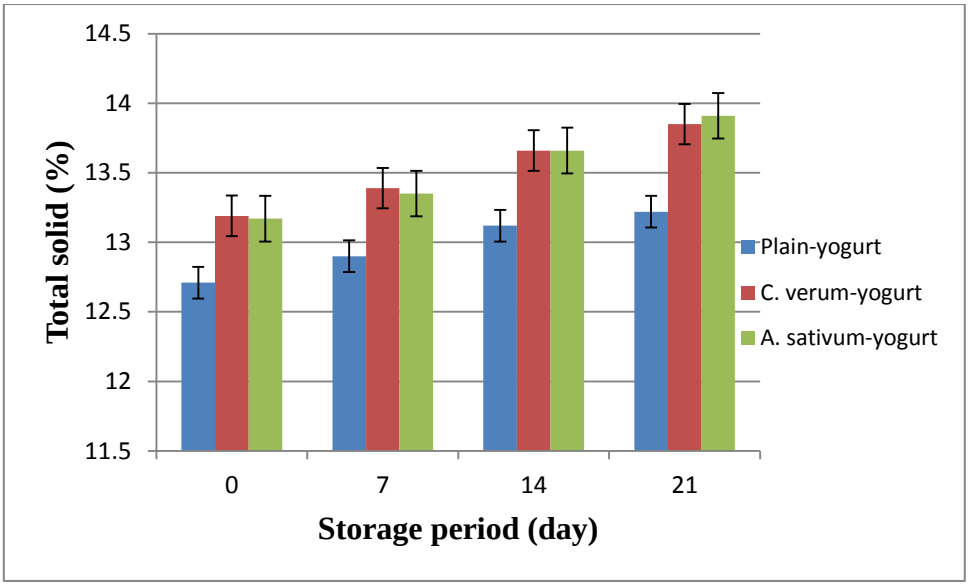


Figure 4.17 Changes of total solids (%) in goat milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

4.3.5 Total moisture content

The moisture content in the mixture of *A. sativum* or *C. verum* water extract with three types of milk was not different from milk alone (Table 4.5). The moisture content in cow milk yogurt reduced gradually during 21 days storage from 87.36±0.5% to 86.83±0.5% (Figure 4.18). The moisture content in yogurt in the presence of *A. sativum* or *C. verum* water extract was significantly unchanged throughout period of storage.

The moisture content in plain- camel milk yogurt reduced from 87.86±0.5% to 87.16±0.05% during 21 days of storage (Figure 4.19) and these were also unchanged ($p>0.05$) in the presence of *A. sativum* or *C. verum*. On the other hand, there was a tendency for lower moisture content in goat milk- yogurt in the presence of *A. sativum* or *C. verum* (~ 86%; $p<0.05$) compared to plain- goat milk yogurt (~ 87%) during 21 days of storage (Figure 4.20).

Table 4.5 Moisture content (%) in cow, camel and goat milk in presence and absence of *A. sativum* and *C. verum* water extracts.

Sample	Moisture content (%)
Cow milk	87.49±0.03
AS+cow milk	87.28±0.01
CV+cow milk	87.26±0.01
Camel milk	87.92±0.01
AS+camel milk	87.83±0.02
CV+camel milk	87.85±0.01
Goat milk	87.45±0.01
AS+goat milk	86.98±0.01
CV+goat milk	87.02±0.01

AS= *A. sativum* and CV= *C. verum*. Cow, camel and goat milk presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

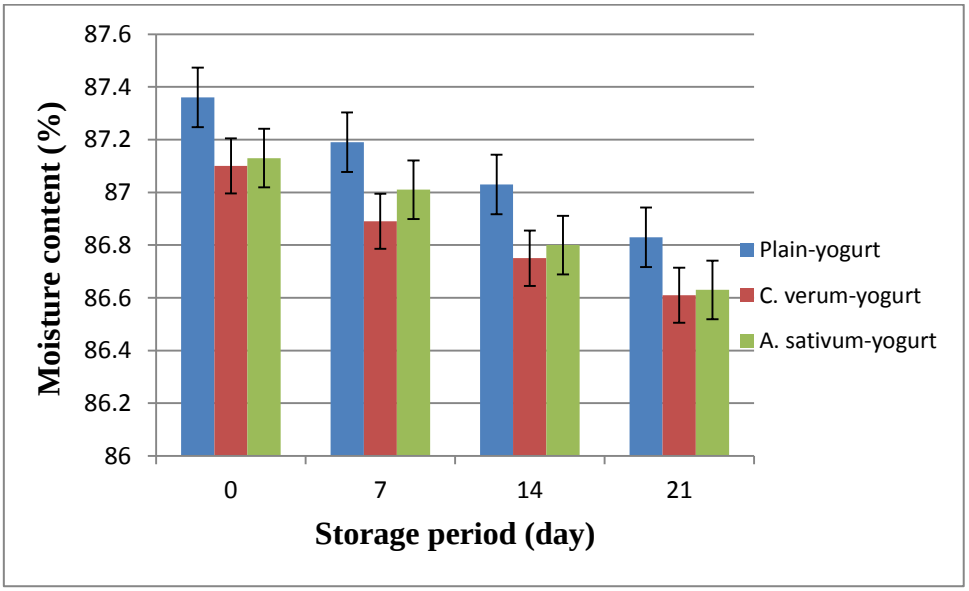


Figure 4.18 Changes of moisture content (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

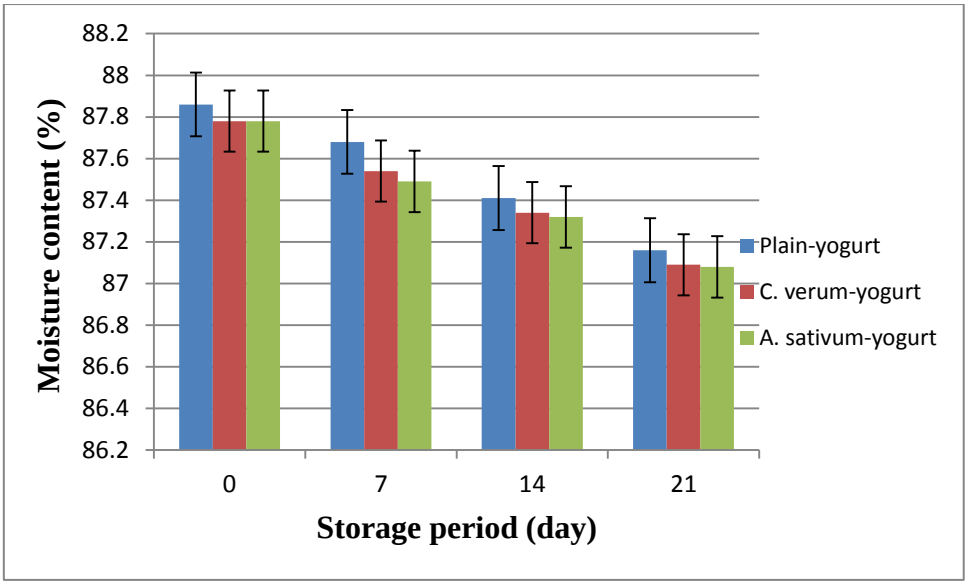


Figure 4.19 Changes of moisture content (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

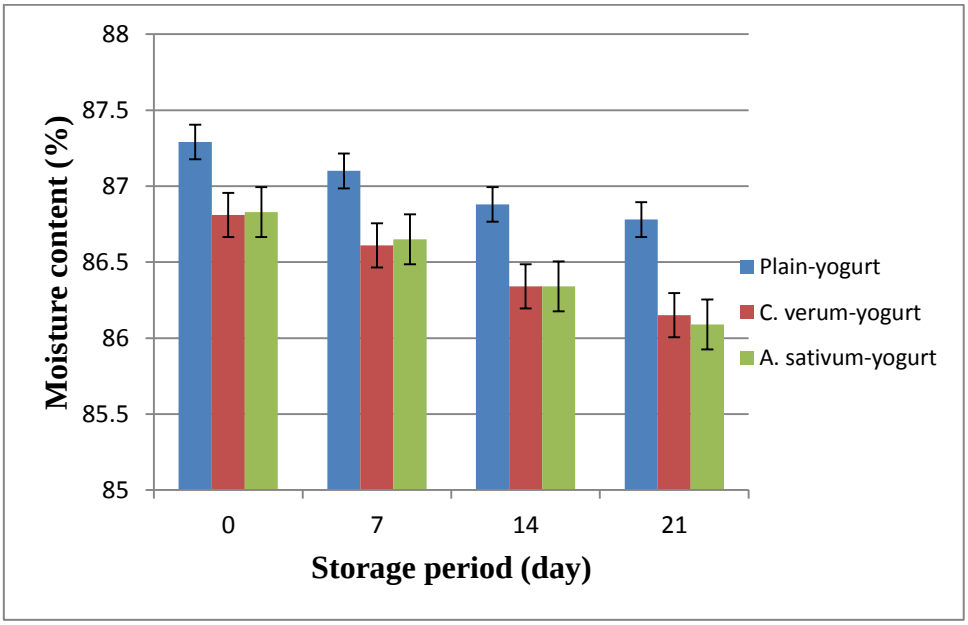


Figure 4.20 Changes of moisture content (%) in goat milk yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

4.3.6 Ash content of yogurt

Ash content in cow, camel and goat milk was about 0.5%. The presence of *A. sativum* or *C. verum* water extract in the three types of milk did not significantly affect the ash content (Table 4.6). The ash content in cow-, camel- and goat- milk yogurt both in the presence and absence of herbal extracts ranged between 0.5% – 0.6% during 21 days of refrigerated storage (Figures 4.21, 4.22 and 4.23 respectively).

Table 4.6 Ash content (%) in cow, camel and goat milk in presence and absence of *A. sativum* and *C. verum* water extracts.

Sample	Ash content (%)
Cow milk	0.51±0.02
AS+cow milk	0.52±0.01
CV+cow milk	0.53±0.02
Camel milk	0.46±0.01
AS+camel milk	0.50±0.01
CV+camel milk	0.52±0.01
Goat milk	0.52±0.01
AS+goat milk	0.55±0.01
CV+goat milk	0.55±0.01

AS= *A. sativum* and CV= *C. verum*. Cow, camel and goat milk presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

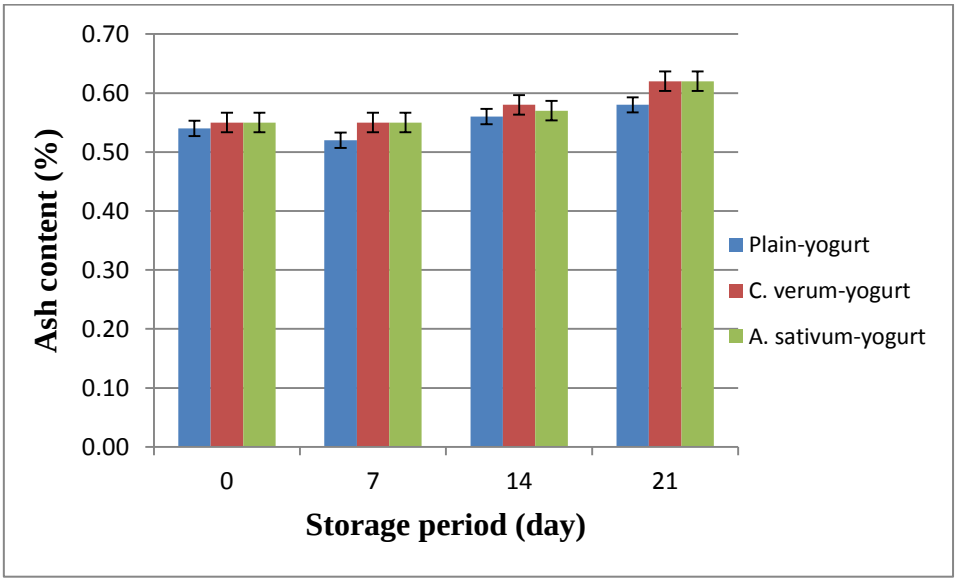


Figure 4.21 Changes of ash content (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

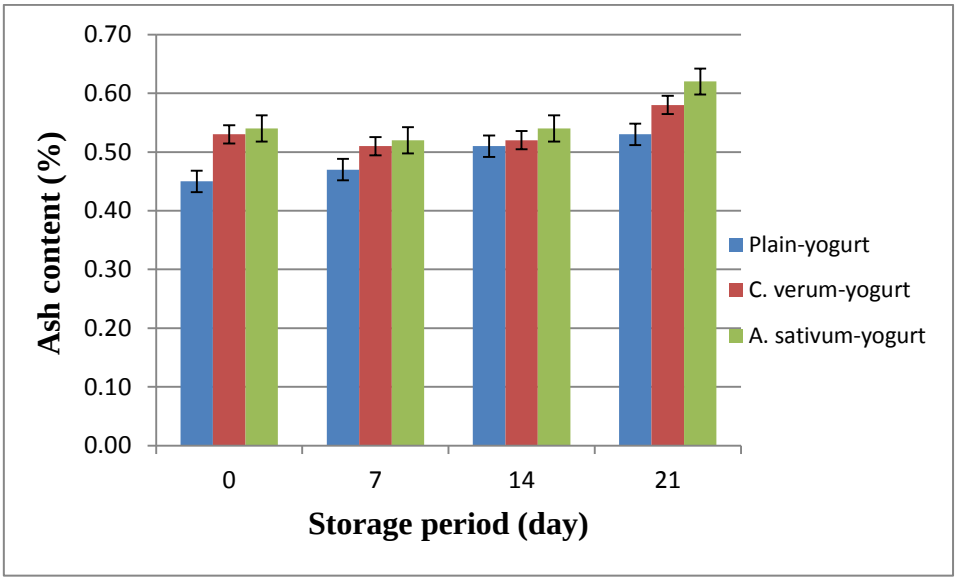


Figure 4.22 Changes of ash content (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

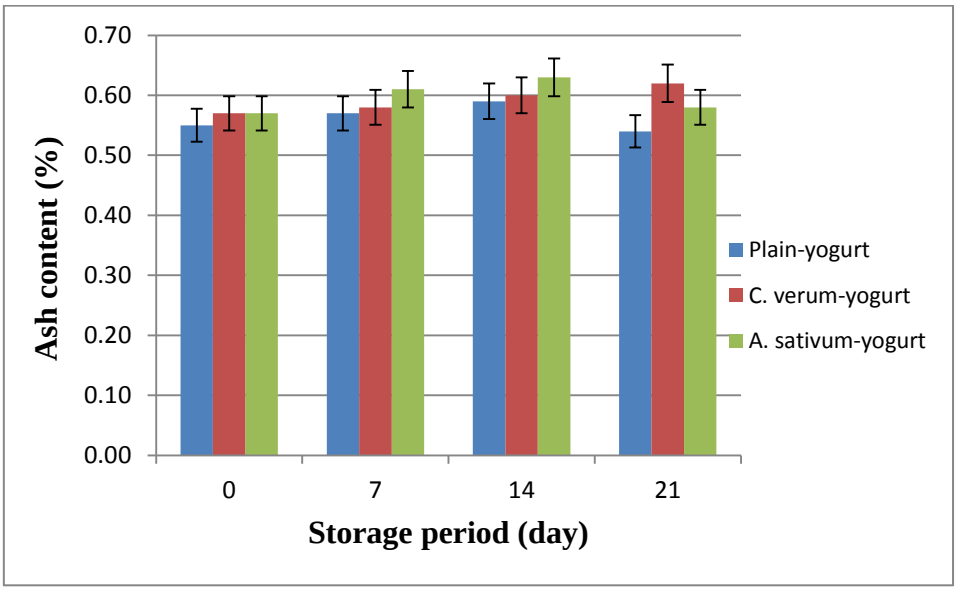


Figure 4.23 Changes of ash content (%) in goat milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

4.3.7 Fat content in yogurt

The fat content in cow, camel and goat milk was 3.40±0.1%, 4.77±0.1% and 5.74±0.1% respectively (Table 4.7). The addition of *A. sativum* and *C. verum* water extracts into milk did not affect fat content in milk (Table 4.7). The fat content of fresh plain-yogurt made from cow milk was 3.44±0.1 % (Figure 4.24). The presence of *A. sativum* or *C. verum* water extract in fresh cow milk- yogurt showed no effect on fat content (3.49±0.1 % and 3.46±0.1 % respectively). Refrigerated storage of yogurt increased ($p<0.05$) the content of fat to 3.81±0.1 %, 3.87±0.1 % and 3.86±0.1 % for plain-, *A. sativum*- and *C. verum*-yogurt respectively on day 14 of storage. Prolonged storage to 21 days decreased ($p<0.05$) the fat content to 3.11±0.1 %, 3.28±0.1 % and 3.33±0.1 % for plain-, *A. sativum*- and *C. verum*-yogurt respectively.

The presence of *A. sativum* or *C. verum* water extract in fresh camel milk yogurt showed no significant increase in the value of fat content (4.87±0.1% and 4.84±0.1% respectively) compared to control (4.78±0.1%; Figure 4.25). Refrigerated storage of yogurt (4°C) increased ($p<0.05$) the fat content in both plain- and herbal-yogurt to similar level after 2 weeks of storage (5.9 %; Figure 4.25).

The fat content of plain-yogurt made from goat milk reduced from 6.8% to 5.5% during 21 days of storage (Figure 4.26). The presence of *A. sativum* or *C. verum* water extract had no effect on fat content after fermentation and during refrigerated storage.

Table 4.7 Fat content (%) in cow, camel and goat milk in presence and absence of *A. sativum* and *C. verum* water extracts.

Sample	Fat content (%)
Cow milk	3.40±0.1
AS+cow milk	3.45±0.1
CV+cow milk	3.42±0.1
Camel milk	4.77±0.2
AS+camel milk	4.85±0.1
CV+camel milk	4.81±0.1
Goat milk	5.74±0.4
AS+goat milk	5.81±0.1
CV+goat milk	5.77±0.3

AS= *A. sativum* and CV= *C. verum*. Cow, camel and goat milk presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

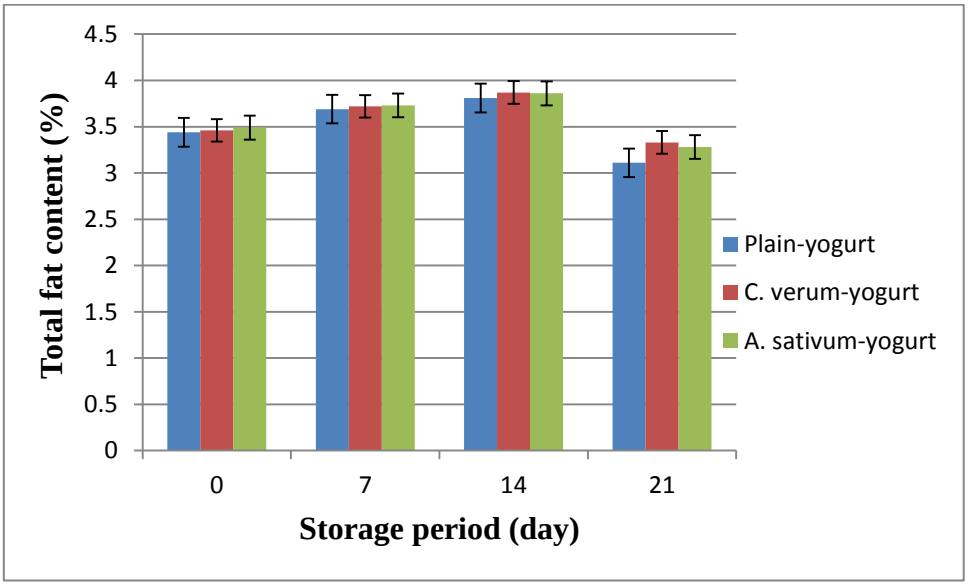


Figure 4.24 Changes of total fat content (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

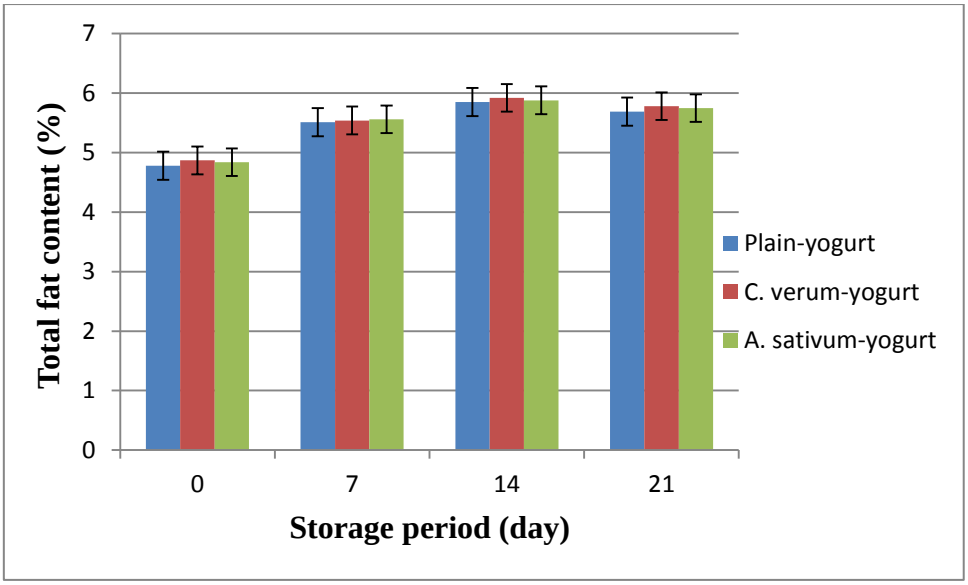


Figure 4.25 Changes of total fat content (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

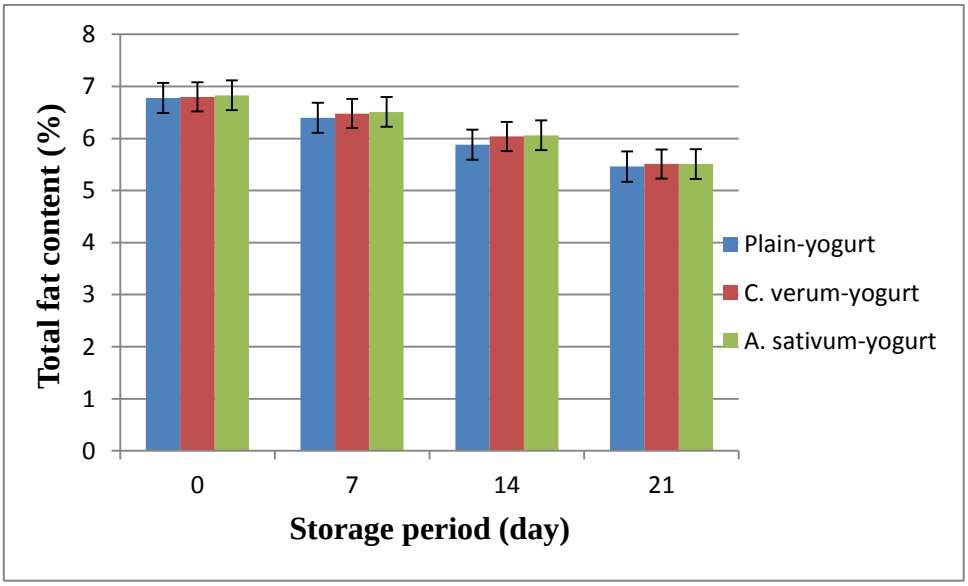


Figure 4.26 Changes of total fat content (%) in goat milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

4.3.8 Solid-not-fat (SNF)

Cow, camel and goat milks showed SNF with averages of 9.11±0.1%, 7.31±0.2% and 5.81±0.1% respectively (Table 4.8). The mixing of *A. sativum* or *C. verum* water extract with each milk did not affect SNF. The SNF in all types of yogurt (with or without herbal extracts) made from cow, camel and goat milk varied between 9% - 10%, 6.7% - 7.4% and 6% - 8% respectively during the 21 days of storage (Figures 4.27, 4.28 and 4.29 respectively).

Table 4.8 Solid-not-fat content (%) in cow, camel and goat milk in presence and absence of *A. sativum* and *C. verum* water extracts.

Sample	Solid-not-fat (%)
Cow milk	9.11±0.1
AS+cow milk	9.27±0.1
CV+cow milk	9.32±0.1
Camel milk	7.31±0.2
AS+camel milk	7.32±0.1
CV+camel milk	7.34±0.1
Goat milk	5.81±0.4
AS+goat milk	6.21±0.1
CV+goat milk	6.21±0.3

AS= *A. sativum* and CV= *C. verum*. Cow, camel and goat milk presented as controls. Results are shown as mean (n = 3) ± standard error. *$p < 0.05$ as compared to control.

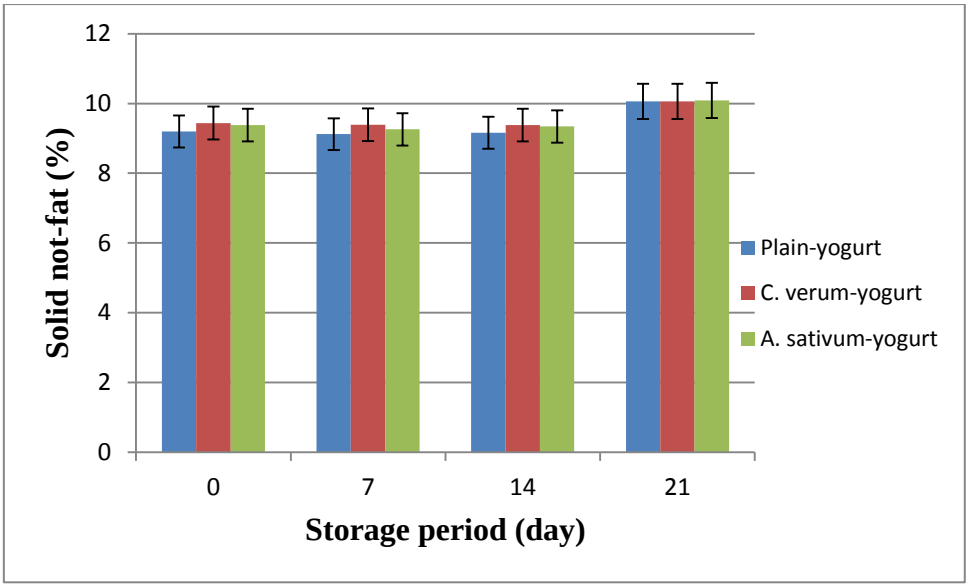


Figure 4.27 Changes of solid-not-fat content (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

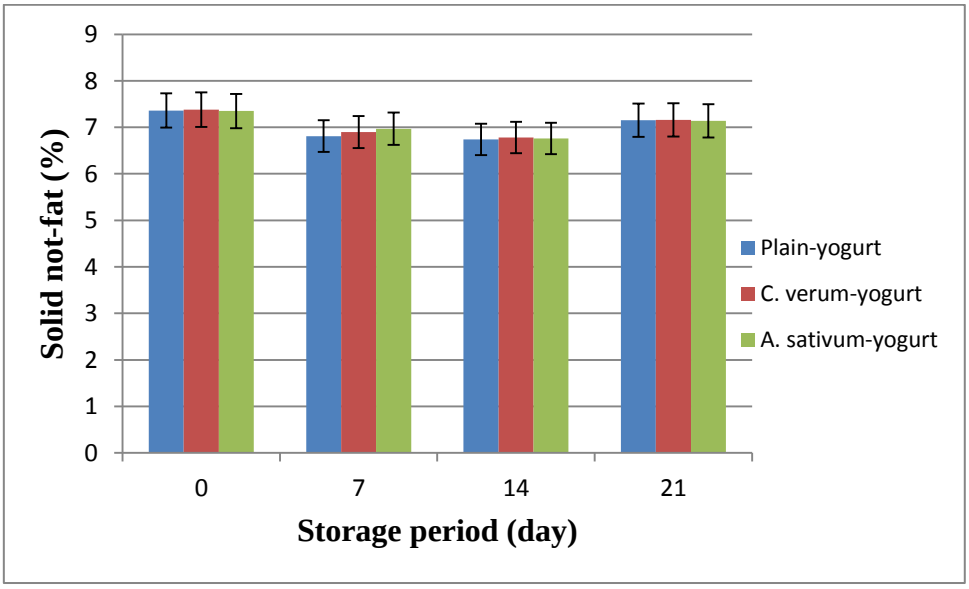


Figure 4.28 Changes of solid-not-fat content (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

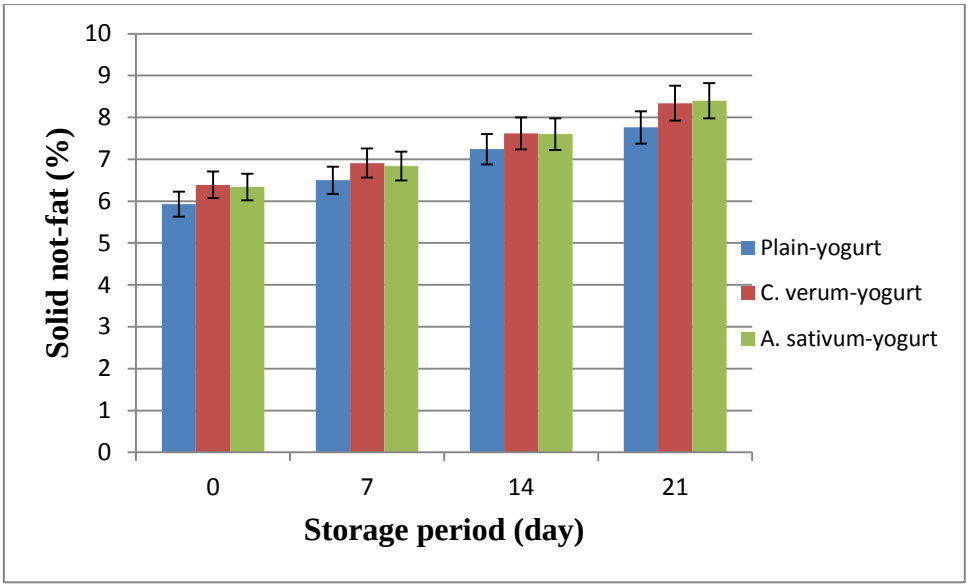


Figure 4.29 Changes of solid-not-fat content (%) in goat milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

4.4 DISCUSSION

4.4.1 Post-acidification in yogurt

The pH of yogurt continued to decline during refrigeration and this could be explained by the residual metabolic activity of yogurt bacteria. The activity of *β*-galactosidase released by the LAB to cleave lactose is still active even at refrigerated storage temperature (0–5°C; Kailasapathy and Sultana, 2003). This is contributed to the accumulation of lactic acid, acetic acid, citric acid, butyric acid, acetaldehyde and formic acid produced by yogurt starter culture as metabolic by-products (Novak and Lubiere, 2000; Ostlie *et al*., 2003; Adolfsson *et al*., 2004). The present study showed no enhancement in pH reduction in the presence of *A. sativum* or *C. verum* water extract in yogurt prepared from three different types of milk. This could be attributed to the ability of milk to resist changes in pH during fermentation (Salaün *et al*., 2005; Ranadheera *et al*., 2012) even in presence of acidic matters in both herbal extracts due

to inherent differential buffering capacity of milk (Salaün *et al*., 2005; Ranadheera *et al*., 2012). This is demonstrated by the degree of pH reduction in both plain- and herbal- yogurt which was the highest in cow milk yogurt followed by goat- and camel- milk yogurt. The production of ammonia from the urease activity of yogurt bacteria (Haque *et al*., 2009) and from the deamination of some amino acids (Analie and Bennie, 2001; Adolfsson *et al*., 2004) may lead to slight alkalinization of the growth medium. Thus the measured yogurt pH value during refrigerated storage can be regarded as the net effect of production of residual acids/ amino groups.

Organic acid is an intermediary product in the biosynthesis of pyrimidine nucleotides. The major organic acid in raw milk is citric acid (Neal-McKinney et al., 2012) with minor contributions from other acids such as benzoic and ascorbic acids, which are present in milk in fewer amounts (Neal-McKinney *et al*., 2012). LAB also produces acetic acid (Ramachandran and Shah, 2009) which affects the flavor profile of yogurt (Chandan and O'Rell, 2006). Amirdivani and Baba, (2011) reported that acidity of yogurt increased during cold storage in parallel to the change in pH. The present study showed that the increase in TA in herbal-yogurt made from different milk did not differ from the plain- yogurt during storage except in *A. sativum*- cow milk yogurt. This could be explained by the differential ability of LAB to grow in cow milk than in camel- and goat- milk during fermentation (i.e. higher ($p<0.05$) viable cell counts of *S. thermophilus* in *A. sativum*- cow milk yogurt than plain-yogurt; Figure 3.15). Goat milk- yogurt showed the highest ($p<0.05$) titratable acidity followed by yogurt made from camel and cow milk which is in support with pervious finding reported by Eissa *et al*., (2010 and 2011) on plain-yogurt.

4.4.2 Proximate composition

4.4.2.1 Lactose and carbohydrates

During fermentation of milk, LAB hydrolyses lactose to its monosaccharide components i.e. glucose and galactose and a portion of glucose is converted to lactic acid (Purnomo and Muslimin, 2012). This hydrolysis lowers lactose concentration in yogurt thus making this fermented milk tolerated better than milk by persons with lactose maldigestion (Shortt *et al*., 2004; De Vrese *et al*., 2001). *A. sativum* or *C. verum* contains no lactose or carbohydrate and thus these herbal extracts have no contribution to yogurt sugar content. The reduction of carbohydrate in yogurt during storage can be attributed to a further decrease in lactose content of yogurt associated with bacterial metabolic activity. This can be correlated with the viability of yogurt bacteria that consumed lactose as essential nutrient for their growth during refrigerated storage (Ramachandran and Shah, 2009). Lactose plays important role in the survival of LAB because it is important source of energy (Ramachandran and Shah, 2009). In the present study, the reduction of lactose and carbohydrates contents in yogurt during refrigerated storage found was in accordance with other reports (Gracia-Fontan *et al*., 2006; Ismaiel *et al*. 2011; Purnomo and Muslimin, 2012). Despite the addition of *A. sativum* or *C. verum* did not markedly change the reduction of carbohydrate during storage, the use of particular milk is instrumental in allowing more viable bacteria to survive the storage. Increase viable bacteria during storage in turn is beneficial with respect to reducing further the lactose content in yogurt and hence made yogurt more suitable for consumption by lactose-intolerant persons.

4.4.2.2 Total solids, moisture content and ash

The present study showed that total solids increase during storage period was due to moisture content loss as previously demonstrated by Kamruzaman *et al*. (2002). The evaporation rate of moisture content is time storage depended and this may explain the variation of moisture content in all types of yogurt made from three different types of milk during storage. Total solids of both plain- and herbal- yogurt made from the three types of milk (12% - 14%) were lower for cow- and camel- milk yogurt than the recommended range in yogurt manufacture (14–15 g/100 g; Kamruzaman, *et al*., 2002). However, herbal- goat milk yogurt showed 14% total solids on the last two weeks of storage. Robinson *et al*. (2002) reported that the best yogurt is made from milk containing 15–16 g/100 g total solids which is higher than the total solids in the three types of milk in the present study (12% - 13%). Since the treated yogurt prepared in the present study did not involve milk powder addition, it can be deduced that the addition of milk powder in further studies could easily correct the solid content in milk. Such an approach is would increase protein content and enhance protein network, thus improving yogurt texture (Lee and Lucey, 2010).

The ash content in the three types of yogurt was not an affected by the addition of herbal extracts. Nevertheless, the lower ash content in yogurt than those in earlier studies (Isanga and Zhang, 2007 and 2009; Eissa *et al*., 2011) could be attributed to variations in feeding regimes that influence mineral contents in milk (Güler and Park, 2009).

4.4.2.3 Fat and solid-not-fat

The addition of herbs such as garlic or other similar additives like fruits (Qureshi *et al*., 2011; Olugbuyiro and Oseh, 2011) did not affect yogurt fat content. However, as demonstrated in the present study and other (Köse and Ocak, 2011) storage time and types of milk could have significant effect on the lipolytic activity of LAB and thus explain the differences in the fat content in all treated yogurt samples during storage. The highest fat content in yogurt was shown in goat milk-yogurt followed by camel- and cow- milk yogurt (Figures 4.24, 4.25 and 4.26). These could however variation in the ranking in fat content as reflowed by the highest fat in camel- (5.78 %) followed by goat- (4.41%) and cow- (3.71 %) milk yogurt (Eissa *et al*., (2010 and 2011). This is not surprising since factors such as lactation period and feeding management of animals (Güler-Akin and Serdar, 2007) contribute significantly to the variance in fat content in milk.

The presence of *A. sativum* or *C. verum* water extract in yogurt made from three milk species had no significant effect on solid-not-fat (SNF). The minimum range of required SNF in yogurt as subjected by international legal standards or indirectly by the manufacturer seeking to produce an end product with certain physical properties is from 8.2 to 8.6% (FDA, 2009; USDA specification, 2001). While SNF in plain- and herbal- yogurt made from cow milk was within the recommended legal range, other treated yogurt made from camel and goat milk were lower than the minimum range. Adjustment of solids content by adding 4 g/100 g full cream milk powder (Isanga and Zhang, 2009) may help to standardize the SNF in plain- and herbal- yogurt made from camel and goat milk.

4.5 CONCLUSIONS

The addition of *A. sativum* or *C. verum* water extract in yogurt had no significant effect on the post-acidification during 21 days of refrigerated storage. Herbal- goat milk yogurt showed the highest total solids and lowest moisture content among the other treated yogurt (cow- and camel- milk yogurt) during refrigerated storage. Herbal-yogurt made from three types of milk showed no significant effect on lactose, carbohydrates, ash, fat and solid-not-fat contents as compared to respective plain-yogurt. Although *A. sativum* and *C. verum* did not significantly affect the physicochemical properties of yogurt that measured in the present study however, the added herbs may still provide characteristic/ unique flavour with some additional nutrition (e.g. phenolic compounds) for the consumers. The exceptionally low lactose content in herbal- yogurt made from camel and goat milk could further justify the suitability of these fermented milk lactose-intolerant people.

5.0 Enrichment of yogurt with *Allium sativum* and *Cinnamomum verum*: Influence on water holding capacity, syneresis, exopolysaccharides production and rheological properties

5.1 INTRODUCTION

Smaller peptides and free amino acids are formed as a result of the proteolytic activity of microbial enzymes produced by the yogurt bacteria (Hayes *et al*., 2007). Proteolysis contribute to yogurt flavour directly, via the formation of peptides and free amino acids (FAAs) and indirectly through precursors such as amines, acids, thiols, aldehydes, ketones, lactones, methyl esters and secondary alcohols (Considine *et al*., 2000). It is important to regulate the enzymatic hydrolysis of casein because the activity of proteases from psychrotrophic bacteria or by milk native plasmin is known to give yogurt with different firmness, viscosity and degree of syneresis (Harte *et al.*, 2003; Welman and Maddox, 2003). This is because proteolysis of casein by native milk proteinases alone is an important factor linked to high moisture levels and low quality of yogurt produced (Jolly *et al*., 2002: Welman and Maddox, 2003).

Rheometry is a useful technique for measuring the quality of a product such as texture. Viscoelastic properties in the food industry play a role for evaluating the gelation system by measuring the extent and strength of internal structures (Sendra *et al*., 2010). Milk coagulation during fermentation by acid occurs as a consequence of removal of calcium bond between casein micelles. This causes the destabilization of casein leading to aggregation and formation of curd (Shaker *et al*., 2000). The gelation process starts with the aggregation of whey proteins associated with caseins (β-lactoglobulin via κ-casein binding; Lee and Lucey, 2010). The reaction between α-lactalbumin and β-lactoglobulin with the micelles through an intermediate formed between the two whey proteins in solution may also contribute to the gelation process (Xu *et al*., (2008). Texture is a very important factor used to define the quality of yogurt (Peng et al., 2009). Rheology is the science of the deformation and flow of materials and oscillatory test is commonly employed to assess the rheological

characteristics of yogurt by provide the necessary information about how yogurt texture changes with time (Remeuf *et al*., 2003; Sodini *et al*., 2005). Whey separation makes yogurt appears watery and it has negative effects on the rheological characteristics of yogurt gels resulting in low quality of yogurt. Susceptibility to syneresis (STS) provides an indication of the non-homogeneities in the gel system of yogurt in which higher whey separation correlates highly with gel instability (Peng *et al*., 2009).

Bacterial exopolysaccharides (EPS) are regarded as more proper substitute for food additives as biothickeners or biostabilizers. EPS can easily solubilize in water and this increase the viscosity of the milk serum surface, thus reducing syneresis and improve the texture and mouthfeel of yogurt (Khurana and Kanawjia, 2007). Bacterial EPS has the potential to be developed as functional food ingredients with favourable impact on both health and economic benefits. Increase viscosity of EPS containing food was reported useful in assisting transient colonization of probiotics in the gastrointestinal tract (Khurana and Kanawjia, 2007). The improvement of yogurt texture by incorporation dietary fibres during fermentation have been studied using gelatine (Gonçalvez *et al*., 2005), pectin (Matia-Merino *et al*., 2004), k-carragenean (Sohrabvandi *et al*., 2013) oat, rice, soy and maize fibers (Fagan *et al*., 2006). However, the use of plant materials with medicinal properties has not been attempted to similar extent. It is anticipate that phytochemicals present in those plants would adversely affect physical properties of yogurt such as water holding capacity, syneresis, EPS and rheology. Nevertheless in view of the enormous potential of increase nutritional and therapeutical values by addition of medicinal plants, thus it is important to establish the changes in physical properties of yogurt in the presence of plants extracts. Therefore, the present work was carried out to study the effect of *A.*

sativum and *C. verum* water extracts on the changes of water holding capacity, syneresis, exopolysaccharides production and rheological properties in yogurt during refrigerated storage.

5.2 MATERIALS AND METHODS

5.2.1 Plant water extraction procedure

The water extract of plant was performed according to the method described in Section 3.2.3.

5.2.2 Yogurt manufacturing process

5.2.2.1 Starter culture and yogurt preparation

The starter culture preparation was carried out using the method described in Section 3.2.4.1. Yogurt was made using either cow or camel milk in the presence and absence of *A. sativum* or *C. verum* water extract (see Section 3.2.4.2).

5.2.3 Isolation and quantification of exopolysaccharides (EPS)

Exopolysaccharides were isolated from yogurt samples based on procedure described by Elin *et al*. (2010). Yogurt sample (25ml) was diluted with dH_2O in the ratio of 1:1. The mixture was boiled (100°C) for 10 min followed by cooling (25°C) for another 10 min. Then, 4ml of 20% (w/v) trichloroacetic acid (TCA; Sigma Chemical Company) were added to the solution and the mixture was allowed to stand at room temperature for 2 hours. After centrifugation (10000 rpm for 30 min at 4°C) to remove the precipitated proteins and bacterial cells, the pH of the supernatant was adjusted to 6.8 using 40% (w/v) NaOH. The solution was subjected to centrifugation again (10000 rpm for 30 min at 4°C) after which the supernatant was mixed with a double volume of cold (4°C) ethanol followed by storage at 4°C for 24 hours. The precipitated EPS was harvested by centrifugation (10000 rpm for 30 min at 4°C) and subsequently dissolved in 10 ml dH_2O. Total EPS (mg/l) was estimated in each

sample by phenol sulphuric method using glucose as a standard (Torino *et al*., 2001; see Section 4.2.7).

5.2.4 Water holding capacity (WHC)

The water holding capacity of yogurt was determined essentially as described by Harte *et al.*, (2003). Briefly, the yogurt (10g) was placed in a centrifuge tube and subjected to centrifugation (10000rpm, 15minutes, at 4°C). The separated whey was pipetted out and weighed. The following formula was used to calculate WHC%:

$$\text{WHC } (\%) = 1 - \left(\frac{W_1}{W_2}\right) x\ 100$$

where: W_1 = Weight of whey after centrifugation, W_2 = Weight of yogurt being centrifuged.

5.2.5 Susceptibility to syneresis (STS)

Susceptibility to syneresis was carried out as described by Isanga and Zhang, 2009. Yogurt sample (10g) was placed on a filter paper placed on top of a funnel. After 6 hours of drainage, the volume of the whey collected in a beaker was measured and used as an index of syneresis. The following formula was used to calculate STS:

$$\text{STS } (\%) = 1 - \left(\frac{V_1}{V_2}\right) x\ 100$$

where V_1 = Weight of whey collected after drainage, V_2 = Weight of yogurt sample.

5.2.6 Rheological measurements

Dynamic oscillatory measurement of yogurt was carried out using Bohlin VOR controlled strain Rheometer (Malvern Instrument UK), with a cone-plate geometry, in which the rotating cone was 40 mm in diameter, and cone angle of 4° with a gap of 0.150 mm (Niraula *et al*., 2003). Temperature was maintained at 20±0.1 °C using Peltier Plate system (- 40 to +180 ^{o}C, Peltier Plate system from Bohlin Instrument Ltd.) which acts as temperature controller. Yogurt samples were gently

stirred 10 times by spoon prior to rheological analysis in order to minimise differences due to structural breakdown during handling of the yogurt. Amplitude sweeps were carried out at 20°C to characterize the viscoelastic linear region of yogurt samples. Strain ranging from 0.0005 to 0.1% was used at a constant frequency of 0.5 Hz. The frequency sweep was performed at a controlled strain mode. A low deformation strain was chosen from the linear viscoelastic profile from amplitude sweep. The elastic modulus (*G*'), the viscous modulus (*G*'') and the loss tangent defined as tan δ = (G''/*G*') were obtained from the equipment software in all cases. Shear viscosity profiles of yogurt samples were measured as a function of shear rate sweep (0.010 - 80.000 s^{-1}).

5.2.7 Sensory evaluation

The sensory evaluation of set-style yogurt was performed after 1 and 21 days of refrigerated storage. An untrained panel of 12 assessors recruited from students and staff members of the Institute of Biological Sciences, Faculty of Science, University of Malaya with age ranging from 20 to 35 years old (mean age was 22). The sensations perceived when evaluating a sample were described according to the following categories: appearance, homogeneity of surface, taste, mouthfeel, aroma and overall preference. Every category was described by certain descriptors (Majchrzak *et al*., 2010) as listed in Table 5.1. The evaluation form was given to each panel with 2 groups of yogurt (cow- milk yogurt and camel- milk yogurt) with each group consisting of 3 coded yogurt samples served in plastic cups (10g for each). The first group contained plain-, *A. sativum*- and *C. verum*-cow milk yogurt. The second group contained plain-, *A. sativum*- and *C. verum*-camel milk yogurt. Water was available for panel members to rinse their mouth between samples eating. The

evaluation was scored (Bodyfelt *et al*., 1988) on the following 1-10 point hedonic scales: (1-2= extremely poor, 3-4= poor, 5-6= fair, 7-8= good, 9-10= excellent).

Table 5.1 Sensory descriptors and definitions.

Categories	Descriptors	Definitions
Appearance	Syneresis/whey separation	Whey separated from the samples during storage.
	Colour	The intensity of the white colour.
Homogeneity of surface	Grainy	The surface is homogenous if not shows no irregularity; surface without grains
	Lumpy	The surface is homogenous if not shows no irregularity; surface without bumps.
	Firmness	Evaluated visually by slowly placing a spoonful of yogurt on the untouched yogurt surface and evaluate how long the structure is retained.
Taste	Overall intensity	Intensity of overall yogurt taste
	Sour	An acidic taste associated with citric acid solutions.
	Sweet	A sweet taste associated with sugar
	Bitter	A basic taste associated with caffeine solutions
Mouthfeel	Texture (Thickness)	Sensation of the sample consistency in the mouth; flow ability of the yogurt (viscosity).
Aroma	Overall intensity	Intensity of overall aroma of the yogurt
Preference	Overall intensity	Overall preference after 1 min having swallowed the yogurt.

(Source: Majchrzak *et al*., 2010).

5.2.8 Statistical analysis

Three batches of yogurt were prepared and analysed for water holding capacity, susceptibility to syneresis and exopolysaccharides. For rheological measurements two batches of yogurt were refrigerated (4°C) for 0, 7, 14 and 21 days. The average was taken and data were expressed as mean ± standard error. The significance was established at $p<0.05$. Data analysis was done using SPSS® version 17.0. The sensory analysis scores were analysed statistically using one way analysis of variance (ANOVA, SPSS 17.0), followed by Duncan's post hoc test for mean comparison.

5.3 RESULTS

5.3.1 Exopolysaccharides (EPS) production

The presence of *A. sativum* or *C. verum* water extract in fresh cow milk yogurt caused small increase in the production of EPS (95.6±0.4 mg/l and 85.4±0.2 mg/l respectively) compared to plain-yogurt (87.2±0.3 mg/l; Figure 5.1). Refrigerated storage of yogurt (7 days) increased EPS in *C. verum*- yogurt (176.8±0.7 mg/l; $p<0.05$) compared to plain-yogurt (81.0±1.4 mg/l). Prolonged storage of yogurt to two more weeks reduced ($p<0.05$) EPS of plain- and *C. verum*-yogurt (47.4±0.6 mg/l and 66.6±1.3 mg/l respectively; Figure 5.1). *A. sativum*-yogurt showed continuous increase in EPS content throughout the 21 days of storage (129.2±0.2 mg/l and 193.6±1.2 mg/l on day 14 and 21 respectively).

Fresh camel milk-yogurt contained 278.6± 0.3 mg/l EPS (Figure 5.2). The presence of *A. sativum* or *C. verum* water extract had no effect ($p>0.05$) on EPS in camel milk yogurt (297±0.2 mg/l and 296.4±0.3 mg/l respectively). Refrigerated storage (7 days) increased EPS in *A. sativum*- and *C. verum*-yogurt (418.2±0.4 mg/l and 472.2±0.2 mg/l respectively; $p<0.05$) compared to plain-yogurt (283.8 ±0.4 mg/; Figure 5.2). Prolonged storage of yogurt for another 7 days increased EPS in plain- and *A. sativum*-yogurt (463.0±1.5 mg/l and 504.4±1.7 mg/l respectively; $p<0.05$) but not in *C. verum*-yogurt (483.8±1.6 mg/l; $p>0.05$). Both plain- and *A. sativum*-yogurt showed decrease in EPS content (416.6±0.9 mg/l and 438.2±1.1 mg/l, $p<0.05$) on day 21 of storage (Figure 5.2).

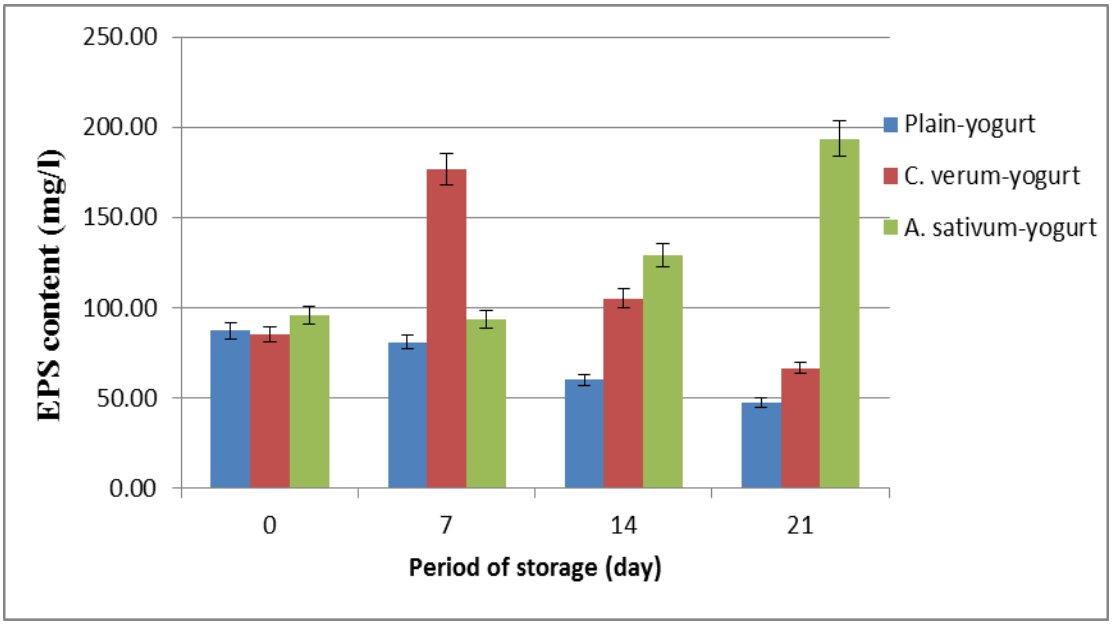


Figure 5.1 Exopolysaccharide (EPS) content in cow milk yogurt (mg/l) in the absence and presence of *A. sativum* or *C. verum* water extract during 21 days of refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

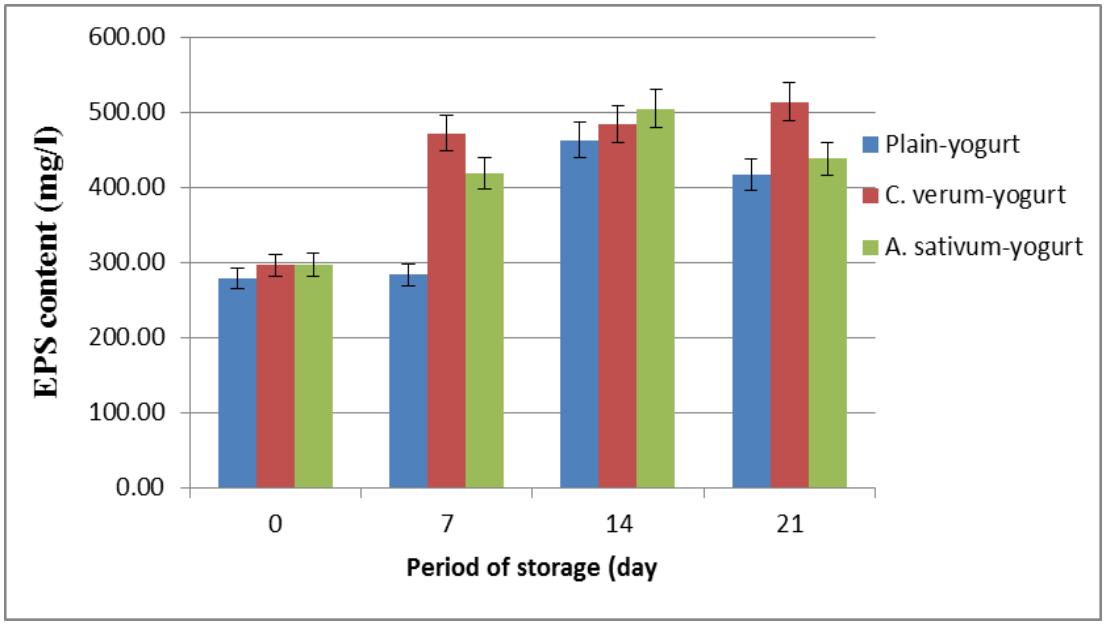


Figure 5.2 Exopolysaccharide (EPS) content in camel milk yogurt (mg/l) in the absence and presence of *A. sativum* or *C. verum* water extract during 21 days of refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain- yogurt at the same storage period.

5.3.2 Water holding capacity (WHC)

Water holding capacity is a critical parameter in yogurt manufacturing because it relates to syneresis (separation of whey) which is an undesirable feature (Kovalenko and Briggs, 2002). WHC was higher ($p<0.05$) in fresh cow milk yogurt (26.35±3.1%) than camel milk yogurt (0.41±0.3%; Figures 5.3 and 5.4 respectively). The presence of *A. sativum* water extract in fresh cow- and camel- milk yogurt resulted in higher ($p<0.05$) WHC (33.85±2.8% and 2.66±0.1% respectively). However, the presence of *C. verum* water extract in both fresh yogurt did not affect WHC (Figures 5.3 and 5.4). Refrigerated storage had remarkable effect ($p<0.05$) on the increase of WHC in plain-camel milk yogurt (8.66±0.9%) on day 7 of storage but not for cow milk yogurt (25.95±2.7%). WHC increased ($p<0.05$) in both *A. sativum*- (38.08±1.2%) and *C. verum*- (36.21±2.6%) cow milk yogurt after 7 and 14 days of storage respectively (Figure 5.3). However, increase WHC due to refrigerated storage to 11.54±0.2%, 17.3±1.4% and 24.5±1.8% for plain-, *A. sativum*- and *C. verum*- camel milk- yogurt respectively on day 21 of storage (Figure 5.4).

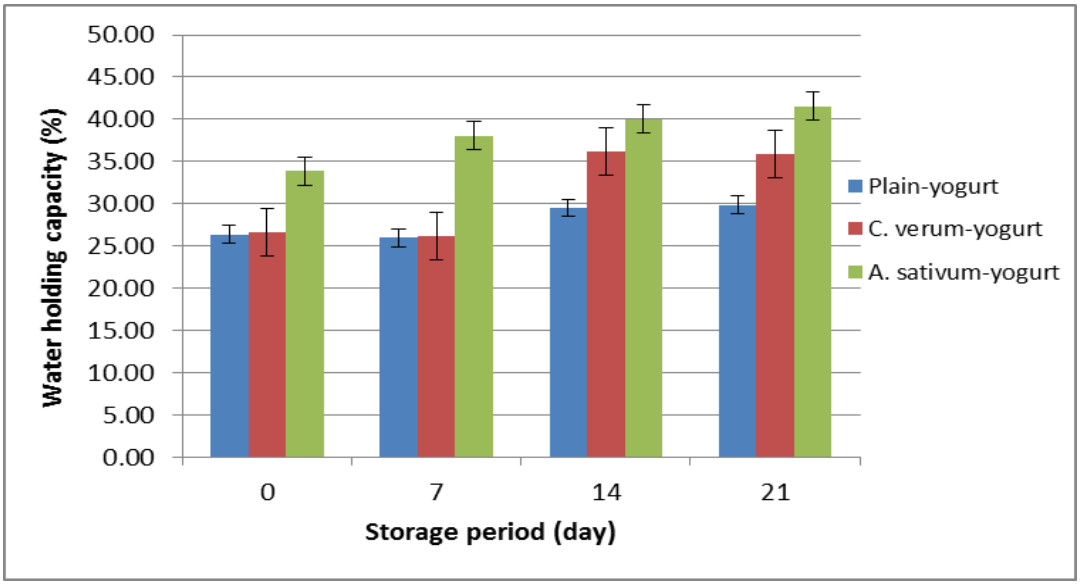


Figure 5.3 Water holding capacity (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days of refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain- yogurt at the same storage period.

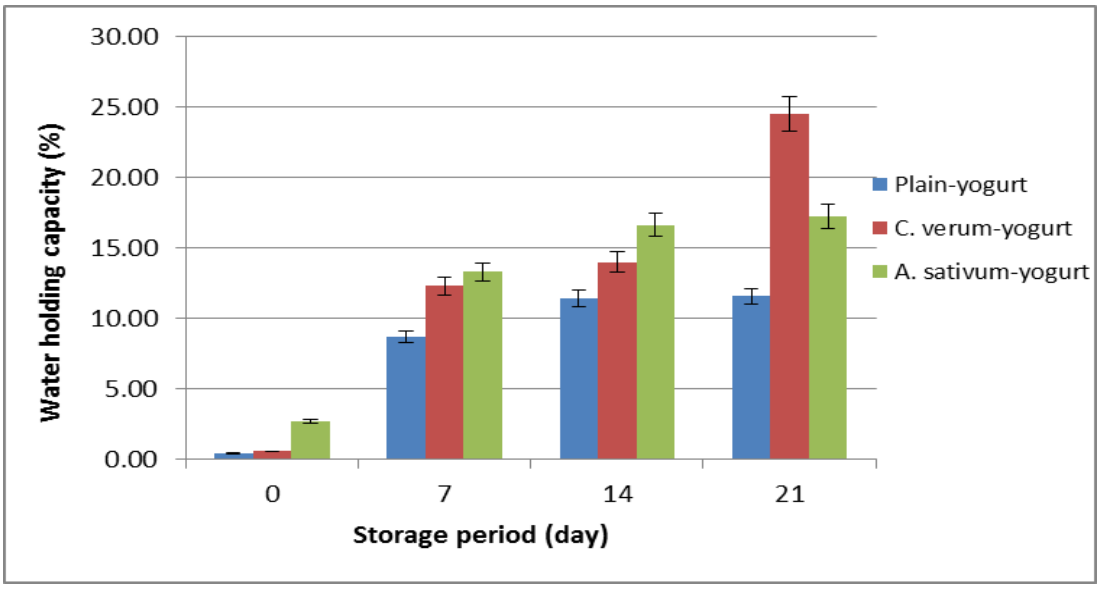


Figure 5.4 Water holding capacity (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days of refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain- yogurt at the same storage period.

5.3.3 Susceptibility to syneresis (STS)

The STS in yogurt made from cow and camel milk are shown in Figures 5.5 and 5.4 respectively. The STS in fresh cow milk yogurt was 38.1±1.7%. The presence of *A. sativum*- and *C. verum* water extract had no effect on STS of yogurt (37.8±0.9% and 35.5±1.9% respectively; Figure 5.5). Refrigerated storage increased ($p<0.05$) STS for plain- and *A. sativum*- yogurt to 47.9±1.3% and 44.2±0.6% respectively but decreased ($p<0.05$) to 23.9±2.4% for *C. verum*-yogurt on the 7th day. Further storage to 14 and 21 days reduced STS to 34.4±1.8% for plain-yogurt and 22.8±1.8% for *A. sativum*-yogurt.

STS in fresh camel milk yogurt was 20.6±0.4% whereas the presence of *A. sativum* or *C. verum* water extract reduced STS to 12.0±0.3% ($p<0.05$) and 18.7±0.7% respectively (Figure 5.6). Refrigerated storage (7, 14 and 21 days) caused sustained increase in STS for plain- and *A. sativum*- yogurt ranging of 32% - 46% and 24% - 34% respectively. *C. verum*-yogurt showed significant reduction in STS (11.1±0.5%) on 7 day of storage. Further storage increased ($p<0.05$) STS to 23.5±0.3% on day 21 of storage (Figure 5.6).

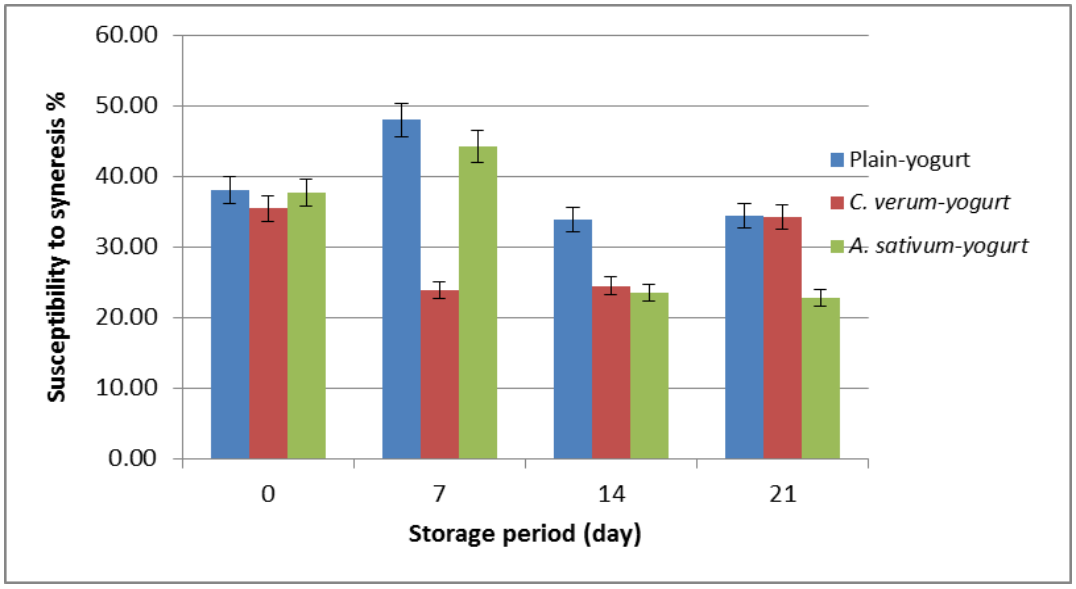


Figure 5.5 Susceptibility to syneresis (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days of refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

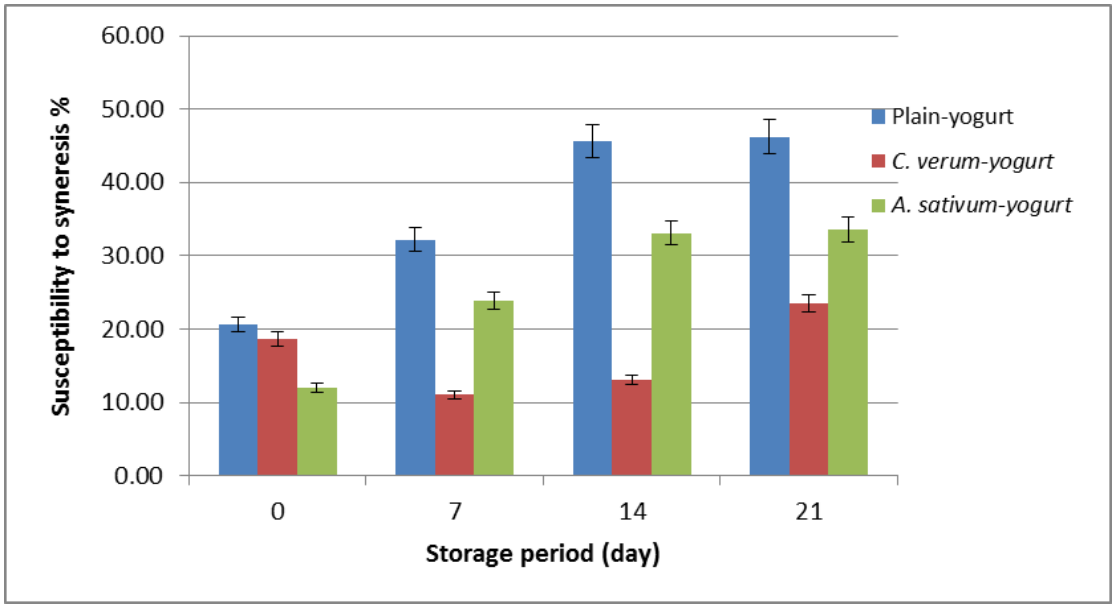


Figure 5.6 Susceptibility to syneresis (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

5.3.4 Dynamic rheology

a) Amplitude sweep

Figure 5.7 shows the changes of elastic modulus (G') and viscous modulus (G'') in cow milk yogurt at different storage period after being subjected to strain. It is observed that both G' and G'' were within the linear viscoelastic domain with typical G' values higher than G'' for both plain- and herbal- yogurt (Figure 5.7 a, b, and c). The values of G' reduced tremendously in the presence of *A. sativum* or *C. verum* in yogurt as compared to plain-yogurt. Refrigerated storage up to 21 days enhanced the structure of plain- yogurt with constant G' values range from 76.98 to 105.43 Pa over a range of strain 0.008-0.009% (Figure 5.7 a). On the other hand, lowest G' values were observed on day 14 and 21 for *A. sativum*-yogurt (7.85 Pa; Figure 5.7 b) and *C. verum*-yogurt (6.29 Pa; Figure 5.7 c) respectively.

Camel milk yogurt in the presence and absence of *A. sativum* or *C. verum* at different storage periods showed a drop in both elastic modulus (G') and viscous modulus (G'') over the whole strain% range measured during 21 days of storage (Figure 5.8 a, b, c). The viscous modulus (G'') was dominant over the elastic modulus (G') during the storage period. However, plain-yogurt showed higher G' values than G'' on 0 day over short range of strain (0.05-0.08%) prior to G' and G'' crossed over each other (Figure 5.8 a). The presence of *A. sativum* or *C. verum* in yogurt showed no changes in the yogurt structure during 14 days of refrigerated storage. However, extended storage to 21 days enhanced the structure for *C. verum*-yogurt (Figure 5.8 c).

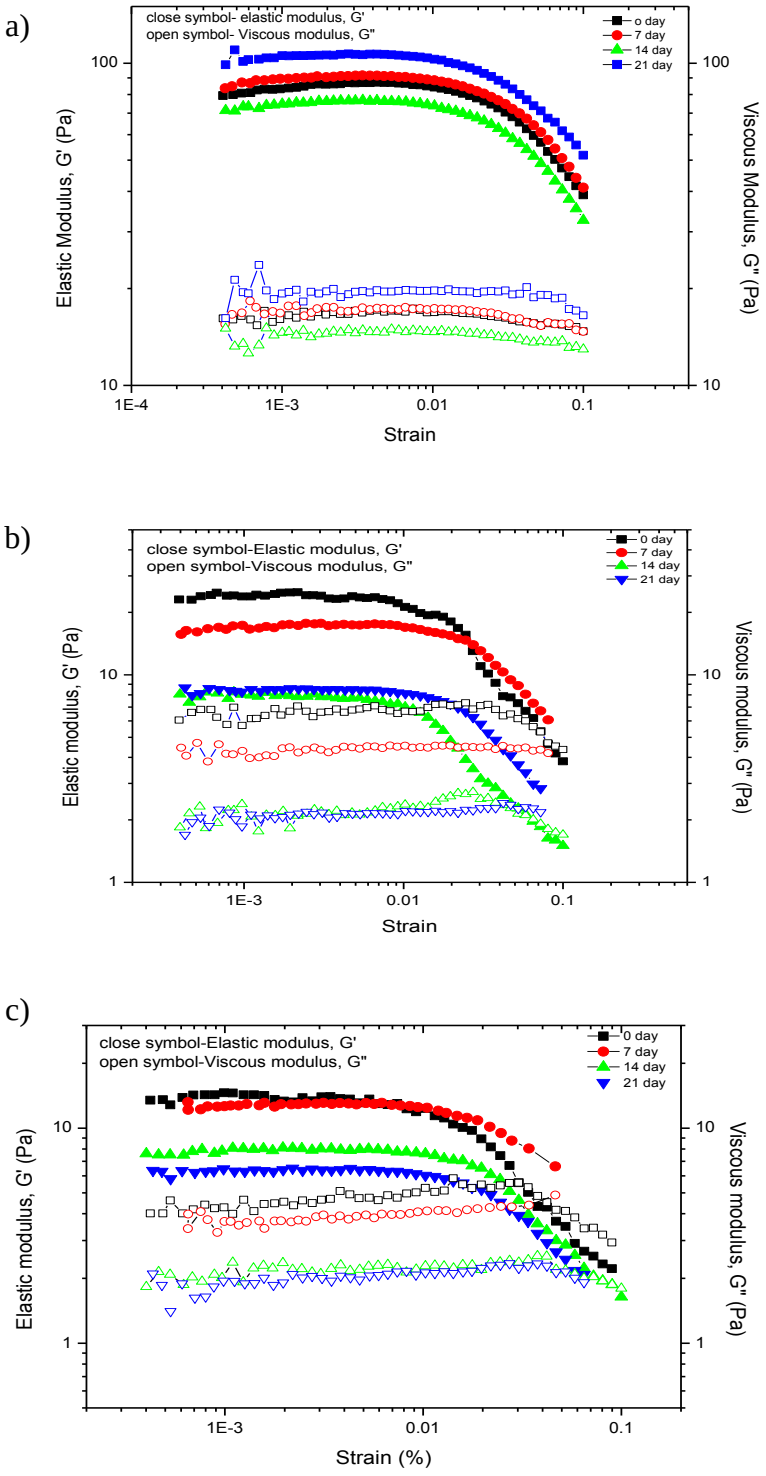


Figure 5.7 Amplitude sweep: elastic modulus (G') and viscous modulus (G'') versus strain % in cow milk-yogurt during 21 days refrigerated storage at 4°C. (a) Plain-yogurt, (b) *A. sativum*- yogurt , and (c) *C. verum*- yogurt. Values are presented as mean (n=2).

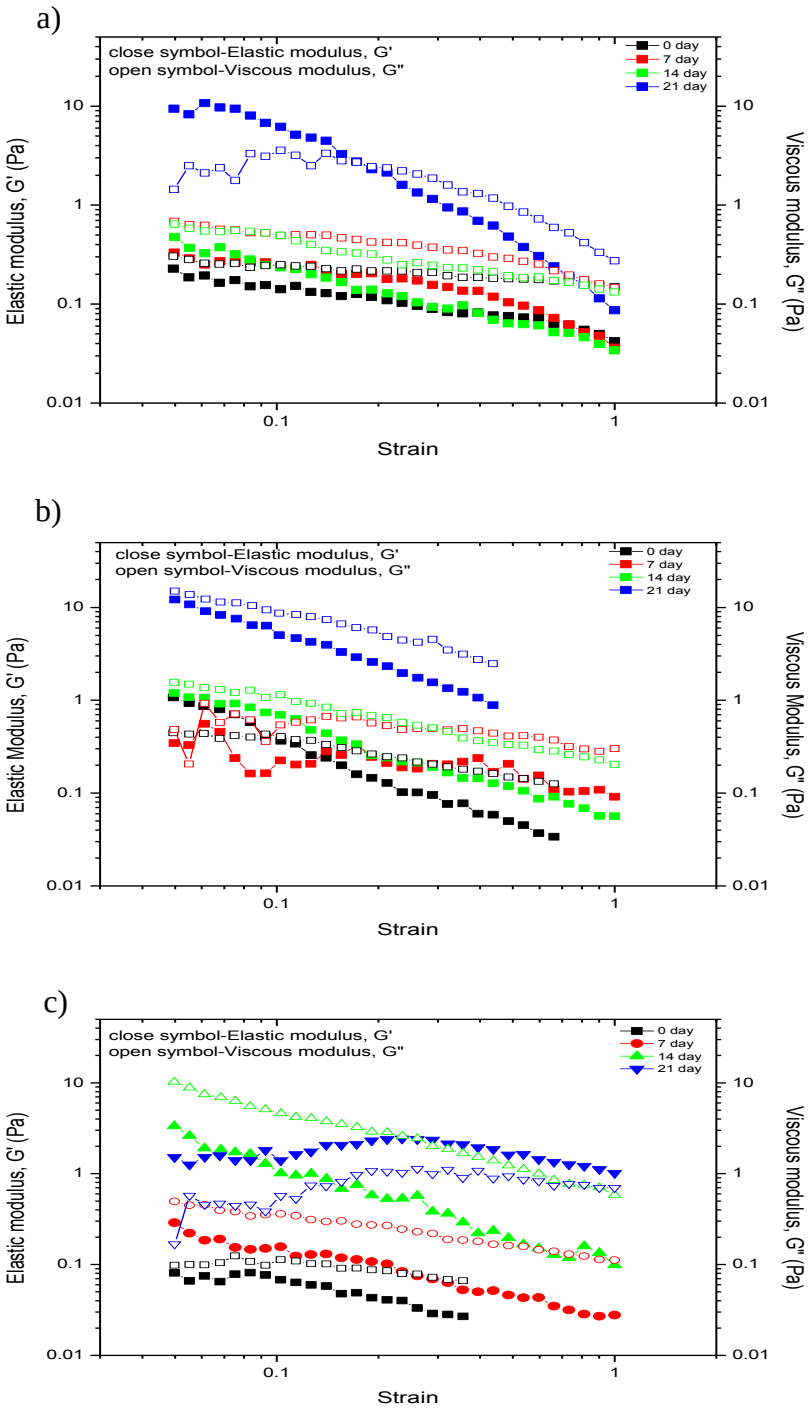


Figure 5.8 Amplitude sweep: elastic modulus (G') and viscous modulus (G'') versus strain % in camel milk-yogurt during 21 days refrigerated storage at 4 °C. (a) Plain-yogurt, (b) *A. sativum*- yogurt , and (c) *C. verum*- yogurt. Values are presented as mean (n=2).

b) Frequency sweep

Cow milk yogurt showed that both G' and G'' were frequency dependence (Figure 5.9). The elastic modulus (G') was found to be larger than the viscous modulus (G'') over the whole frequency range measured for both plain- and herbal-yogurt at all storage periods studied. However, both G' and G'' values were higher in plain-yogurt (Figure 5.9 a) than in *A. sativum* -yogurt (Figure 5.9 b) and *C. verum*-yogurt (Figure 5.9 c). In addition, high G' values in the presence of *A. sativum* or *C. verum* in yogurt on day 0 and 7 of storage were observed but extended storage period for another 2 weeks resulted in pronounced reduction of these values (Figure 5.9 b and c respectively). It should be noted that all yogurt samples showed elastic behaviour (G' > G'') over the whole range of frequencies tested which indicated solid like behaviour.

Camel milk yogurt showed weakly- structured system (liquid-like behaviour) due to the dominance of the viscous modulus (G'') over the elastic modulus (G') throughout the storage period (Figure 5.10). The presence of *A. sativum* or *C. verum* water extract in yogurt showed no enhancement in the yogurt structure compared to plain-yogurt (Figure 5.10). In contrast to unchanged *A. sativum*-yogurt (Figure 5.10 b), refrigerated storage improved the yogurt structure of plain- and *C. verum*-yogurt as evidenced by increased G' and G'' values on day 21 of storage (Figure 5.10 a and c). The tan (δ) values in cow milk yogurt (0.2 - 0.3) were less than camel milk yogurt (1 – 2) during the entire storage period (Figures 5.11 and 5.12 respectively). In addition, all camel milk yogurt showed unstable gel network which were reflected in the unsteady tan (δ) values during all storage periods (Figure 5.12).

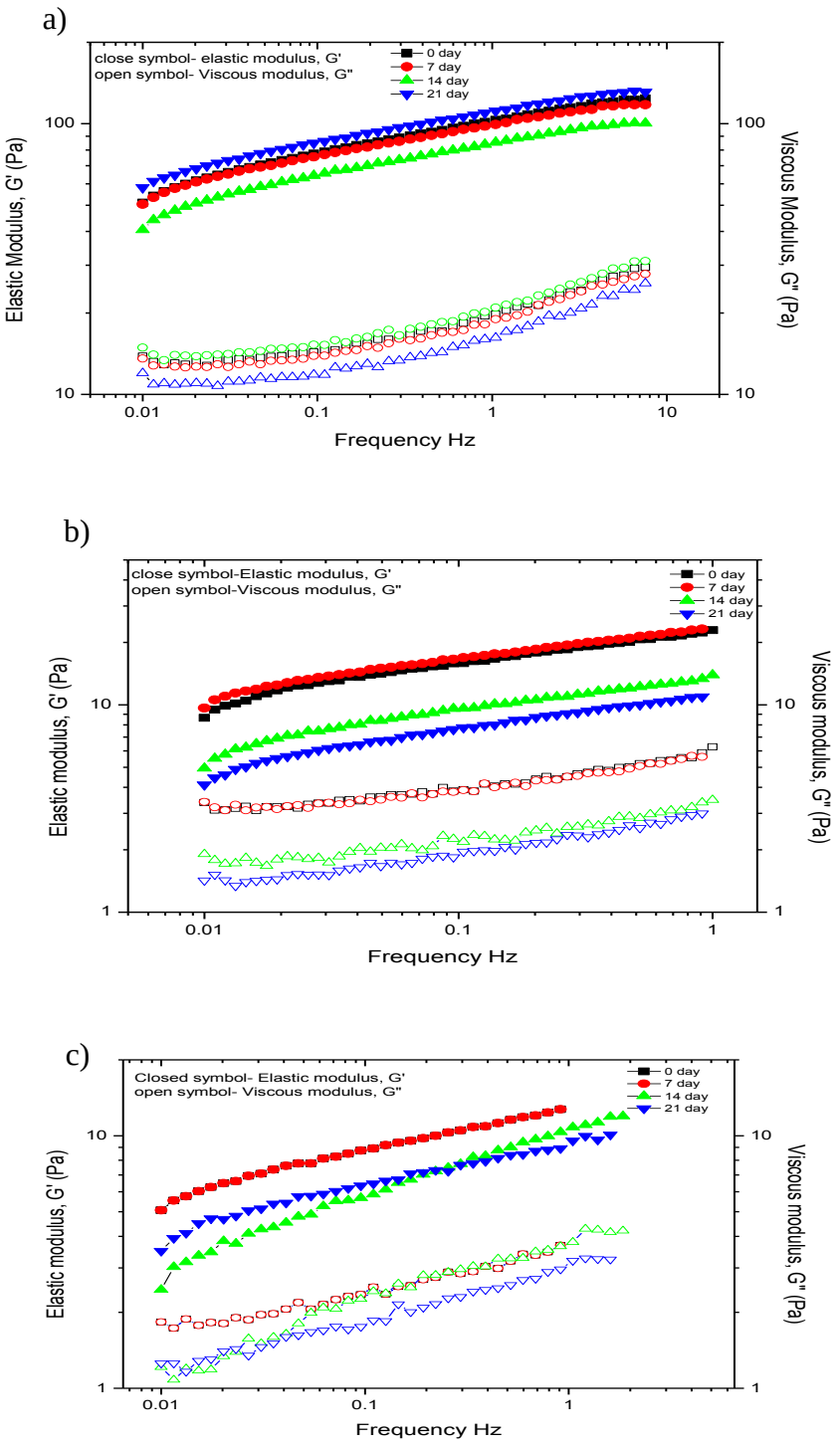


Figure 5.9 Frequency sweep: elastic modulus (G') and viscous modulus (G'') versus strain % in cow milk-yogurt during 21 days refrigerated storage at 4°C. (a) Plain-yogurt, (b) *A. sativum*- yogurt , and (c) *C. verum*- yogurt. Values are presented as mean (n=2).

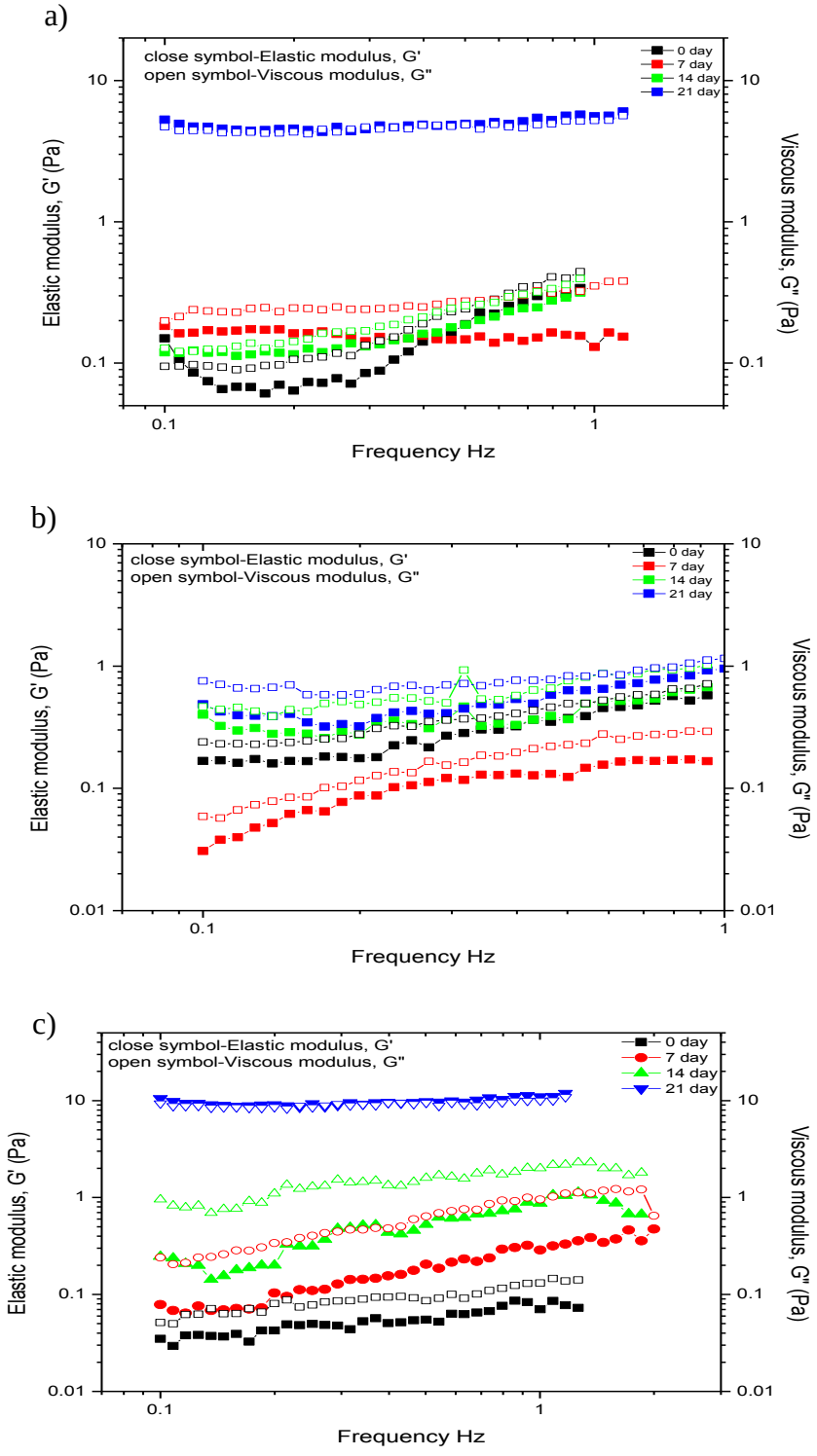


Figure 5.10 Frequency sweep: elastic modulus (G') and viscous modulus (G'') versus strain % in camel milk-yogurt during 21 days refrigerated storage at 4°C. (a) Plain-yogurt, (b) *A. sativum*- yogurt , and (c) *C. verum*- yogurt. Values are presented as mean (n=2).

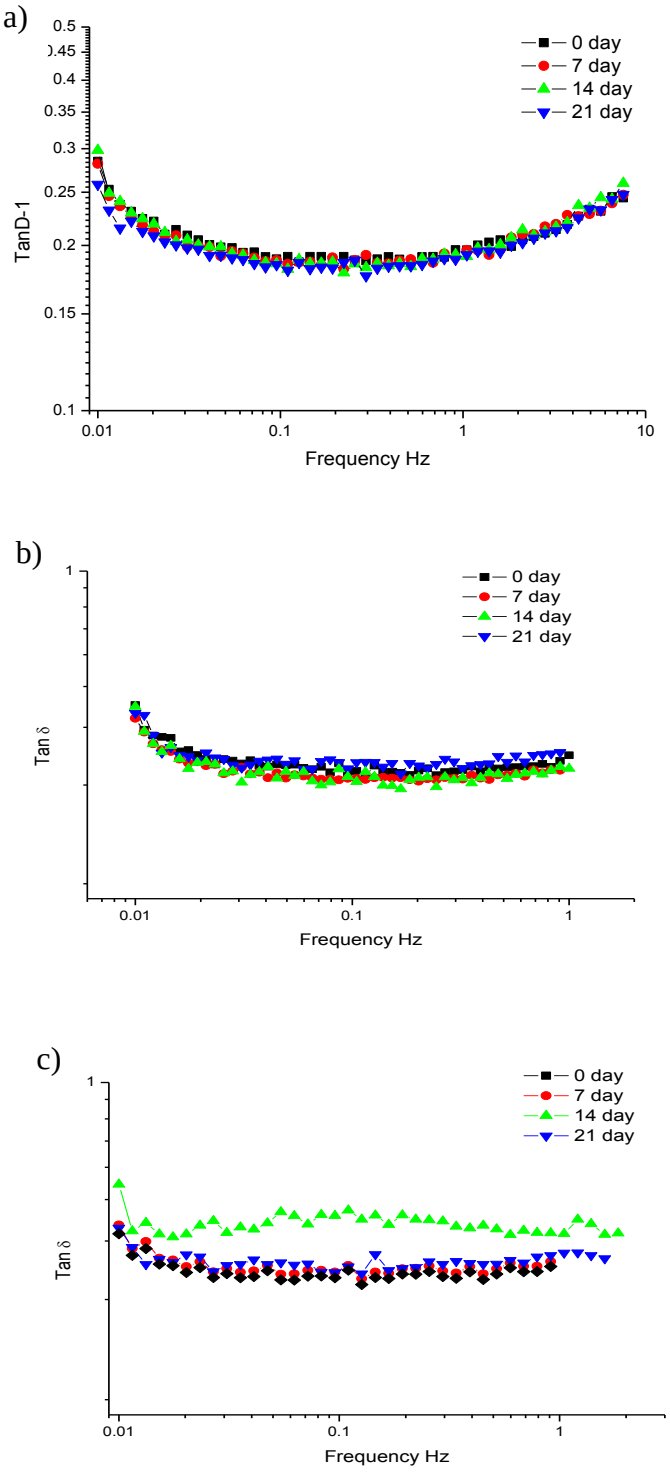


Figure 5.11 Frequency sweep: tan δ versus strain % in cow milk-yogurt during 21 days refrigerated storage at 4°C. (a) Plain- yogurt, (b) *A. sativum*- yogurt, and (c) *C. verum*- yogurt. Values are presented as mean (n=2).

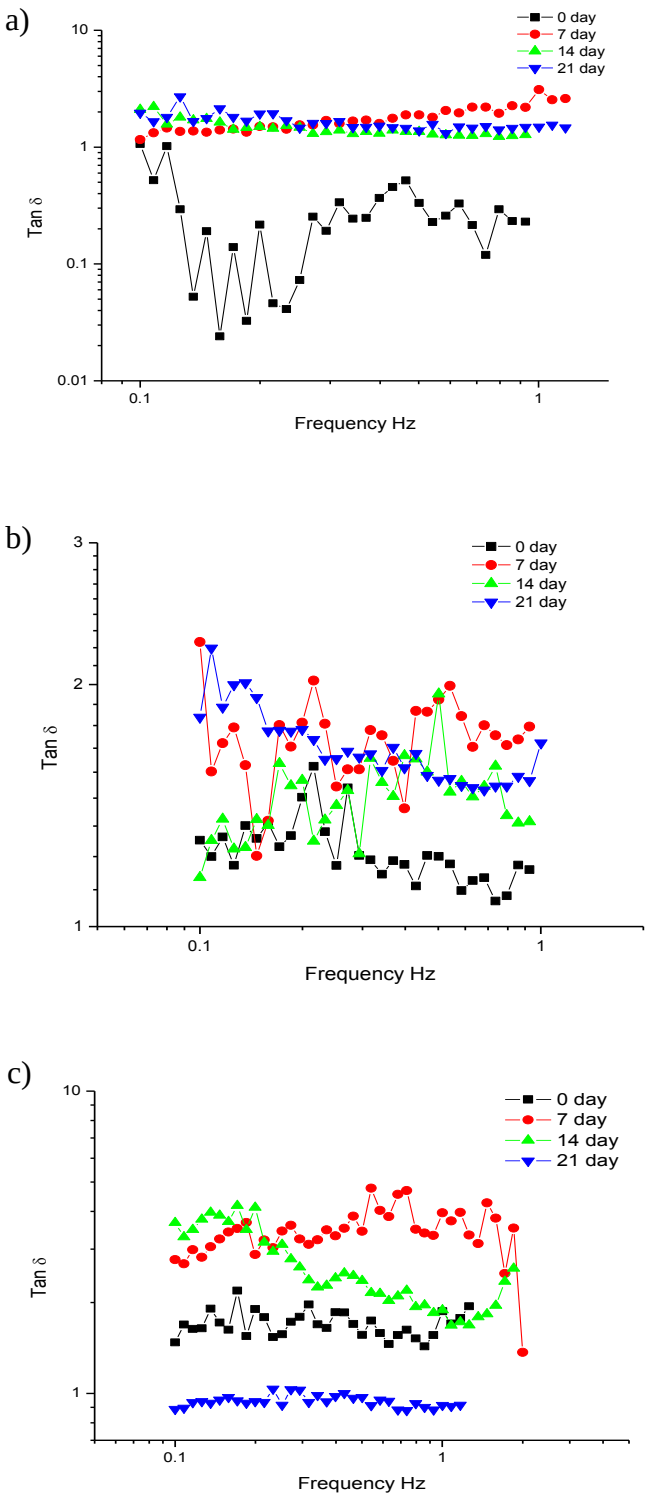


Figure 5.12 Frequency sweep: tan δ versus strain % in camel milk-yogurt during 21 days refrigerated storage at 4°C. (a) Plain- yogurt, (b) *A. sativum*- yogurt , and (c) *C. verum*- yogurt. Values are presented as mean (n=2).

5.3.5 Apparent viscosity

The apparent viscosity profiles of cow- and camel- milk yogurt were measured during storage as a function of shear rate sweep (Figures 5.13 and 5.14 respectively). Both plain- and herbal-yogurt prepared from cow and camel milk exhibited shear-thinning behaviour, i.e., the apparent viscosity decreases with shear rate. Prolonged refrigerated storage to 21 days resulted in lower viscosity for *A. sativum*- and *C. verum*-cow milk yogurt than plain-yogurt (Figure 5.13). In contrast, refrigerated storage of plain-camel milk yogurt increased viscosity profiles on day 21 of storage (Figure 5.14 a) which was enhanced further by *C. verum* on the last 3 weeks of storage and *A. sativum* on day 14 of storage (Figure 5.14 c).

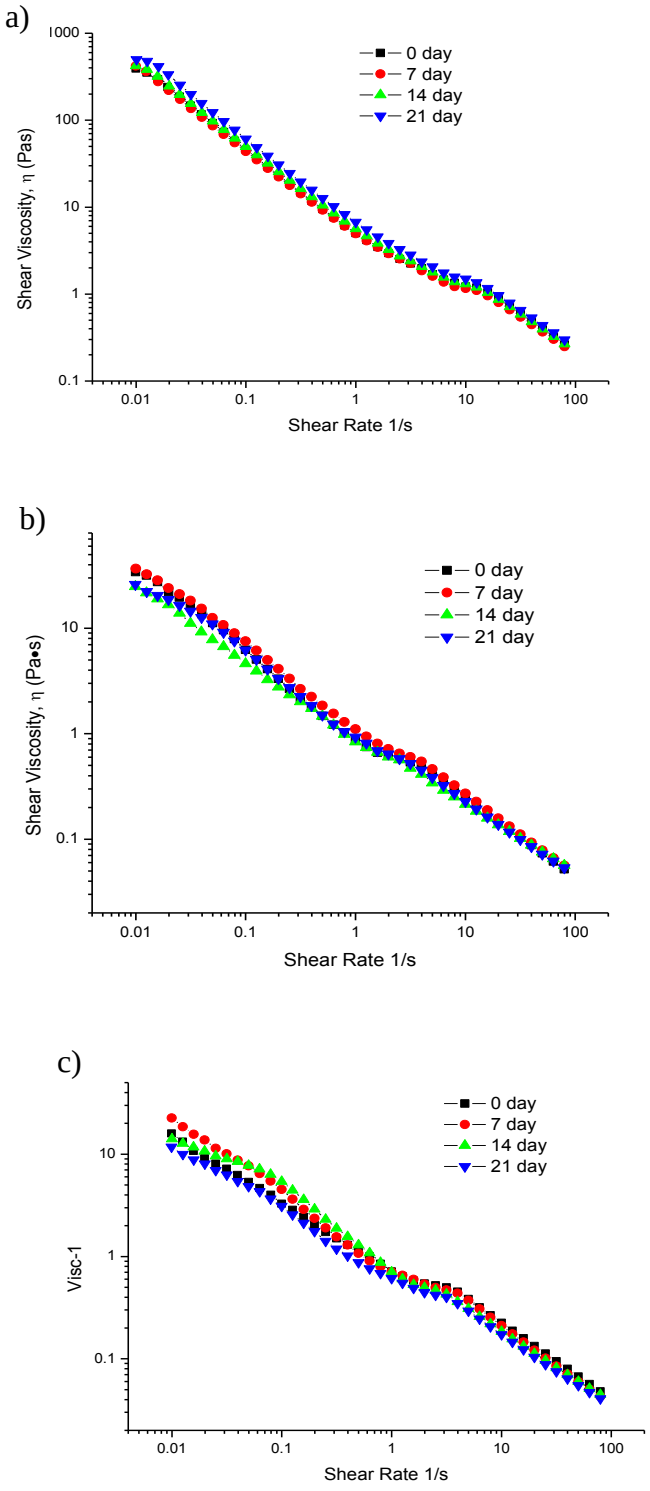
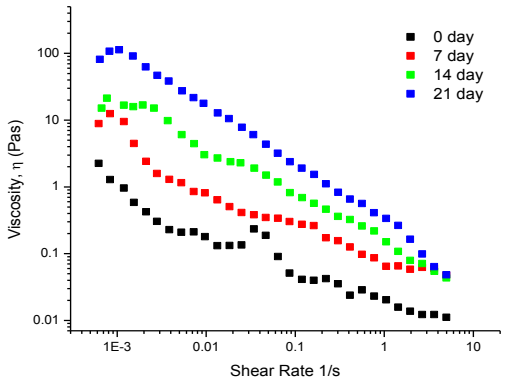
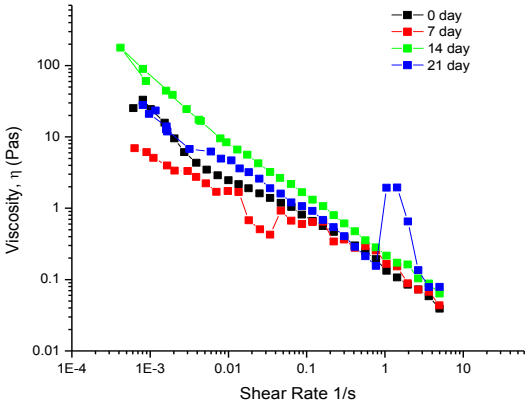


Figure 5.13 Apparent viscosity versus shear rate (1^{-s}) in cow milk-yogurt during 21 days refrigerated storage at 4°C. (a) Plain- yogurt, (b) *A. sativum*- yogurt , and (c) *C. verum*- yogurt. Values are presented as mean (n=2).

a)



b)



c)

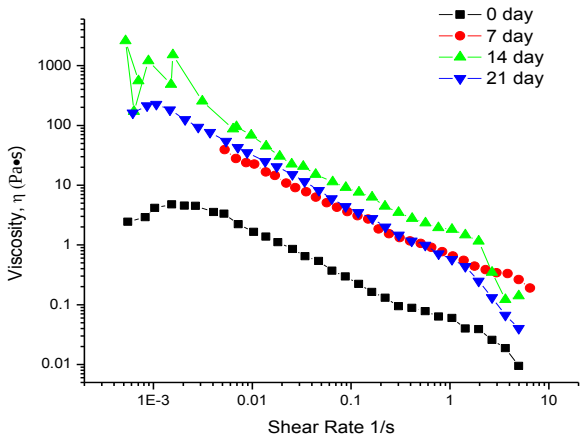


Figure 5.14 Apparent viscosity versus shear rate (1^{-s}) in camel milk-yogurt during 21 days refrigerated storage at 4 °C. (a) Plain- yogurt, (b) *A. sativum*- yogurt , and (c) *C. verum*- yogurt. Values are presented as mean (n=2).

5.3.6 Sensory evaluation

The sensory evaluation of cow- and camel- milk yogurt is as shown in Tables 5.2 and 5.3 respectively. There were no significant ($p>0.05$) differences in the overall quality scores for both cow- and camel- milk yogurt in the presence of *A. sativum*- or *C. verum* as compared with respective plain- yogurt after days 1 and 21 of storage. No differences in syneresis for *A. sativum*- and *C. verum*- cow milk yogurt compared to plain-yogurt after 1 and 21 days of storage. Syneresis in fresh camel milk- yogurt ranged from 3.03 to 3.33 but these values reduced to 1.23-1.88 after 3 weeks refrigerated storage. *C. verum*-yogurt prepared from cow or camel milk showed the lowest colour score (3-4) compared to plain- and *A. sativum*- yogurt (6-7) after 1 and 21 days of storage. Yogurt homogeneity (grain/lump) was considered higher (3-5; $p<0.05$) in cow milk yogurt than those in camel milk yogurt (1-4). Furthermore, higher firmness in cow milk yogurt (5-6; $p<0.05$) than camel milk yogurt (1-2). Cow milk yogurt also scored higher mouthfeel (2-3) than camel milk yogurt (1-2). There were no significant differences in sourness, sweetness, bitterness and overall preference scores in herbal-yogurt prepared from cow- and camel- milk as compared to respective plain-yogurt after 1 and 21 days of storage. The presence of *A. sativum* reduced ($p<0.05$) aroma score in cow milk- yogurt (2.33 ± 0.7) but not in camel milk yogurt (5.42 ± 1.0) compared to respective plain-yogurt (6.08 ± 1.83 and 5.50 ± 1.4 respectively) after day 1 of storage. However, the aroma score of *C. verum*- cow- or camel- milk yogurt was considered no different from respective plain-yogurt.

Table 5.2 Results of sensory evaluation of *A. sativum*- and *C. verum*- yogurt made from cow milk after 1 and 21 days of refrigerated storage.

Attributes	Plain-yogurt	*A. sativum*-yogurt	*C. verum*-yogurt
Day 1			
Overall sensory quality*	5.62±0.12	5.55±1.38	5.32±1.59
Appearance			
Syneresis	4.66±1.69	4.98±1.52	4.85±1.22
Color	6.60±0.22	6.13±0.34	4.10±1.12*
Surface homogeneity			
Grainy	3.32±1.34	4.53±1.41	4.84±1.59
Lumpy	3.18±0.19	3.49±1.09	3.02±1.32
Firmness	5.42±0.49	5.54±0.27	5.57±0.59
Taste			
Overall intensity	6.50±1.45	5.08±1.24	5.92±1.56
Sourness	5.83±1.95	6.42±1.73	5.67±1.72
Sweetness	4.83±1.19	3.42±1.16	3.58±1.83
Bitterness	2.92±1.51	3.08±1.88	2.42±1.24
Mouthfeel	5.31±1.99	3.11±0.90*	2.71±1.28*
Aroma			
Overall intensity	6.08±1.83	2.33±0.65*	6.33±1.50
Preference			
Overall intensity	6.61±0.45	6.38±0.5	6.06±1.0
Day 21			
Overall sensory quality*	5.24±0.22	5.01±1.72	4.99±0.36
Appearance			
Syneresis	6.37±0.46	5.88±0.34	5.63±1.23
Color	6.57±0.81	5.86±1.93	3.00±0.12*
Surface homogeneity			
Grainy	3.60±1.03	4.12±0.37	3.72±1.06
Lumpy	3.27±0.90	3.10±0.36	3.10±1.11
Firmness	5.78±0.40	6.09±0.45	6.23±0.54
Taste			
Overall intensity	6.53±0.37	6.33±0.41	6.53±1.20
Sourness	6.36±0.45	5.78±0.33	6.41±1.70
Sweetness	2.70±0.36	2.73±0.27	2.81±0.32
Bitterness	1.07±0.07	2.13±0.07	2.15±0.05
Mouthfeel	2.7±1.20	2.20±1.71	2.52±1.62
Aroma			
Overall intensity	7.14±0.58	6.12±0.52	7.72±1.57
Preference			
Overall intensity	6.92±0.26	6.86±0.33	7.55±1.31

The evaluation of the intensity of the descriptors was done using a 10-unit scale.
Each value of plain-, *A. sativum*- and *C. verum*- yogurt were a mean of 12 results± standard error.
*$p < 0.05$ as compared to control.
* Overall sensory quality = overall impression of the product.

Table 5.3 Results of sensory evaluation of *A. sativum*- and *C. verum*- yogurt made from camel milk after 1 and 21 days of refrigerated storage.

Attributes	Plain-yogurt	*A. sativum*-yogurt	*C. verum*-yogurt
Day 1			
Overall sensory quality*	1.04±0.05	1.08±0.03	1.00±0.00
Appearance			
Syneresis	3.03±0.06	3.21±0.43	3.33±0.02
Color	6.73±1.23	6.52±0.96	4.35±1.51*
Surface homogeneity			
Grainy	2.33±1.60	2.05±1.22	2.42±0.45
Lumpy	1.25±0.97	2.15±0.07	2.99±1.23*
Firmness	1.11±0.03	2.03±0.32	2.14±1.20
Taste			
Overall intensity	6.43±1.61	5.67±0.96	6.11±0.43
Sourness	6.42±1.93	5.92±1.68	5.67±1.15
Sweetness	3.83±1.75	4.92±1.38	4.83±1.27
Bitterness	2.42±1.31	2.17±1.27	2.83±1.64
Mouthfeel	1.11±0.32	1.96±0.67	1.75±1.63
Aroma			
Overall intensity	5.50±1.44	5.42±1.00	5.75±1.48
Preference			
Overall intensity	6.17±1.64	5.75±1.71	6.00±1.21
Day 21			
Overall sensory quality*	1.20±1.32	1.22±1.26	1.11±0.96
Appearance			
Syneresis	1.23±1.64	1.29±1.49	1.88±0.77
Color	6.77±0.03	6.43±1.43	4.13±1.93*
Surface homogeneity			
Grainy	3.50±1.23	4.11±0.44	4.78±1.68
Lumpy	1.31±0.64	1.02±1.22	1.79±1.79
Firmness	1.32±1.52	1.44±0.71	1.65±0.18
Taste			
Overall intensity	5.55±1.16	5.36±0.05	5.48±1.01
Sourness	5.12±0.04	5.66±1.11	5.41±0.02
Sweetness	2.86±1.52	2.79±0.11	3.30±1.63
Bitterness	1.08±0.01	1.14±0.17	1.72±0.09
Mouthfeel	1.42±1.33	1.25±0.32	1.61±0.06
Aroma			
Overall intensity	5.40±1.62	5.45±0.81	6.15±1.10
Preference			
Overall intensity	6.05±1.22	5.78±1.51	6.62±0.69

The evaluation of the intensity of the descriptors was done using a 10-unit scale.
Each value of plain-, *A. sativum*- and *C. verum*- yogurt were a mean of 12 results± standard error. *p < 0.05 as compared to control.
* Overall sensory quality = overall impression of the product.

5.4 DISCUSSION

5.4.1 Crude EPS content

Exopolysaccharides are produced by lactic acid bacteria (LAB) during fermentation and play an important industrial role in the texture development of yogurt (Savadogo *et al*., 2004). However, the presence of EPS can critically influence the development of texture of the final product because of their interaction with the free water in the gel-like structure (Girard and Schaffer-Lequart, 2007; Purwandari *et al*., 2007). The production of EPS by yogurt bacteria is affected by strain and species of cultures used and growth condition (Degeest *et al*., 2002; Mozzi *et al*., 2003; Aslim *et al*., 2005). In the current study, the yield of EPS in the presence of *A. sativum* and *C. verum* water extracts in cow- or camel- milk yogurt was higher than that in the absence. This could be due to the enhanced numbers of EPS producer (*S. thermophilus*) observed in our study (Figures 3.12 and 3.14). The EPS production by yogurt culture is growth-associated where the optimum EPS production was observed during the maximum of bacteria cell production (Degeest *et a*l., 2002; Elin *et al*., 2010). The decrease in EPS of *C. verum*-cow milk yogurt (14 and 21 days) and *A. sativum*- camel milk yogurt (21 days), may be associated with increase presence of enzymes capable of degrading EPS (Degeest *et al*., 2002). The higher ($p<0.05$) EPS observed in treated camel milk yogurt than cow milk yogurt during period of storage was also shown associated with higher ($p<0.05$) *S. thermophilus* in the former than in the latter (Figure 3.14).

5.4.2 Water holding capacity and susceptibility to syneresis

Lee and Lucey, (2010) reported that an increase in total solid content can result in higher water holding capacity with subsequent reduction in syneresis. This was not generally observed in the present study except in *C. verum*- cow milk yogurt.

Increase acidification prevents network rearrangement during whey expulsion, thereby reducing the occurrence of wheying off (Castillo *et al*., 2006). In the present study, the extent of pH reduction in cow milk yogurt was higher than camel milk yogurt during 21 days of storage (Figure 4.3) which associated with higher WHC in the former than in the latter. However, low pH in cow milk yogurt was associated with lower STS only on day 14 and 21 of refrigerated storage. On the other hand, camel milk yogurt showed lower STS during the first week of storage for plain- and *A. sativum*- yogurt and during the entire period of storage for *C. verum*- yogurt. EPS which acts as a hydrocolloid may partially explain the differences in STS in cow- and camel- milk yogurt during storage. In fact the present study showed higher ($p<0.05$) EPS in camel milk- yogurt than in cow milk- yogurt either with or without herbal extracts (Figure 5.2). The instability of gel network causes loss of the ability to entrap all the serum phase that results in whey separation (Lee and Lucey, 2010). Thus, it can be suggested that unstable gel network of camel milk yogurt could be the main factor for lower levels of WHC.

5.4.3 Dynamic rheology

The cross-linking capacity of denatured whey proteins is one of the important factors in the organization of the yogurt structure because it enhances the degree of bridging between protein particles (Remeuf *et al*., 2003). Oscillatory measurements revealed the internal structure of the sample by the elastic and viscous portions of flow. The G′ and G″ are the elastic and viscous contribution to flow and tan (δ) which is equal to G″/G′ reflects the viscoelastic behaviour (Singh and Muthukumarappan, 2008). Amplitude sweep showed the ability of cow milk yogurt (either with or without herbal extracts) to become more fluid with increased the strain to 0.04% throughout the 21 days of refrigerated storage which resulted in the values of G′ and

G″ crossed over each other (Figure 5.7). The dominance of elastic modulus over viscous modulus in *C. verum*- camel milk yogurt on day 21 of storage (Figure 5.8) indicate that *C. verum* improved the consistency of the yogurt. This occurs possibly by decreasing STS in yogurt (Figure 5.6), which is associated with an increase in EPS amounts (Figure 5.2). Subsequently, this increased the colloidal linkage between milk proteins micelles leading to more intense network of yogurt (Pyo and Song, 2009). This supports the fact that the structure of *C. verum*- camel milk yogurt on day 21 of storage can better withstand the breakdown during strain% increase more than *A. sativum*-yogurt. The latter showed fragile structure and failed to withstand breakdown during increase the shear strain% (Figure 5.8). The absence of linear region in elastic modulus in camel milk yogurt (Figure 5.8) compared to in cow milk yogurt (Figure 5.7) could be because of the weakness in the network of camel milk yogurt (Haque *et al*., 2001).

During frequency sweep cow milk yogurt showed elastic behaviour (G′ > G″) whereas camel milk yogurt showed viscous behaviour (G′ < G″) (Figures 5.9 and 5.10). The low values of G′ and G″ in both types of yogurt in the presence of *A. sativum* or *C. verum* water extract as compared to the absence (Figure 5.9) suggest the disruptive effect of these herbal extracts (phenolic compounds bind with milk protein) on the interactions between the protein aggregates. This observation is in agreement with other studies (Alexandropoulou *et al*., 2006; Argyri *et al*., 2006; Cilla *et al*., 2009). The lower G′ values than G″ in camel milk yogurt during 21 days of storage (Figure 5.10) reconfirmed the weakness observed in the network of these yogurts (Figure 5.8). In the present study, it was found that cow milk yogurt had lower tan (δ) values than camel milk yogurt (Figures 5.11 and 5.12 respectively) and this

establishes the solid-like behaviour for cow milk yogurt and liquid-like behaviour for camel milk yogurt.

5.4.5 Apparent viscosity

Both cow- and camel- milk yogurt exhibited shear-thinning behaviour resulted in decrease in the apparent viscosity of yogurt with shear rate increase over 21 days of storage. This occurs because of weekly gel structure of yogurt. The lower viscosity in camel milk yogurt than cow milk yogurt is in agreement with Jumah *et al*. (2001) who suggested that the low amount of casein such as κ-casein and β-lactoglobulin (744.00 and 248.00 mg/l respectively; Al-Alawi and Laleye, 2011) in camel milk as compared to cow milk (3,554.67 and 2,397.33 mg/l respectively; Al-Alawi and Laleye, 2011) is not sufficient to make a complete matrix. Since this two milk proteins play important role in gelation process of yogurt (Xu *et al*., 2008). The improvement of *C. verum*- and *A. sativum*- camel milk yogurt viscosity during storage may be associated with higher EPS production by LAB that might enhance the rearrangement of the protein network.

The viscosity of camel milk yogurt could be enhanced via an increase in total solids of milk by evaporation or addition of milk powder, increasing protein content by ultrafiltration or addition of milk proteins, increasing fat content by addition of full cream milk, increasing calcium content by calcium fortification and addition of stabilizers such as starch/ gelatin etc. (Hashim *et al*., 2009). On the other hand, the requirement of these additions to make camel-milk yogurt having more gel-like properties would increase the cost of yogurt production.

5.4.6 Sensory evaluation

The sensory quality of yogurt is an important criterion for consumers to making the purchasing decision whereas yogurt type influenced consumers' preferences to a low extent (Majchrzak *et al*., 2010). The extents of growth of lactic acid bacteria during milk fermentation are associated with changes in sensory parameters and these are widely used to evaluate the final product of yogurt (Donkor, *et al*., 2007). The differences in acidity during fermentation and refrigerated storage of cow- and camel- milk yogurt upon the inclusion of *A. sativum* or *C. verum* water extract can be regarded crucial in affecting the sensory evaluation (Tables 5.2 and 5.3). The sourness taste character described by both groups of yogurt could be as a result of accumulation of lactic acid, acetic acid, citric acid, butyric acid, acetaldehyde and formic acid produced by yogurt starter culture as metabolic by-products resulting in pH reduction (Novak and Lubiere, 2000; Ostlie *et al*., 2003; Adolfsson *et al*., 2004). For the appearance, the panelists observed that all yogurts made from cow milk showed less syneresis after fermentation and during refrigerated storage (Table 5.2). However, more distinctive syneresis was noted for yogurt made from camel milk both in the presence and absence of herbal extract (Table 5.3) and these were reflected in higher WHC in cow milk yogurt than camel milk yogurt (Figure 5.4). Yogurt appeared to be favoured in white colour and thus the darker colour of the extracts of *C. verum* may be responsible for the lower colour scores of *C. verum*-yogurt than plain-yogurt for both groups. Surface homogeneity (grainy and lumpy) scores were found higher ($p<0.05$) in camel- than in cow- milk yogurt (Table 5.3). Thus the poor texture noted for camel milk yogurt associated with lower ($p<0.05$) firmness and mouthfeel scores than cow milk yogurt. Low aroma score was reported by the panellists for *A. sativum*-cow milk yogurt and this could be due to the odor of diallyl

disulfide (DADS) and allyl methyl sulfide (AMS) which is responsible for the distinctive strong smelling sulfur (Block, 2009). Surprisingly, the presence of *A. sativum* water extract in camel milk yogurt received high aroma score (Table 5.3). Hansanugrum and Barringer (2010) demonstrated that milk proved effective in the deodorization of DADS and AMS because of its fat content. The higher fat content (5 - 6 %) in *A. sativum*- camel milk yogurt than *A. sativum*- cow milk yogurt (3 - 4 %) may thus provide more available fat for DADS and AMS to dissolve resulting in deodorization of DADS and AMS in the former more than in the latter.

5. 5 CONCLUSIONS

The presence of *A. sativum* and *C. verum* water extracts in yogurt made from either cow or camel milk enhanced the yield of EPS as compared to the absence. Changes in fermentation of milk due to *A. sativum* and *C. verum* contribute to higher WHC were observed in treated cow milk yogurt than treated camel milk yogurt. Lower STS was occurred in the presence of *A. sativum* and *C. verum* water extracts in cow- and camel- milk yogurt than respective plain-yogurt. However, both herbs affected yogurt rheology properties by showing weekly gel structure and exhibited shear thinning behaviour. However, prolonged refrigerated storage of *C. verum*- and *A. sativum*-camel milk yogurt enhanced the viscosity of yogurt. The addition of *A. sativum* and *C. verum* did not affect the sensory evaluation of cow- and camel- milk yogurt but camel milk yogurt can reduce the aroma due to *A. sativum*.

6.0 Viability of lactic acid bacteria and *Bifidobacterium bifidum* in *Cinnamomum verum*- and *Allium sativum*- yogurt during refrigerated storage

6.1 INTRODUCTION

Probiotic is a dietary supplement of live microorganism that contributes to the health of the host. It is defined as "live microorganisms which when administered in enough amounts will confer a health benefits on the host" (Guarner *et al.,* 2005). The most common types of microbes that used as probiotic are lactic acid bacteria (LAB).

LAB play a pivotal role in most fermented foods by virtue of its ability to produce various antimicrobial compounds promoting probiotic properties (Temmerman *et al*., 2002) such as antitumoral activity (Hilde *et al*., 2003), reduction of serum cholesterol (Jackson *et al*., 2002), alleviation of lactose intolerance (De Vrese *et al*., 2001), stimulation of the immune system (Isolauri *et al*., 2001), and stabilization of gut microflora (Adolfsson *et al*., 2004). LAB strains also synthesize short chain fatty acids and vitamins which directly increase yogurt's nutritional values. Certain strains of LAB also synthesize exopolysaccharides (EPS) which, when employed in the manufacturing of fermented milk improve its texture and viscosity (Ruas- Madiedo *et al*., 2002).

Commercially produced food biotechnology products may contain either a single probiotic strain or bacterial mixtures of various complexities. Thus, the addition of probiotic increases yogurt's nutritional and therapeutic properties (Güler-Akın and Serdar Akin, 2007). It is highly desirable that the viable number of probiotics in the final product to be at least 10^6–10^7 cfu/g to be accepted as the therapeutic minimum (Madureira *et al*., 2011). Various ways were carried out to enhance the viability of probiotics by the addition of certain ingredients such as cocoa powder and stabilizers which were shown able to increase the survival of probiotic bacteria during passage through gastric tract (Ranadheera *et al*., 2012). In addition, lipid fraction of cocoa butter found to protect *Bifidobacterium longum* from environment stress (Lahtinen *et al*., 2007). Chocolate can also enhance the survival of *Lactobacillus helveticus* and *B.*

longum (91% and 80% respectively) compared to milk (20% and 30%) in low pH environment (Possemiers *et al*., 2010).

In the present study, starter culture containing a mixture of yogurt bacteria and probiotics was used to ferment cow or camel milk. Camel milk contains all essential nutrients and the composition is similar to that of cows' milk (Yagil and Van, 2000). However, camel milk has special properties not found in cow milk with respect to higher antibacterial and antiviral properties (El Agamy, 2000), higher vitamin C content (Wernery *et al*., 2005; Kumar *et al*., 2009) and higher levels of immunoglobulin, lactoferrin, lysozyme, lactoperoxidase and peptidoglycan recognition protein (Agrawal *et al*., 2004; El- Sayed *et al*., 2010). These differences also contribute to camel milk's higher buffering capacity than cow milk (Anonymous, 2003). This may be advantageous to product stability because high buffering capacity of food matrix promote the viability of probiotics during storage (Kailasapathy and Chin, 2000; Mainville *et al*., 2005). Water extracts from certain plants had stimulatory effects on viable yogurt bacteria (Behrad *et al*., 2009), but the effects of *Allium sativum* and *Cinnamomum verum* on yogurt bacterial growth and their metabolism in cow or camel milk yogurt during fermentation and refrigerated storage are not known. Therefore, the objective of the present study was to investigate the effects of these two herbal water extracts on the viability of LAB and *Bifidobacterium bifidum* in cow and camel milk yogurt.

6.2 MATERIALS AND METHODS

6.2.1 Substrates and chemicals

All the substrates and chemicals used in this study are as described in Section 3.2.1.

6.2.2 Plant water extraction procedure

The water extract of plant was performed according to the methods described in Section 3.2.3.

6.2.3 Yogurt manufacturing process

6.2.3.1 Starter culture and yogurt preparation

The starter culture preparation was carried out using the methods described in Section 3.2.4.1. The two groups of bio-yogurt made from cow- or camel-milk both in the presence and absence of *A. sativum*- or *C. verum* were prepared as described in Section 3.2.4.2.

6.2.4 Microbial viable cell counts (VCC) in yogurt

Sample preparation and VCC of *Lactobacillus spp*, *S. thermophilus* and *B. bifidum* were performed as described in Sections 3.2.7.1, 3.2.7.2, 3.2.7.3 and 3.2.7.4 respectively.

6.2.5 Statistical analysis

Statistical analysis of all data obtained was performed as described in Section 3.2.8.

6.3 RESULTS

6.3.1 Viable cell counts (VCC) of LAB and *B. bifidum*

6.3.1.1 VCC in milk before fermentation (BF)

Both cow and camel milk were inoculated with the same amount of bacteria. However, camel milk tended to have greater number of initial VCC than that from cow milk for all the three bacterial species (Table 6.1). The presence of *A. sativum* or *C. verum* water extract did not affect initial VCC in cow milk but appeared to stimulate VCC for *Lactobacillus* spp. and *B. bifidum* in camel milk.

Table 6.1 Viability of LAB and *B. bifidum* in cow and camel milk in the absence or presence of *A. sativum* and *C. verum* water extracts.

Samples	*S. thermophilus* counts ($x10^8$ cfu/ml)	*Lactobacillus* spp. counts ($x10^6$ cfu/ml)	*B. bifidum* counts ($x10^9$/ml)
cow milk	0.62±0.3	1.19±0.1	0.24±0.1
AS + cow milk	0.73±0.1	1.78±0.1	0.87±0.2
CV + cow milk	0.66±0.3	1.65±0.02	0.38±0.1
camel milk	1.14±0.2	3.05±0.4	1.13±0.02
AS + camel milk	1.34±0.2	3.68±0.1*	1.74±0.1*
CV + camel milk	1.31±0.01	3.29±0.2*	1.83±0.1*

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as a mean (n = 3) ± standard error. *$p < 0.05$ as compared to control.

6.3.1.2 VCC in yogurt during storage (4°C)

a) VCC of *Lactobacillus* spp. in yogurt

Lactobacillus spp. VCC for *C. verum*- and *A. sativum*-cow milk yogurt (2.1 x 10^6 cfu/ml and 1.7 x 10^6 cfu/ml respectively) were marginally higher ($p>0.05$) than plain-cow milk yogurt (1.4x 10^6 cfu/ml) on day 0 of storage (Figure 6.1). Refrigerated storage (4°C) for 7 days increased *Lactobacillus* spp. VCC to about 2.3 x 10^6 cfu/ml for cow milk yogurt with significant effects ($p<0.05$) seen in plain- and *A. sativum*- cow milk yogurt. Plain- and *A. sativum*-cow milk yogurt showed gradual reduction in VCC to 1.7 x 10^6 cfu/ml and 1.6 x 10^6 cfu/ml on day 14 of storage. However, *C. verum*-cow milk yogurt showed the same VCC on day 14 and 21 of

storage (1.7 x10^6 cfu/ml). In contrast, the VCC in plain-camel milk yogurt were ~10 fold higher than in plain-cow milk yogurt (Figure 6.2). The presence of *C. verum* or *A. sativum* increased ($p<0.05$) VCC of *Lactobacillus* spp. to 19.2 x10^6 cfu/ml and 26.9 x10^6 cfu/ml respectively compared to plain-camel milk yogurt 13.2 x10^6 cfu/ml (Figure 6.2). VCC of *Lactobacillus* spp. in both plain- and *A. sativum*-camel milk yogurt decreased at faster rates than in *C. verum*-camel milk yogurt from 7 day to 21 day. The lowest VCC was recorded on day 14 for plain-camel milk yogurt (1.3 x10^6 cfu/ml) and on day 21 for *C. verum*- and *A. sativum*-camel milk yogurt (4.3 x10^6 cfu/ml and 1.7 x10^6 cfu/ml respectively).

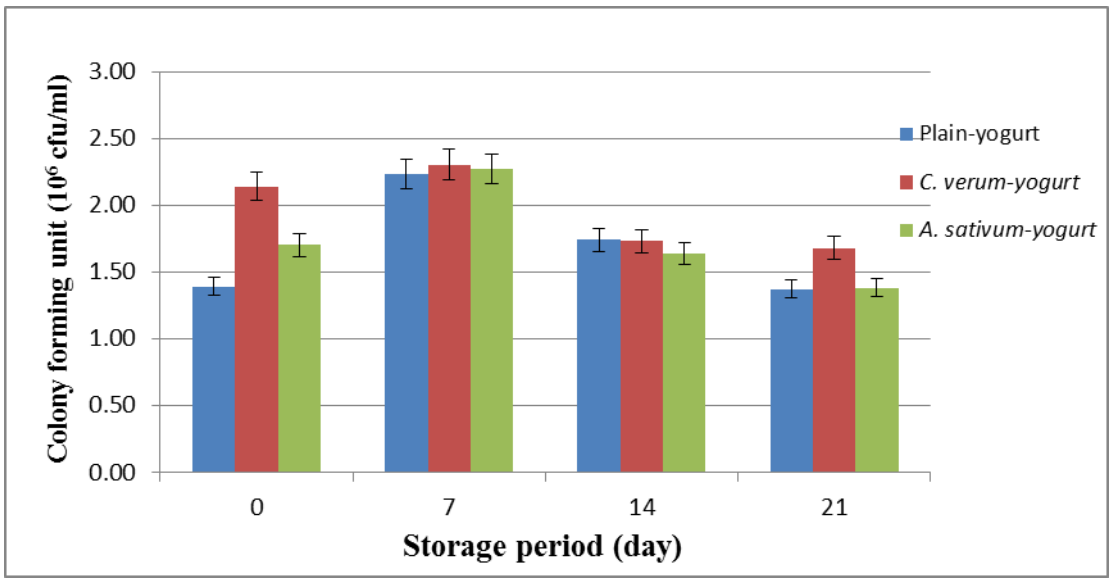


Figure 6.1 Changes in viable cell counts of *Lactobacillus* spp. (x10^6cfu/ml) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at $p = 0.05$ compared to plain-yogurt at the same storage period.

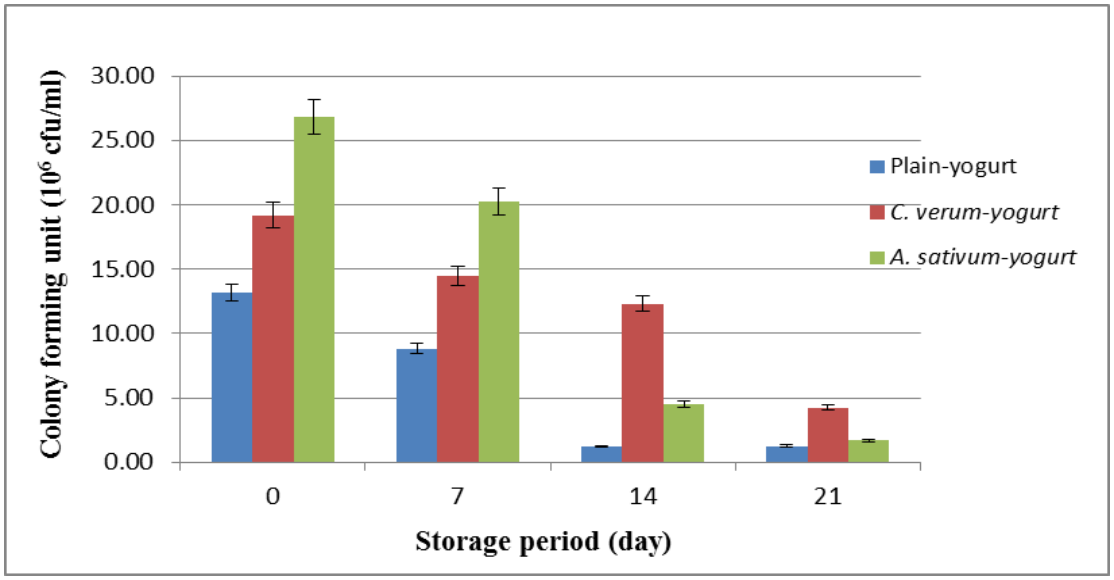


Figure 6.2 Changes in viable cell counts of *Lactobacillus* spp. (x10^6cfu/ml) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

b) VCC of *S. thermophilus* in yogurt

Figures 6.3 and 6.4 show the VCC of *S. thermophilus* in cow- and camel- milk yogurt respectively during refrigerated storage (4°C). Fresh yogurt (0 day) showed no differences in VCC of *S. thermophilus* in both *A. sativum*- (2.6 x 10^8 cfu/ml) and *C. verum*- (2.7 x 10^8 cfu/ml) cow milk yogurt compared to plain-cow milk yogurt (2.4 x 10^8 cfu/ml; Figure 6.3). The VCC increased ($p<0.05$) to 4.3 x 10^8, 4.9 x 10^8 and 5.3 x 10^8 cfu/ml for plain-, *A. sativum*- and *C. verum*- cow milk yogurt respectively by day 14 of storage. These values were followed by a small reduction to 3.7 x 10^8, 4.5 x 10^8 and 4.7 x 10^8 cfu/ml for plain-, *A. sativum*- and *C. verum*- cow milk yogurt respectively by day 21 of storage.

The presence of *A. sativum* or *C. verum* in fresh camel milk yogurt also had no significant effects on *S. thermophilus* VCC compared to fresh plain-camel milk yogurt (Figure 6.4). However, the VCC in camel milk yogurt increased ($p<0.05$) almost 3 folds higher by day 14 of storage (9.5 x 10^8, 11.7 x 10^8 and 9.9 x 10^8 cfu/ml for plain-,

A. sativum- and *C. verum*- camel milk yogurt respectively). Extension of storage to 21 days resulted in a decrease in VCC of S. *thermophilus* to 7.0 x 10^8 cfu/ml for both plain- and *C. verum*- camel milk yogurt but not for *A. sativum*- camel milk yogurt (12.5 x 10^8 cfu/ml; Figure 6.4). The presence of *A. sativum* or *C. verum* in camel milk yogurt showed 2-3 folds higher in VCC of *S. thermophilus* than in cow milk yogurt.

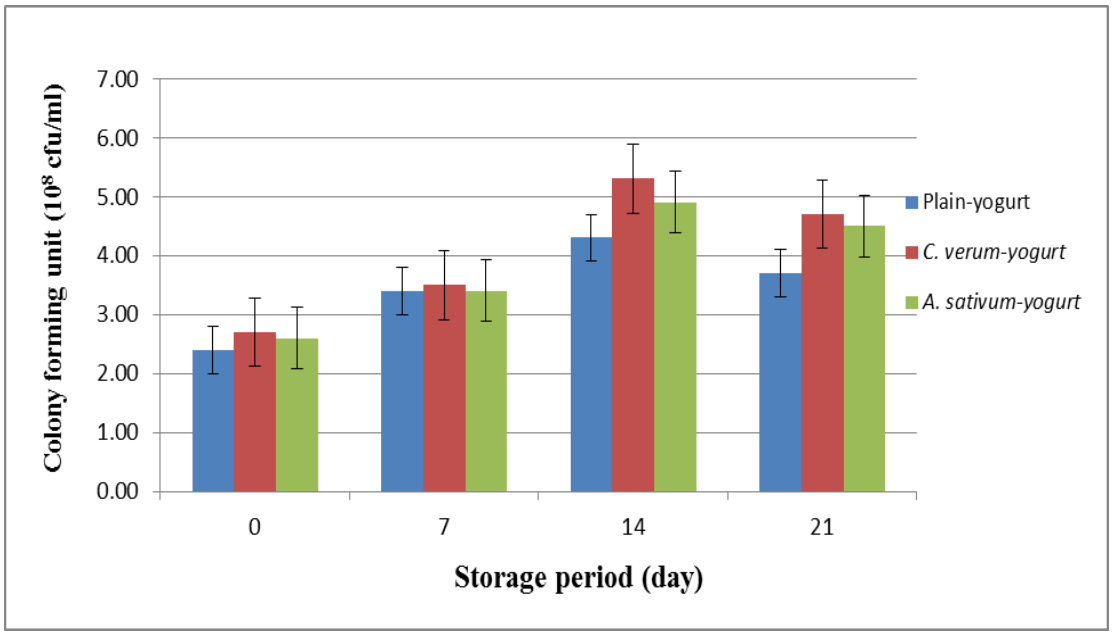


Figure 6.3 Changes in viable cell counts of *Streptococcus thermophilus* (x10^8cfu/ml) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

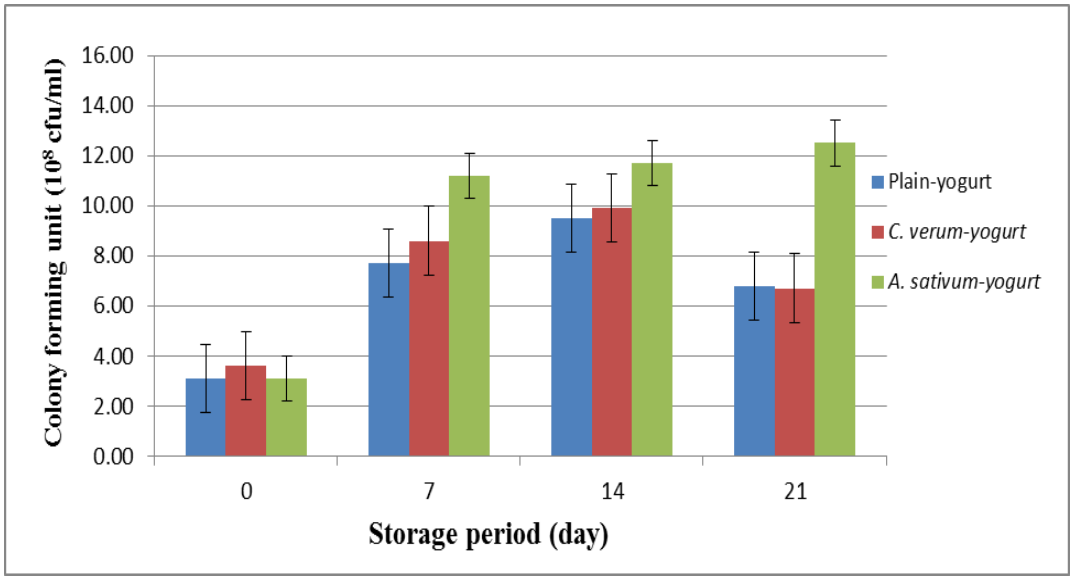


Figure 6.4 Changes in viable cell counts of *Streptococcus thermophilus* (x10^8cfu/ml) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

c) VCC of probiotic (*B. bifidum*) in yogurt

Fresh *A. sativum*- or *C. verum*- yogurt made from cow milk showed higher ($p<0.05$) VCC of *B. bifidum* (8.10 x 10^9cfu/ml and 6.59 x 10^9cfu/ml respectively) than plain-yogurt (1.89 x 10^9cfu/ml; Figure 6.5). Refrigerated storage of plain-yogurt increased ($p<0.05$) *B. bifidum* VCC to the highest counts (2.68 x 10^9cfu/ml) on day 7 followed by significant decrease to 0.26 x 10^9cfu/ml by day 21 of storage. The VCC of *B. bifidum* in *A. sativum*- and *C. verum*- yogurt decreased ($p<0.05$) during refrigerated storage but they were still higher than plain- yogurt even on day 21 of storage (0.48 x 10^9cfu/ml and 1.01 x 10^9cfu/ml respectively; Figure 6.5).

The VCC of *B. bifidum* in fresh plain-yogurt made from camel milk was 1.99 x 10^9cfu/ml (Figure 6.6). The presence of *A. sativum* or *C. verum* in yogurt increased ($p<0.05$) the VCC to 19.61 x 10^9cfu/ml and 25.55 x 10^9cfu/ml respectively. Refrigerated storage of yogurt up to 7 days increased the VCC of *B. bifidum* in plain-yogurt (6.05 x 10^9cfu/ml) followed by reduction ($p<0.05$) to 0.75 x 10^9cfu/ml on day

21 of storage (Figure 6.6). The VCC of *B. bifidum* in both *A. sativum*- and *C. verum*-yogurt decreased ($p<0.05$) to 1.41 x 10^9cfu/ml and 1.11 x 10^9cfu/ml for *A. sativum*- and *C. verum*- yogurt respectively on day 21 of storage.

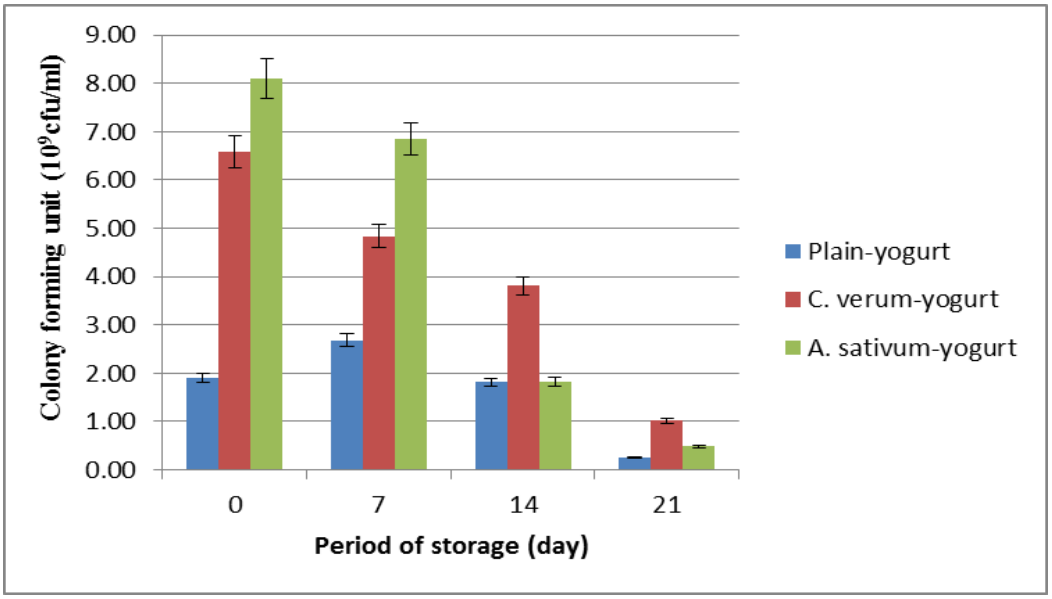


Figure 6.5 Changes in viable cell counts of *Bifidobacterium bifidum* (x10^9cfu/ml) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

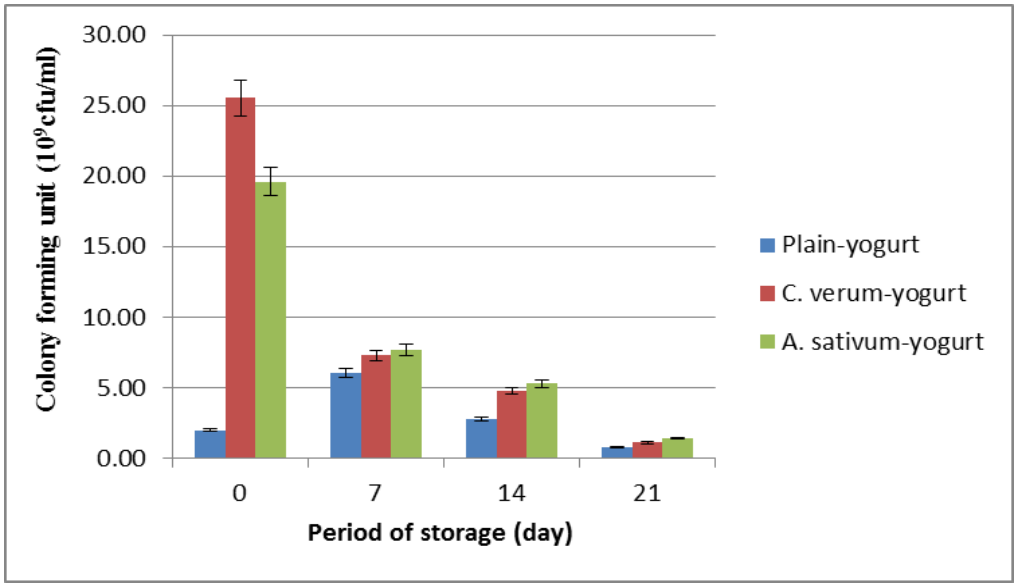


Figure 6.6 Changes in viable cell counts of *Bifidobacterium bifidum* (x10^9cfu/ml) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

6.4 DISCUSSION

6.4.1 Viability of yogurt bacteria

Yogurt containing live cultures confer health benefits to the host when they are consumed in appropriate quantity (Saxelin *et al*., 2003). Thus it is necessary for most of these live cultures to survive during their shelf life prior being consumed. Plant ingredients such as guar gum and cocoa or compound from plant (dextrose) were found to enhance the viability of probiotics in dairy products (Ranadheera *et al*., 2012). Behrad *et al*. (2009) reported that the addition of licorice in yogurt did not affect the LAB population during storage. In the present study, the inclusion of *A. sativum* or *C. verum* water extract in milk or yogurt increased LAB counts compared to controls. This was despite the presence of compounds with antimicrobial activities in both *A. sativum* (e.g. allicin and diallyl thiosulphinic acid, Milner, 2006) and *C. verum* (e.g. cinnamaldehyde, Jayaprakasha *et al*., 2003). LAB is known to thrive in milk even in the presence of antimicrobial constituents such as non-immunological

proteins (lactoperoxidase, lysozyme and xanthine oxidase) that may inhibit their growth (Kei-ichi, 2002). This was possible due to the presence of an exopolysaccharide matrix produced by LAB that may act as barrier to slow down the diffusion of antimicrobial compounds in yogurt (Amrouche *et al.*, 2006). Most lactic acid bacteria require a wide range of growth factors including carbon/nitrogen source, vitamins, minerals, fatty acids, purines, and pyrimidines for their growth and biological activity (Nancib *et al*., 2005). Thus, the presence of *A. sativum* or *C. verum* water extract may have provided some of these nutrients.

The VCC of *Lactobacillus* spp for both types of yogurt reduced during refrigerated storage by day 14 for cow milk-yogurt and day 7 for camel milk-yogurt. This is in agreement with previous study whereby the VCC of *Lactobacillus* spp significantly decreased by the 14th day of refrigerated storage (Haynes and Playne, 2002; Kailasapathy and Sultana, 2003; Laniewska-Trokenheim *et al.*, 2010). Increase concentration of organic acids is one of the important factors that can dramatically affect bacterial growth. Camel milk which has higher buffering capacity (Anonymous, 2003) is able to resist changes in pH during fermentation of milk hence the higher viability of LAB compared to cow milk. The reduction of *Lactobacillus* spp cell counts during storage for both types of yogurt could be associated with the post-acidification which causes a further reduction in pH (Shah, 2000; Eissa *et al.*, 2010). In addition, the increase hydrogen peroxide produced by yogurt bacteria may affect the survival of *Lactobacillus* spp (Talwalkar and Kailasapathy, 2004). The further reduction of *Lactobacillus* spp cell counts in camel milk-yogurt than in cow milk-yogurt observed in our study (Figure 6.2) may not only occur as a result of pH decline but also due to higher antibacterial activities of camel milk than cow milk (Anonymous, 2003). Lysozyme in milk is a protein with lytic activity towards Gram-

positive bacteria. This protein is present in higher concentrations in camel milk (288 μg 100/ ml) than in cow milk (13 μg 100/ ml; Agrawal *et al*., 2005). In addition, lactoperoxidase system in camel milk was shown to be bacteriostatic against the Gram-positive strains (Anonymous, 2003). Despite the faster rate of *Lactobacillus* spp reduction in camel milk yogurt during storage (Figure 6.2). The viable cell counts in camel milk yogurt were still higher than in cow milk yogurt. This result is in agreement to previous report by Singh and Sharma, (2009) who stated that higher *Lactobacillus* number in camel milk than in cow milk. Moreover, the higher VCC of *Lactobacillus* spp may reflect the higher free amino acids present in camel milk than in cow milk (Merin *et al*., 2001; Al-Alawi and Laleye, 2011). In addition, the higher milk protein proteolysis by *L. delbrueckii* spp *bulgaricus* in camel milk than in cow milk (Abu-Tarboush, 1996) should provide more digestible proteins which readily support yogurt bacteria growth during fermentation (El-Zahar *et al*., 2003) and storage (Donkor *et al*., 2006). Therefore, despite the higher 'mortality' of *Lactobacillus* spp in camel milk yogurt during refrigerated storage this should not affect its functional values because the VCC of these bacteria on the 3rd week of storage were still much higher than those in cow milk yogurt which was the highest in the 2nd week of storage.

The presence of *A. sativum* or *C. verum* water extract did not affect the growth of *S. themophilus* during fermentation of milk as demonstrated by similar VCC in fresh cow- and camel- milk yogurt. This could be due to the nature of *S. themophilus* which is acid sensitive (Marteau *et al.*, 1997). The increase in the VCC of *S. thermophilus* in both types of yogurt during 14 days refrigerated storage is in agreement to previous studies (Birollo *et al.*, 2000; Donkor *et al*., 2006). LAB is known to have high demand for peptides and amino acids as essential growth factors

(Donkor *et al*., 2006). Proteinases and peptidases constitute the primary enzymes in LAB responsible for milk proteins proteolysis as a source of amino acids and nitrogen (Shihata and Shah, 2000). The reduction in *S. thermophilus* by day 21 of storage may be attributed to the accumulation of organic acids (Ostlie *et al.*, 2003) and waste products produced by bacterial activity such as hydrogen peroxide (Shah, 2000) known to affect VCC. The sustained survival of *S. thermophilus* in *A. sativum*-camel milk-yogurt during 21 day of storage (Figure 6.4) was found to be associated with higher proteolytic activity in yogurt (Figure 3.11).

6.4.2 Viability of probiotic (*B. bifidum*) in yogurt

Food containing probiotic are claimed to provide several health benefits such as the improvement in lactose utilisation in lactose malapsorbtion (De Vrese *et al*., 2001), prevention of cancer (Isolauri *et al*., 2001), maintenance of intestinal microflora balance (Analie and Bennie, 2001; Hasler, 2002) and reduction in the level of serum cholesterol (Jackson *et al*., 2002). Moreover, yogurt containing *B. bifidum* Bb-12 can enhance the immunoglobulin A (IgA) production in the intestine and thus enhance the immune system especially in infants (Fukushima *et al*., 1998). It also has inhibitory effects on commonly known food-borne pathogens (Schiffrin and Blum, 2001; Rafter, 2003; Goderska and Czarnecki, 2007) and ability to control intestinal infections by producing inhibitory/antimicrobial substances such as organic acids, hydrogen peroxide, deconjugated bile acids, antibiotics and bacteriocins (Rafter, 2003). The survival of probiotic microflora in yogurt is governed by physicochemical factors such as yogurt acidity, dissolved oxygen, species interaction, inoculation practice and storage conditions (Rybka and Kailasapathy, 1995). Our results showed that the presence of *A. sativum* or *C. verum* water extract in both cow and camel milk yogurt increased ($p<0.05$) the VCC of *B. bifidum* compared to respective plain-yogurt

during 21 days of storage. This could be related to the essential growth factors present in *A. sativum* or *C. verum* such as vitamins, minerals and amino acids (Abdullah *et al*., 1988; Augusti and Sheela, 1996; Al-Numair *et al*., 2007). The presence of higher free amino groups in herbal yogurt made from camel than cow milk (Figure 3.11) may explain the higher VCC of *B. bifidum* in the former than in the latter. Furthermore, the higher buffering capacity in camel milk than cow milk (Anonymous, 2003) may help to stabilize the pH in yogurt (4.5-4; Figure 4.4) thus allowing more *B. bifidum* growth prior to the development of inhibitory acidic environment.

The present study showed significant reduction in *B. bifidum* VCC of *A. sativum* and *C. verum* yogurt made from either cow or camel milk during refrigerated storage. This observation was in agreement with Vinderola *et al*. (2000) whereby the reduction of VCC of *B. bifidum* was shown to be yogurt type and the starter culture used dependent. Thus, the faster reduction ($p<0.05$) of these bacteria in herbal- camel milk- than cow milk- yogurt after 7 days of storage would suggest that specific milk composition in the former may be responsible for the reduction. In particular, the antimicrobial compounds are present in higher concentrations in camel milk than other ruminants milk (Agrawal *et al*., 2005; Anonymous, 2003).

It is important to ensure sufficiently high VCC of products containing probiotics. This is to account for the large proportion of cell mortality during transition from the point of food ingestion to arrival in the colon. Since the acceptable minimum therapeutic number of probiotic VCC in the food by the time colon is reached range between 10^6– 10^7 cfu/g, a much higher probiotic VCC (10^8–10^9 cfu/g) should be present in food immediately before ingestion (Madureira *et al*., 2011). Thus, the present results demonstrated that *A. sativum*- and *C. verum*- yogurt made from either cow or camel milk provided higher viable *B. bifidum* over two weeks of

storage. Therefore, these types of yogurt may be considered as probiotic yogurt with promising therapeutic properties upon daily consumption.

6.5 CONCLUSIONS

A. sativum and *C. verum* stimulated *Lactobacillus* spp growth more in camel-milk yogurt than in cow milk yogurt during fermentation. Despite *Lactobacillus* spp could not sustain survival in camel milk yogurt during refrigerated storage, however the VCC of these bacteria were still higher than that in cow milk yogurt. The presence of these two herbal extracts in both types of yogurt did not affect the viability of *S. thermophilus* during 21 days of storage except in *A. sativum*- camel milk yogurt. The growth of *B. bifidum* was improved in the presence of *A. sativum* or *C. verum* water extract in both cow- and camel- milk yogurt during fermentation and they continued to survive even during refrigerated storage. Thus, *A. sativum* and *C. verum* may be used to support the survival of LAB in yogurt during refrigerated storage.

7.0 The effect of *Cinnamomum verum* or *Allium sativum* on the survival of LAB and *Bifidobacterium bifidum* after simulated gastrointestinal digestion of yogurt

7.1 INTRODUCTION

Probiotics are live microorganisms that when present in sufficient amounts in the digestive tract may confer health benefits on the host (Lourens-Hattingh and Viljoen, 2001). Combination of lactic acid starter bacteria with probiotic (*Bifidobacterium*, *Lactobacillus*) is widely used in yogurt manufacture (Vinderola *et al*., 2000, Vinderola *et al*., 2011). Lactic acid starter bacteria are acid sensitive (Marteau *et al.*, 1997) and are unable to resist the bile salts. Thus these bacteria show very poor ability to survive through the passage of the stomach and the intestinal tract. For this reason lactic acid starter bacteria were not originally considered to be probiotics (Analie and Bennie, 2001). A recent review proposed that yogurt containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* (Guarner *et al*., 2005) could be regarded as members of the probiotic because both bacteria provide health benefits to the host (Guarner *et al*., 2005). These bacteria are able to release β-galactosidase enzymes that improve the digestion of nutrients in the intestine and modulate immune responses for human health (Lee *et al*., 2001). To be effective as probiotics, the viable cell counts (VCC) of the final yogurt product should be in the range 10^8–10^9 cfu/g in food right before ingestion so as to ensure sufficient therapeutic minimum of 10^6– 10^7 cfu/g could reach the colon. This equates to daily consumption of about 100-200g of yogurt.

Several novel approaches have been considered to increase the survival of bacteria as they pass through the acidic gastric and alkaline intestinal tract environment (Kailasapathy and Chin, 2000; Huang and Adams, 2004). These include dairy products such as yogurt (Wattiaux and Howard, 2000; Brinques and Ayub, 2011; Ranadheera *et al*., 2012; Opatha Vithana *et al*., 2012), cheese (Madureira *et al*., 2011) and ice cream (Ranadheera *et al*., 2012). The ability of probiotic bacteria to

survive through the gastrointestinal tract varies according to species and even strain-dependent (Wattiaux and Howard, 2000). In addition, functional properties of this probiotic can be affected by the food matrix used in delivery (Lahtinen *et al*., 2007; Ranadheera *et al*., 2010) because the buffering capacity of food would help to enhance the viability of probiotics during gastric transit (Kailasapathy and Chin, 2000; Mainville *et al*., 2005). The objective of this study was to evaluate the influence of *A. sativum* or *C. verum* in yogurt on the survival of LAB and *Bifidobacterium bifidum in vitro* gastrointestinal digestion. Three types of milk (cow, camel and goat milk) were used to further establish the differences in benefits of the addition of these herbs on microbial survival.

7.2 MATERIALS AND METHODS

7.2.1 Substrates and chemicals

Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$), hydrogen chloride (HCl), lysozyme, pepsin, bile salts and pancreatin were purchased from Sigma Chemical Company (St Louis, MO USA). Other substrates and chemicals used in this study are as described in Section 3.2.1.

7.2.2 Experimental designs

This study examined the viability of bio-yogurt starter culture (LAB and probiotic) after subjecting yogurt to simulated gastrointestinal digestion. Yogurt was either fresh (0 day) or stored (7 days) and each was either made with or without *A. sativum* or *C. verum* extract. In addition, the mixture of milk and *A. sativum* or *C. verum* water extract before fermentation (BF) was also prepared. Yogurt was prepared using cow, camel and goat milks. Samples of different types of yogurt after stomach (1 hour) and intestinal (1 and 2 hours) digestion were diluted and subsequently plated

onto agar in petri dishes to evaluate viable cell counts (VCC) of *Lactobacillus* spp, *S. thermophilus* and *B. bifidum*.

7.2.3 Plant water extraction procedure

The water extraction of *A. sativum* or *C. verum* was carried out according to the method described in Section 3.2.3.

7.2.4 Yogurt manufacturing process

7.2.4.1 Starter culture and yogurt preparation

The starter culture was prepared using the method described in Section 3.2.4.1. Bio-yogurt was made using cow, camel and goat milk in the presence and absence of *A. sativum*- or *C. verum* as described in Section 3.2.4.2.

7.2.5 *In vitro* gastrointestinal model

7.2.5.1 Preparation of gastric and duodenum juices

The gastric and duodenum solutions were freshly prepared according to the protocols described by Marteau *et al*., (1997) and Huang and Adams, (2004). To simulate the *in vivo* saliva, 100 ml of a sterile electrolyte solution (6.2 g/l NaCl, 2.2 g/l KCl, 0.22 g/l $CaCl_2$, 1.2 g/l $NaHCO_3$) were added to lysozyme (10 mg) to obtain a final concentration of 100ppm. To simulate the stomach environment (gastric juice), the electrolyte solution was added to 0.3% pepsin and the pH was adjusted to 3 by adding 5M HCl. To simulate the intestinal digestion (duodenum juice), the electrolyte solution (6.4g/l $NaHCO_3$, 0.239g/l KCl, 1.28g/l NaCl) containing 0.3% bile salts and 0.1% pancreatin (v/w concentrations) was adjusted to pH 7.2 by using 5M NaOH.

7.2.5.2 Simulation of gastrointestinal digestion (SGD)

Yogurt samples were mixed with the artificial saliva solution in the ratio of 1:1 followed by incubation at 37^{o}C for 5 minutes. The samples were then mixed with artificial gastric fluid solution in the ratio of 3:5 prior to a second incubation at 37^{o}C for 1 hour. After 1 hour, 30ml of samples from the "stomach digestion" were taken out for analysis. The remaining solutions from "stomach digestion" were then mixed with artificial duodenal secretion in the ratio of 1:4 followed by a third incubation at 37^{o}C for 2 hours. Samples (30ml) were taken out for analysis after each one hour of "intestinal digestion" (Figure 7.1).

The yogurt samples were agitated and stirred intermittently during the incubation time in order to ensure adequate enzymes-digestion using to mimic gastrointestinal digestion.

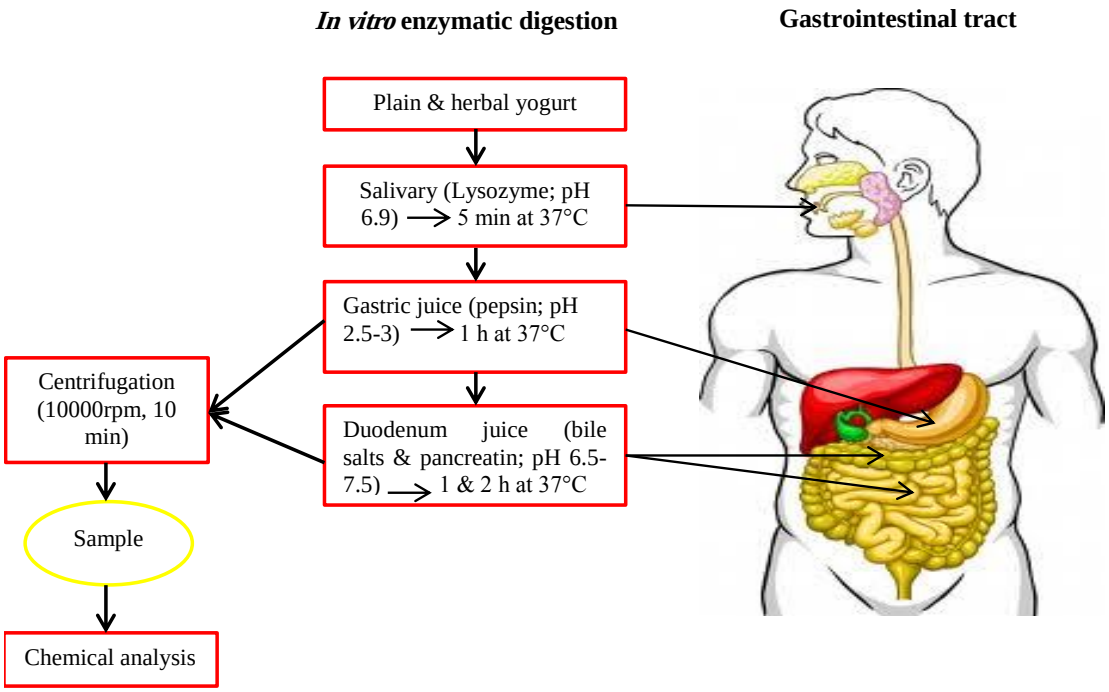


Figure 7.1 The sites of simulated gastrointestinal tract by *in vitro* enzymatic digestion.

7.2.6 Microbial viable cell counts (VCC) in yogurt

Sample preparation and enumeration of *Lactobacillus spp*, *S. thermophilus* and *B. bifidum* were performed as described in Sections 3.2.7.1, 3.2.7.2, 3.2.7.3 and 3.2.7.4 respectively.

7.2.7 Statistical analysis

Statistical analyses of all data obtained were performed as described in Section 3.2.8.

7.3 RESULTS

7.3.1 VCC of *Lactobacillus* spp. after SGD

A) VCC in cow milk and cow milk- yogurt

The VCC of *Lactobacillus* spp. in cow milk after the 1st hour of gastric digestion were 1.85 x 10^6cfu/ml (Figure 7.2) which were not affected by either the presence of *C. verum* or *A. sativum* water extract in yogurt (1.34 x 10^6cfu/ml and 1.83 x 10^6cfu/ml respectively). Intestinal digestion decreased VCC for *Lactobacillus* spp. in *A. sativum* + milk but not for *C. verum* + milk. Extended digestion to another hour in intestinal tract resulted in decrease ($p<0.05$) in *Lactobacillus* spp. VCC of milk + *C. verum* water extract to 1.74 x 10^6cfu/ml whereas ~ 0.1 survival was seen in plain- and *A. sativum* + milk. The VCC of *Lactobacillus* spp. post- gastric digestion of fresh yogurt was the highest in *C. verum*- yogurt (10.56 x 10^6cfu/ml) followed by *A. sativum*- (2.53 x 10^6cfu/ml) and plain- (2.47 x 10^6cfu/ml) yogurt respectively (Figure 7.2). The VCC of *Lactobacillus* spp. were higher ($p<0.05$) after 1 hour of intestinal digestion for plain- (39.35 x 10^6cfu/ml) and *C. verum*- (32.08 x 10^6cfu/ml) yogurt but not for *A. sativum*-yogurt (1.69 x 10^6cfu/ml). Prolonged digestion (2 hours) in intestine for plain- and herbal- yogurt resulted in reduction in *Lactobacillus* spp. VCC ($p<0.05$; Figure 7.2). Refrigerated (7 days) plain- and *A. sativum*- yogurt showed similar VCC of *Lactobacillus* spp. after 1 hour of gastric digestion (2 x 10^6cfu/ml).

However, intestinal digestion resulted in diminished VCC of *Lactobacillus* spp in plain- and *A. sativum*- yogurt. *C. verum*- yogurt showed the highest VCC of *Lactobacillus* spp. (9.25 x 10^6cfu/ml) after 1 hour gastric digestion. However, intestinal digestion reduced VCC to 7.37 x 10^6cfu/ml and 1.66 x 10^6cfu/ml after 1 and 2 hours respectively.

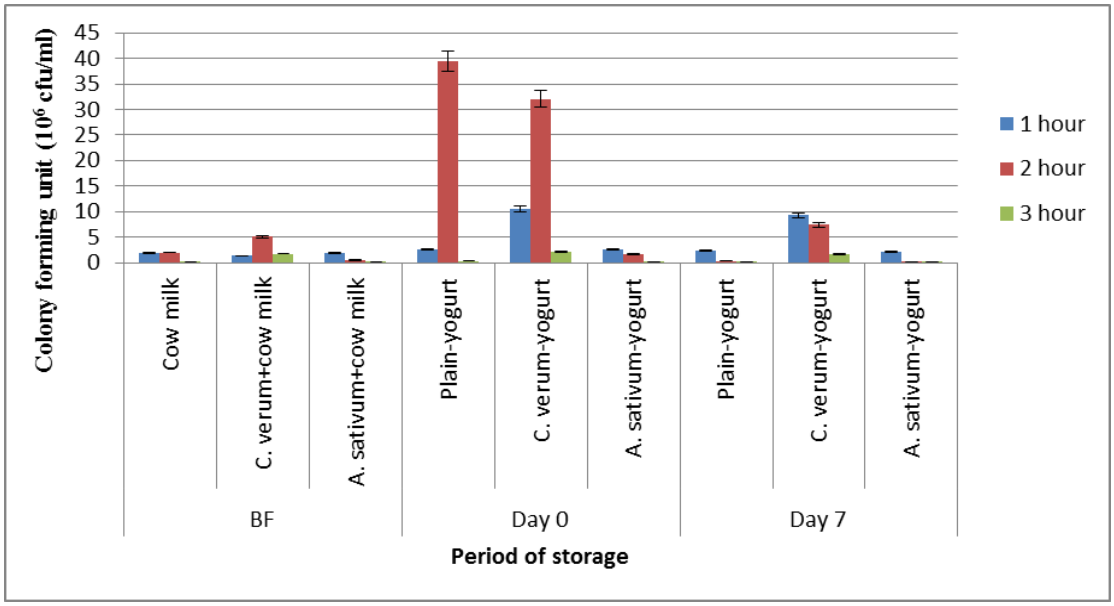


Figure 7.2 VCC of *Lactobacillus* spp. (x10^6 cfu/ml) in cow milk before and after fermentation (BF and 0 day respectively) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars present a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

B) VCC in camel milk and camel milk- yogurt

The VCC of *Lactobacillus* spp. in camel milk after 1 hour gastric digestion was higher in the presence of *A. sativum* and *C. verum* water extracts (2.92 x 10^6cfu/ml and 12.4 x 10^6cfu/ml ($p<0.05$) respectively) than camel milk alone (1.06 x 10^6cfu/ml; Figure 7.3). There was no difference in VCC of *Lactobacillus* spp. in all treatments after 1 and 2 hours intestinal digestion.

The VCC of *Lactobacillus* spp. in fresh plain- and *C. verum*-yogurt after 3 hours of *in vitro* gastrointestinal digestion was 24.07 x 10^6cfu/ml and 59.01 x 10^6cfu/ml respectively (Figure 7.3). However, VCC for *A. sativum*-yogurt decreased from 19.49 x 10^6cfu/ml to 8.8 x 10^6cfu/ml during 3 hours *in vitro* gastrointestinal digestion. Refrigerated (7 day) plain-yogurt showed the highest VCC (79.8 x 10^6cfu/ml) of *Lactobacillus* spp. after 1 hour gastric digestion but this value decreased to 18.3 x 10^6cfu/ml by the 2nd hour intestinal digestion. *A. sativum*- and *C. verum*-yogurt had similar VCC of *Lactobacillus* spp. after gastric- and 1 hour intestinal-digestion. However, the VCC reduced more for the former (1.1 x 10^6cfu/ml) than the latter (13.9 x 10^6cfu/ml) after the 2nd hour intestinal digestion (Figure 7.3).

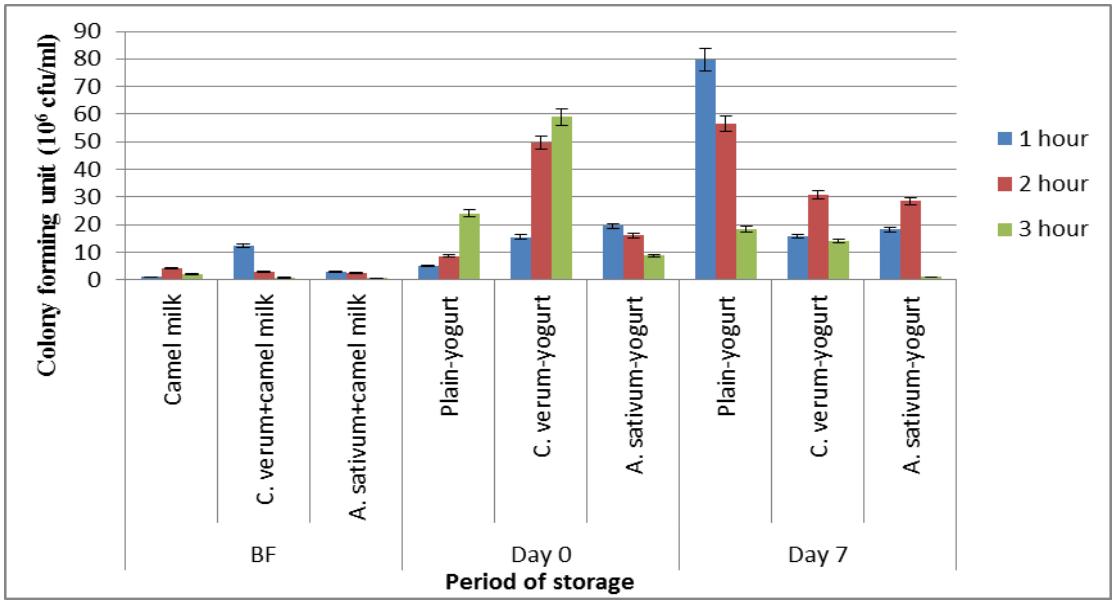


Figure 7.3 VCC of *Lactobacillus* spp. (x10^6 cfu/ml) in camel milk before and after fermentation (BF and 0 day respectively) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars present a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) VCC in goat milk and goat milk- yogurt

The VCC of *Lactobacillus* spp. in goat milk was higher (6.87 x 10^6cfu/ml; $p<0.05$) than goat milk in the presence of *A. sativum* (1.32 x 10^6cfu/ml) and *C. verum* (1.12 x 10^6cfu/ml; Figure 7.4) after 1 hour gastric digestion. Low survival of *Lactobacillus* spp. was seen in all treatments after intestinal digestion.

The VCC of *Lactobacillus* spp. in fresh goat milk yogurt after 1 hour gastric digestion was 25.85 x 10^6cfu/ml (Figure 7.4). The VCC increased ($p<0.05$) to 48.17 x 10^6cfu/ml and 42.69 x 10^6cfu/ml for fresh *A. sativum*- and *C. verum*- yogurt respectively. However, the VCC of *Lactobacillus* spp. decreased ($p<0.05$) to 6.33 x 10^6cfu/ml, 27.8 x 10^6cfu/ml and 16.93 x 10^6cfu/ml for plain-, *A. sativum*- and *C. verum*-yogurt respectively after two hours of intestinal digestion. Refrigerated storage of *A. sativum*- and *C. verum*-yogurt for 1 week showed lower VCC of *Lactobacillus* spp. (3.17 x 10^6cfu/ml and 5.55 x 10^6cfu/ml respectively) than plain-yogurt (12.43 x 10^6cfu/ml) after the 1^{st} hour of gastric digestion (Figure 7.4). VCC of *Lactobacillus* spp. increased ($p<0.05$) in 7 days old *A. sativum*- and *C. verum*-yogurt (20.39 x 10^6cfu/ml and 31.44 x 10^6cfu/ml respectively) after 1 hour intestinal digestion compared to control (12.52 x 10^6cfu/ml). Prolonged digestion to another 1 hour reduced ($p<0.05$) the VCC of *Lactobacillus* spp. in both *A. sativum*- and *C. verum*-yogurt.

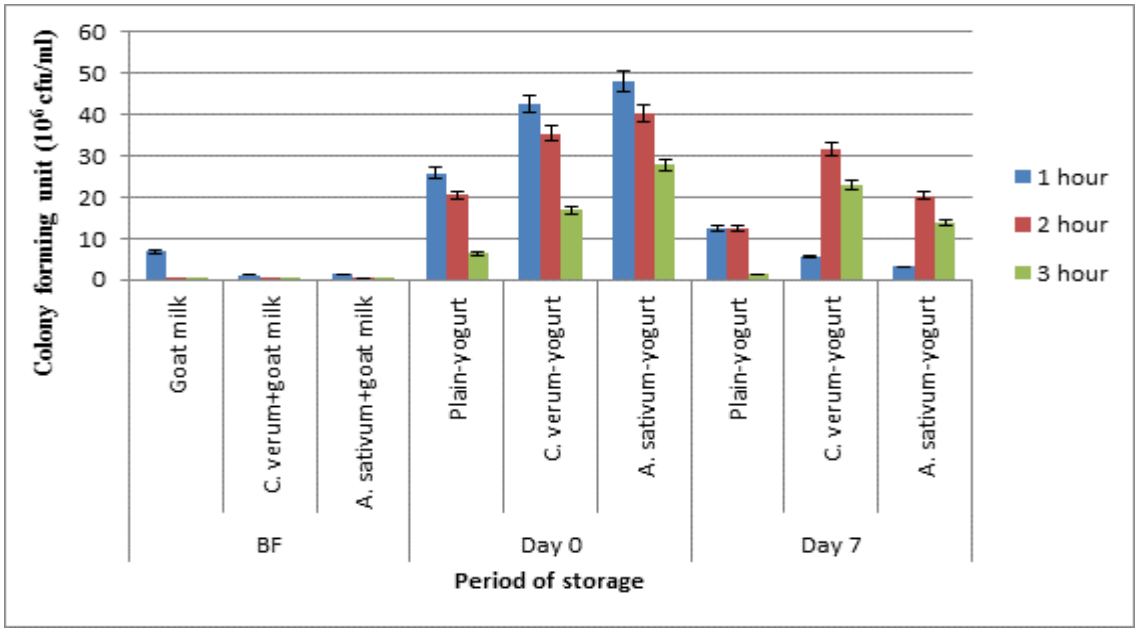


Figure 7.4 VCC of *Lactobacillus* spp. (x10^6 cfu/ml) in goat milk before and after fermentation (BF and 0 day respectively) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars present a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

7.3.2 VCC of *S. thermophilus* after SGD

A) VCC in cow milk and cow milk- yogurt

The VCC of *S. thermophilus* in cow milk was 1.97 x 10^6cfu/ml after the 1 hour gastric digestion (Figure 7.5). The VCC were higher in the presence of *A. sativum* or *C. verum* (2.05 x 10^6cfu/ml and 2.79 x 10^6cfu/ml respectively) than plain cow milk after gastric digestion. The VCC of *S. thermophilus* in all treatments showed significant reduction after 2 hours intestinal digestion. The VCC of *S. thermophilus* in fresh yogurt after 1 hour gastric digestion was similar for all treatments (8 x 10^6cfu/ml). The VCC after 1 hour intestinal digestion was the highest in *C. verum*- yogurt (12.1 x 10^6cfu/ml) followed by *A. sativum*- (6.8 x 10^6cfu/ml) and plain- (2.7 x 10^6cfu/ml) yogurt. *C. verum*- yogurt contained the highest VCC after 2 hours intestinal digestion (Figure 7.5). Similar VCC of *S. thermophilus* were shown in

refrigerated (7 days) plain- and *C. verum*- yogurt (~7 x 10^6cfu/ml) after 1 hour gastric digestion whereas *A. sativum*- yogurt showed lower VCC (4.1 x 10^6cfu/ml; $p<0.05$) than plain-yogurt. The survival of *S. thermophilus* in both herbal- yogurt reduced to ~ 1 x 10^6cfu/ml after 2 hours intestinal digestion whereas it increased to 14.5 x 10^6cfu/ml in plain-yogurt.

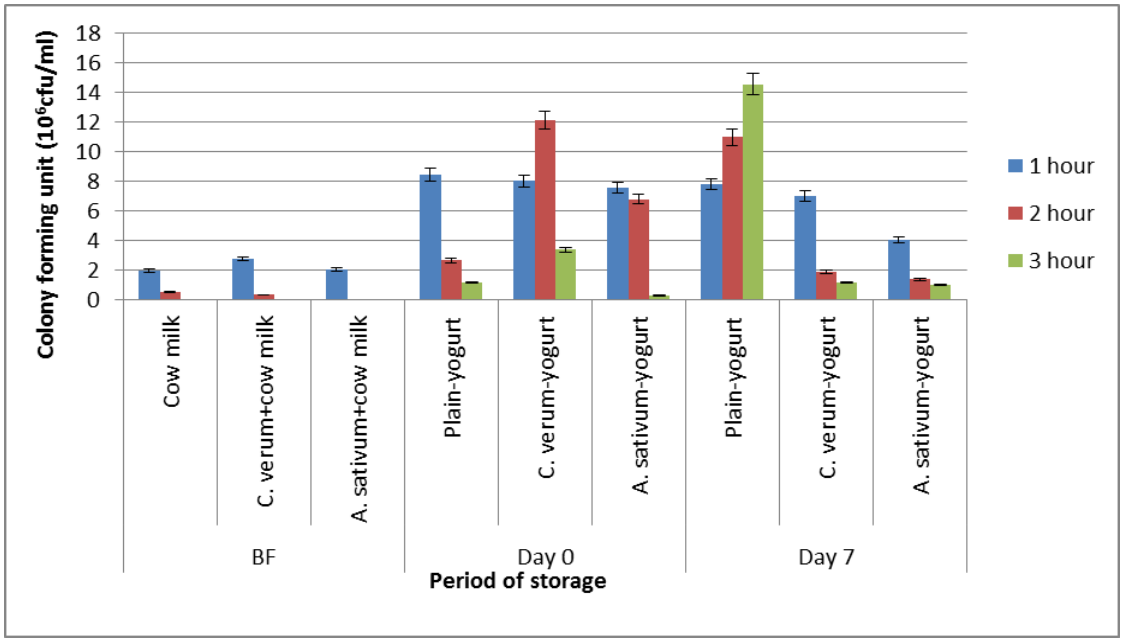


Figure 7.5 VCC of *S. thermophilus* (x10^6 cfu/ml) in cow milk before and after fermentation (BF and 0 day respectively) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars present a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

B) VCC in camel milk and camel milk- yogurt

The VCC of *S. thermophilus* in camel milk both in the presence and absence of herbal extracts was <1 x 10^6cfu/ml during 3 hours of gastrointestinal digestion (Figure 7.6). The VCC of *S. thermophilus* in fresh *A. sativum*- and *C. verum*- yogurt after 1 hour gastric digestion was higher (2.13 x 10^6cfu/ml and 2.21 x 10^6cfu/ml respectively; $p<0.05$) than plain-yogurt (0.78 x 10^6cfu/ml). The VCC of *S. thermophilus* increased in plain- and *C. verum*-yogurt after 2 hours intestinal digestion

to 2.78 x 10^6cfu/ml and 3.42 x 10^6cfu/ml respectively whereas decreased in *A. sativum*-yogurt to 0.02 x 10^6cfu/ml. Refrigerated storage of yogurt (7 day) showed higher ($p<0.05$) VCC of *S. thermophilus* in plain-yogurt (6.97 x 10^6cfu/ml) than in *A. sativum*- and *C. verum*-yogurt (2.24 x 10^6cfu/ml and 3.59 x 10^6cfu/ml respectively) after 1 hour gastric digestion (Figure 7.6). Intestinal digestion of plain-, *A. sativum*- and *C. verum*- yogurt decreased ($p<0.05$) *S. thermophilus* VCC to 1.2 x 10^6cfu/ml, 2.0 x 10^6cfu/ml and 2.7 x 10^6cfu/ml respectively after the 1st hour. Prolonged intestinal digestion to another hour resulted in lower VCC of *S. thermophilus* in *A. sativum*- and *C. verum*- yogurt (0.08 x 10^6cfu/ml and 0.32 x 10^6cfu/ml respectively; $p<0.05$) than plain- yogurt (2.30 x 10^6cfu/ml; Figure 7.6).

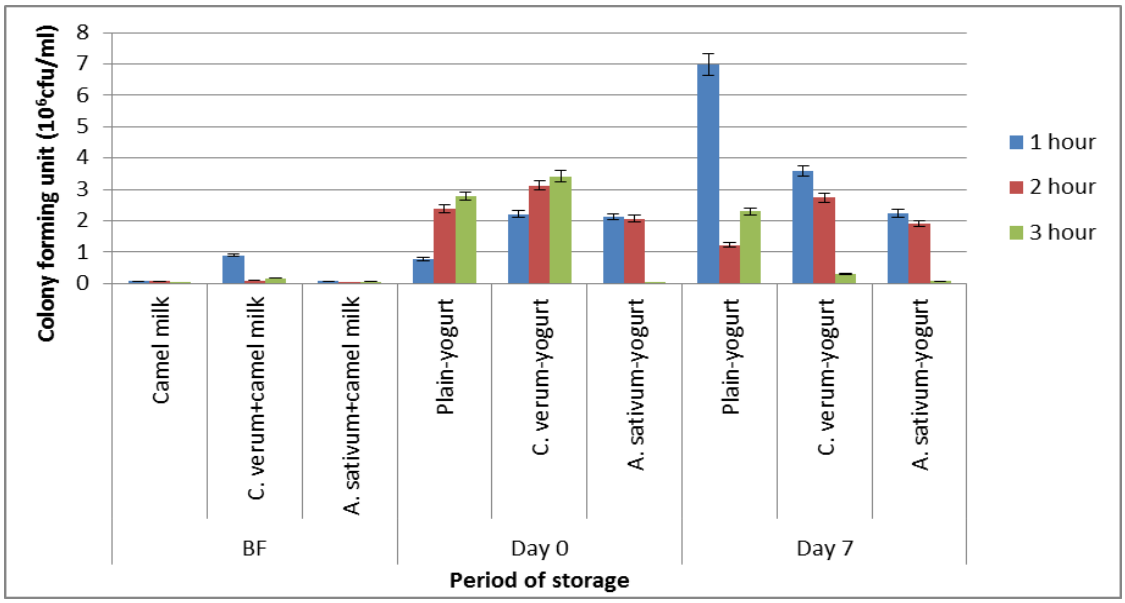


Figure 7.6 VCC of *S. thermophilus* (x10^6 cfu/ml) in camel milk before and after fermentation (BF and 0 day respectively) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars present a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) VCC in goat milk and goat milk- yogurt

There were higher VCC of *S. thermophilus* in *A. sativum* or *C. verum* + goat milk (1.20 x 10^6cfu/ml and 2.44 x 10^6cfu/ml respectively) than plain milk (0.25 x 10^6cfu/ml; Figure 7.7) after 1 hour gastric digestion. Similar VCC of *S. thermophilus* was shown after 2 hours intestinal digestion in goat milk in the presence of *A. sativum* or *C. verum* water extract (~2 x 10^6cfu/ml).

The VCC of *S. thermophilus* in fresh plain-yogurt (8.79 x 10^6cfu/ml) was the highest after 1 hour gastric digestion, but this value decreased to 5.67 x 10^6cfu/ml and 4.96 x 10^6cfu/ml after 1 and 2 hours intestinal digestion respectively. Fresh *A. sativum*-yogurt had the highest VCC of *S. thermophilus* (7.27 x 10^6cfu/ml) after 3 hours SGD whereas *C. verum*- yogurt had the lowest VCC (2.11 x 10^6cfu/ml; Figure 7.7). Seven days old *C. verum*-yogurt had the highest *S. thermophilus* VCC (1.98 x 10^6cfu/ml) after 3 hours SGD compared to plain- and *A. sativum*- yogurt (0.10 x 10^6cfu/ml and 0.44 x 10^6cfu/ml respectively).

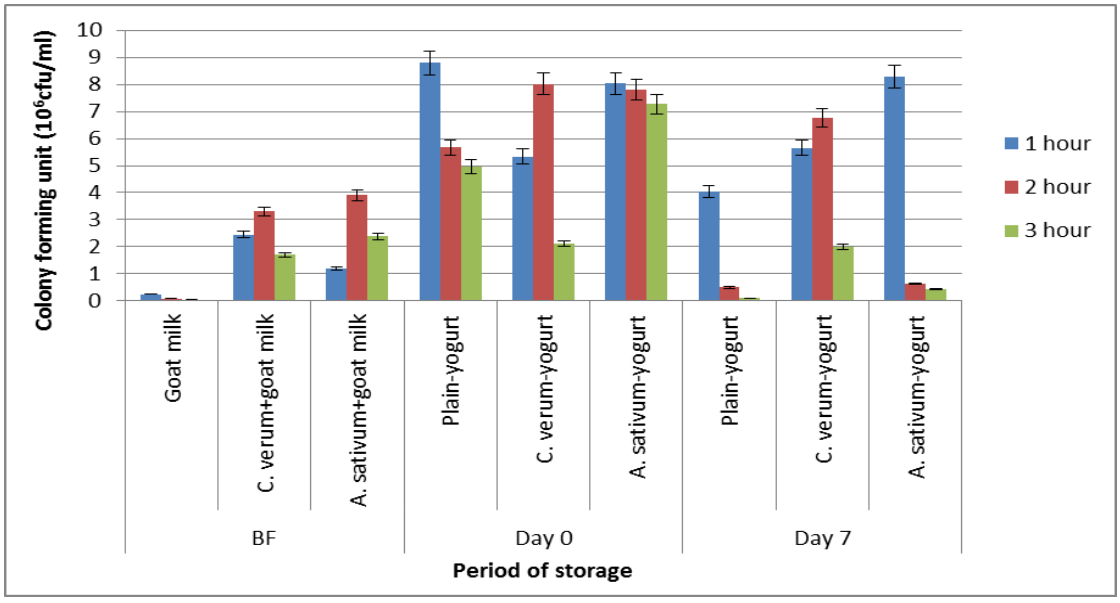


Figure 7.7 VCC of *S. thermophilus* (x10^6 cfu/ml) in goat milk before and after fermentation (BF and 0 day respectively) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars present a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

7.3.3 VCC of *B. bifidum* after SGD

A) VCC in cow milk and cow milk- yogurt

The VCC of *B. bifidum* in cow milk both in the presence and absence of *A. sativum* or *C. verum* water extract were less than 1 x 10^9cfu/ml during 3 hours SGD (Figure 7.8). Similar VCC was shown in all fresh types of yogurt (~ 1.3 x 10^9cfu/ml) after 1 hour gastric digestion. Intestinal digestion (1 hour) increased the VCC of *B. bifidum* to the highest value in plain-yogurt (33.4 x 10^9cfu/ml) followed by *C. verum*- (30.4 x 10^9cfu/ml) and *A. sativum*- (6.6 x 10^9cfu/ml) yogurt. Prolonged digestion for another 1 hour reduced ($p<0.05$) the VCC of *B. bifidum* to 2.4 x 10^9cfu/ml for both plain- and *C. verum*- yogurt and to 0.8 x 10^9cfu/ml for *A. sativum*-yogurt (Figure 7.8). The VCC of *B. bifidum* in refrigerated storage (7 days) plain- yogurt was 49.9 x 10^9cfu/ml after 1 hour gastric digestion. The VCC was not affected by either the presence of *A. sativum* or *C. verum* in yogurt (0.6 x 10^9cfu/ml and 12.4 x 10^9cfu/ml

respectively). Intestinal digestion (1 hour) decreased VCC of *B. bifidum* in plain- and *A. sativum*- yogurt (32.9 x 10^9cfu/ml and 0.2 x 10^9cfu/ml respectively) but not in *C. verum*- yogurt (44.5 x 10^9cfu/ml; $p<0.05$). A further one hour digestion in intestinal section decreased VCC to the lowest value in *A. sativum*- yogurt (0.04 x 10^9cfu/ml) followed by plain- (13.69 x 10^9cfu/ml) and *C. verum*- (19.04 x 10^9cfu/ml) yogurt (Figure 7.8).

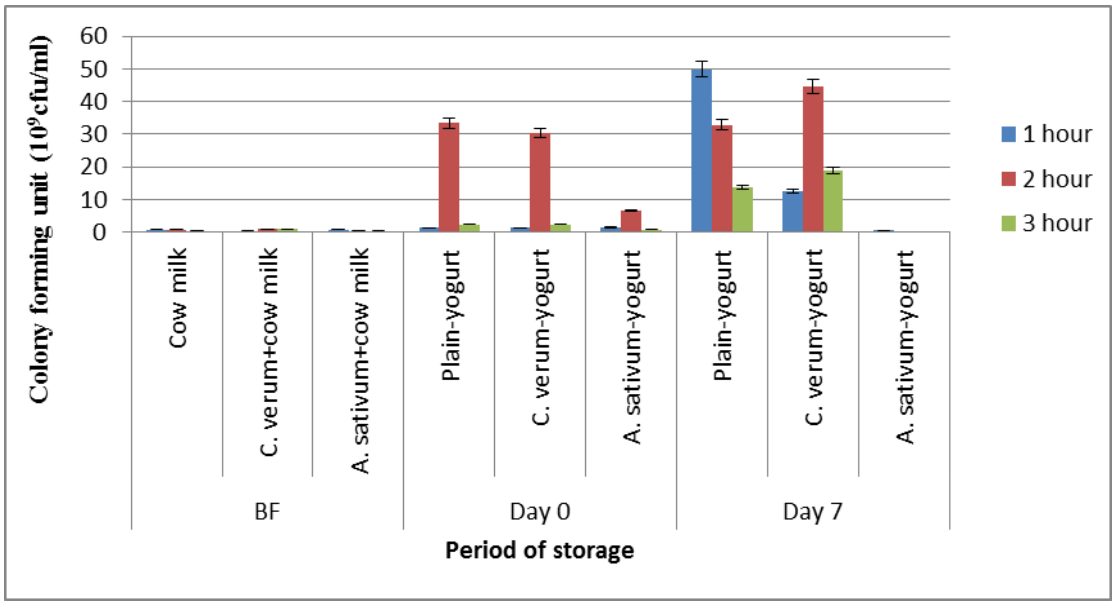


Figure 7.8 VCC of *B. bifidum* (x10^9 cfu/ml) in cow milk before and after fermentation (BF and 0 day respectively) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars present a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

B) VCC in camel milk and camel milk- yogurt

The VCC of *B. bifidum* in all treatments before (0 hour) and after fermentation (0 day) were ≤ 1 x 10^9cfu/ml (Figure 7.9). Refrigerated storage (7 days) of plain- and *C. verum*- yogurt showed similar VCC of *B. bifidum* after 1 hour gastric digestion (66.0 x 10^9cfu/ml) whereas *A. sativum*- yogurt had only 9.7 x 10^9cfu/ml VCC of *B. bifidum*. Intestinal digestion for 2 hours reduced ($p<0.05$) VCC to 4.85 x 10^9cfu/ml,

0.50 x 10^9cfu/ml and 5.55 x 10^9cfu/ml for plain-, *A. sativum*- and *C. verum*-yogurt respectively.

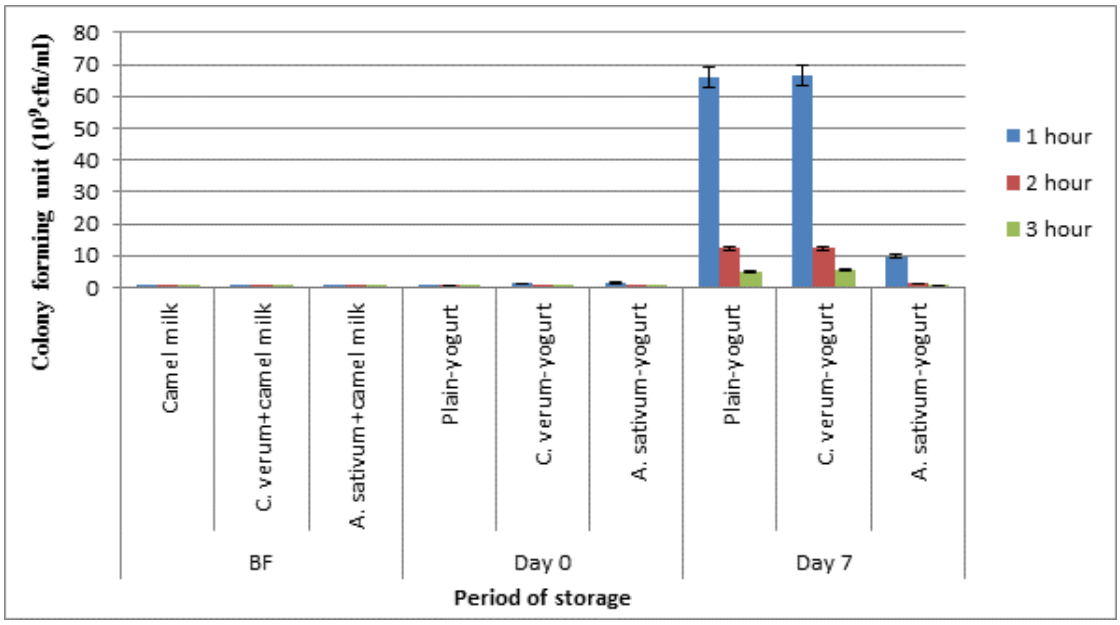


Figure 7.9 VCC of *B. bifidum* (x10^9 cfu/ml) in camel milk before and after fermentation (BF and 0 day respectively) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars present a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) VCC in goat milk and goat milk- yogurt

The VCC of *B. bifidum* in goat milk and refrigerated storage (7 days) yogurt both in the presence and absence of *A. sativum* or *C. verum* water extract were < 1 x 10^9cfu/ml during 3 hours SGD (Figure 7.10). The VCC of *B. bifidum* in fresh *A. sativum*- and *C. verum*- yogurt after 1 hour gastric digestion was higher (10.77 x 10^9cfu/ml and 8.81 x 10^9cfu/ml respectively; $p<0.05$) than plain-yogurt (5.95 x 10^9cfu/ml). Intestinal digestion for 2 hours reduced VCC of *B. bifidum* to 0.46 x 10^9cfu/ml, 3.73 x 10^9cfu/ml and 1.63 x 10^9cfu/ml for plain-, *A. sativum*- and *C. verum*-yogurt respectively (Figure 7.10).

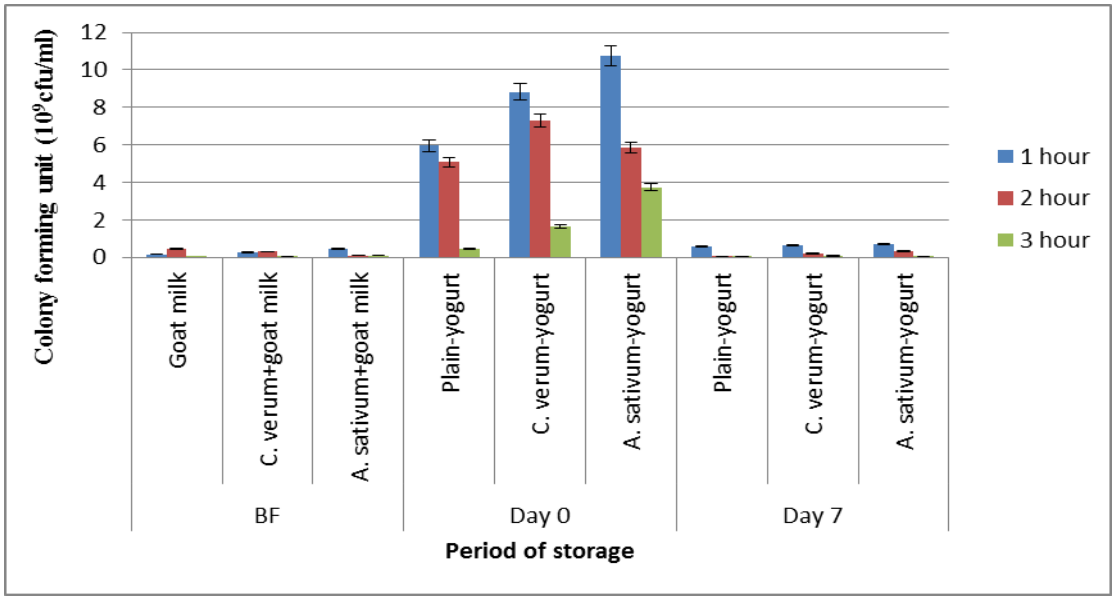


Figure 7.10 VCC of *B. bifidum* (x10^9 cfu/ml) in goat milk before and after fermentation (BF and 0 day respectively) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars present a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

7.4 DISCUSSION

7.4.1 Survival of LAB after SGD

Lactic acid bacteria (LAB) especially lactobacilli are normal populations of intestinal tract of humans and animals and are found in milk and dairy products (Madureira *et al*., 2011). The probiotic effects on gastrointestinal health have been reported (Mishra and Prasad, 2005; Vinderola *et al*., 2011; Madureira *et al*., 2011; Ranadheera *et al*, 2012) and these include stabilization of gut microflora and enhance immune system. Gastrointestinal model (*in vitro*) can be applied to test semisolid foods digestion such as yogurt. The viability of LAB during gastrointestinal digestion of yogurt depends on numerous factors such as strain used, interactions between selected microorganisms, final acidity of product, and the production of hydrogen peroxide via bacterial activity (Mishra and Prasad, 2005; Madureira *et al*., 2011, Vinderola *et al*., 2011). In the present study, the survival of yogurt LAB in the presence of *A. sativum* or *C. verum* water extract was found to be different in all the three types of yogurt. A key phytochemical component contributing to their differences is the phenolic compounds which provide varying protection properties or antibacterial activity depending on the type of milk used. Ranadheera *et al*. (2012) suggested that addition of ingredients such as cocoa powder and stabilizers (guar gum and dextrose) in ice cream provided some protection towards probiotic survival during simulated gastric and intestine transit. On the other hand, this may be complicated by the fact that the amount of bioactive polyphenols compounds with antibacterial activity released during digestion could cause some reduction in LAB cells counts. For instance, cinnamon contains 0.5 to 1.0% volatile oil that composed of several compounds with antibacterial activity such as cinnamyldehyde (50.5%), eugenol (4.7%), cinnamyl acetate (8.7%), methoxycinnamaldehyde (MOCA) and

cinnamic acid (Gupta *et al*., 2008; Ranjan *et al*., 2012). Likewise, the active compounds in garlic such as allicin and thiosulfinate compound have been reported to possess antibacterial activity against some strains of Gram-negative and Gram-positive bacteria (Chung, 2006; Ranjan *et al*., 2012). It is anticipated that during fermentation of milk, compounds with antibacterial activity could be released from the herbal extracts and subsequently affect LAB growth and functions. However, these bacteria were shown able to grow better than those in plain yogurt prompting the possibility that adaptation strategies were engaged. This may include exopolysaccharide matrix released from LAB that acts as a barrier to slow the diffusion of antibacterial compounds (Amrouche *et al*., 2006).

Prior to reaching the intestinal tract, LAB must survive the injurious action of gastric juice during passage through the stomach. Tolerance levels of LAB to acidic environment are variable (Ashraf *et al*., 2009). LAB have different profile of acid tolerance depends on their H^+-ATPase enzymes, composition of their cytoplasmic membrane and exogenous conditions (Analie and Bennie, 2001). In the present study, the highest *Lactobacillus* spp. VCC was shown in fresh and 7 days old herbal yogurt made from goat milk followed by those made from camel and cow milks. Such an observation in the behaviour of *Lactobacillus* spp. in herbal-yogurt made from 3 different types of milk after gastric digestion provides a good base for comparison and help to assess the protective effect of the milk which may depend on the differences in milk chemical composition. Ranadheera *et al*. (2012) reported that high fat content in ice cream has improved protection system of probiotics by acting as buffer to reduce their exposure to gastric acid. The presence study showed that goat milk yogurt had the highest fat content followed by camel- and cow- milk yogurt (see Section 4.3.7) which explains the highest *Lactobacillus* spp. VCC of herbal goat milk yogurt. Since

even slight changes in pH may have a great influence on the LAB survival in low pH environments (Saarela *et al*., 2006). The other factor affecting the survival of *Lactobacillus* spp. in yogurt is increased pH in gastric content after addition of the three types of yogurt associated with yogurt buffering capacity (Salaün *et al*., 2005; Ranadheera *et al*., 2012). The present study showed that the pH of the gastric mixture rose between 3.68 - 3.82, 4.09 - 4.13 and 3.79 - 3.86 as a result of addition of herbal yogurt made from cow, camel and goat milks respectively (Appendix 1) both in fresh and in 7 days refrigerated yogurt.

The lowest VCC of *S. thermophilus* was shown in fresh and 7 days old herbal-camel milk yogurt compared to herbal yogurt made from cow and goat milks after gastric digestion. This could be related to higher antibacterial activities of camel milk (Anonymous, 2003).

Upon exposure to intestinal digestion, the increase of *Lactobacillus* spp. VCC in the presence of *C. verum* extract in fresh cow- and camel- milk yogurt as well as 7 days old *A. sativum*- and *C. verum*- camel milk yogurt indicated that the neutral pH 7.0 appeared to be favourable for some *Lactobacillus* spp. to grow. In addition, recovery of sub lethally-injured cells could have occurred resulted in higher *Lactobacillus* spp. VCC (Marth and Steele, 2001). Similar observation has been seen by Madureira *et al*., (2011) on some strains of probiotic bacteria. The abrupt decrease ($p<0.05$) in *Lactobacillus* spp. VCC at the second hour of intestinal digestion could be due to prolonged bacteria exposure to bile salts present in duodenum juice which is known to negatively affect bacterial survival (Mishra and Prasad, 2005; Ranadheera *et al*, 2012). Since, bile salts are natural sterilizers that assist digestion and absorption of hydrophobic components of the diet; it possesses antimicrobial nature that dissolves bacterial membranes thus strongly inhibit the bacteria from surviving throughout the

intestinal tract (Madureira *et al*., 2011). The improved survival of *Lactobacillus* spp. in *C. verum*- camel milk yogurt at the second hour of intestinal digestion indicated that the presence of *C. verum* in camel milk yogurt may somehow provide some protection to these bacteria. Similar observation was shown in *S. thermophilus* survival in the presence of *C. verum* extract in fresh yogurt made from cow, camel and goat milks at first hour of intestinal digestion (see Figures 7.5, 7.6 and 7.7).

7.4.2 Survival of probiotic (*B. bifidum*) after SGD

Probiotic bacteria grow slowly in milk due to lack of proteolytic activity and thus milk supplemented with peptides and amino acids such as cysteine improved the survival of bifidobacteria (Shihata and Shah, 2000). In addition, *in vitro* limited tolerance of probiotics strains to acid has been demonstrated elsewhere (Mishra and Prasad, 2005; Madureira *et al*., 2011). In the present study, the effect of *A. sativum* or *C. verum* on the survival of *B. bifidum* showed dependence on milk type and phenolic compounds with protection properties or antibacterial activity. *A. sativum* or *C. verum* water extract increased ($p<0.05$) VCC of *B. bifidum* only in fresh and 7 days old goat milk yogurt after gastric digestion. This is in agreement with Ranadheera *et al*. (2011) who found higher survival of probiotics in fruit- goat milk yogurt after gastric digestion. It is possible that the interaction between phenolic compounds and goat milk proteins could provide considerable protection for *B. bifidum* against exposure to low gastric acid. This possibility shown also from Ranadheera *et al*. (2011) suggestion that the addition of ingredients as cocoa powder and stabilizers (guar gum and dextrose) in goat milk ice cream have provided protection towards probiotic survival during simulated gastric digestion. In addition, higher fat content and higher pH in gastric content after addition of herbal goat milk yogurt (Figure 4.26 and Appendix 1) could also contribute to *B. bifidum* growth during gastric digestion. The lower VCC of

B. bifidum of 7 days refrigerated goat milk yogurt compared to those in cow- and camel- milk yogurt after gastric digestion, could be explained by the low ability of *B. bifidum* to survive in goat milk yogurt during refrigerated storage (Güler-Akın and Serdar Akın, 2007) which subsequently affect the VCC of these bacteria after gastric digestion

During intestinal digestion only *B. bifidum* in plain and herbal cow milk yogurt showed ability to grow in such condition and/or recover from sub lethally-injured cells. Such observation was shown also by Madureira *et al*., (2011) on some strains of probiotic bacteria. In addition, camel and goat milk were reported to have higher activity of antimicrobial lactoperoxidase system (Anonymous, 2003) which may be caused further inhibitory effect on *B. bifidum* growth. Prolonged exposure to intestinal digestion (2 hour) showed substantial reduction of *B. bifidum* VCC in all three types of yogurt. This is in agreement with other studies (Saxelin *et al*., 2010; Vinderola *et al*., 2011; Ranadheera *et al*., 2012) who explained that to antimicrobial nature of bile salt that arises mainly from its detergent property.

7.5 CONCLUSIONS

The survival of LAB in yogurt in the presence of *A. sativum* or *C. verum* water extract was differ among the three types of yogurt made from cow, camel and goat milks after *in vitro* gastrointestinal digestion. However, the inclusion of *C. verum* in yogurt enhanced the growth of yogurt bacteria more than that caused by the presence of *A. sativum.* Thus, *C. verum* may act as a protective/ stimulatory agent to enhance LAB/probiotic survival in the digestive tract.

8.0 *Allium sativum* and *Cinnamomum verum* yogurt proteolysis and *in vitro* angiotensin-I converting enzyme activity during refrigerated storage

8.1 INTRODUCTION

Proteolysis in yogurt is the result of symbiotic relationship between two yogurt bacteria *L. bulgaricus* and *S. thermophilus.* The proteinase of *L. bulgaricus* hydrolyzes casein to yield polypeptides which are subsequently broken down further to small molecular weights peptides and free amino acids by the peptidases of *S. thermophilus* (Robinson and Tamime, 2002). The latter also has the ability to metabolize excess amino acids liberated by *L. bulgaricus* (Fox *et al*., 2000; Gobbetti *et al*., 2002; Pescuma *et al*., 2011). *L. bulgaricus* has greater proteolytic activity than *S. thermophilus* (Singh and Sharma, 2009). Thus, the total amino groups in yogurt reflects the balance between those produced through proteolysis and those consumed during *S. thermophilus* and *L. bulgaricus* growth (Singh and Sharma, 2009).

Hypertension is one of the most common cardiovascular diseases. It is a worldwide problem of epidemic proportions, which affects 10% – 20% in the adult population and 40% - 50% in people aged 50 or older (Karakurt and Kasikci, 2012). It is the most common serious chronic health problem because it carries a high risk factor for arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (Ranade *et al*., 2001). The role of the rennin–angiotensin system (RAS) in cardiovascular physiology is well established. The angiotensin-I converting enzyme (ACE- I; EC. 3.4.15.1), a component of RAS catalyzes the formation of the strong pressor agent angiotensin II from angiotensin I contributing to the maintenance of normal blood pressure (Unger, 2002; Coates, 2003). Captopril, enalapril, lisinopril and temocapril are ACE inhibitors drug used to treat hypertension. These drugs produced side effects (Miller *et al*., 2007) thus, justifying the search for natural ACE inhibitors for safe and economical use (Coates, 2003; Kang *et al*., 2003). The consumption of yogurt has increased rapidly owing to the fact that this product fulfills

many of the current dietary needs. Hernadez-Ledesma *et al*. (2004) reported that consumption of yogurt is associated with the reduction of blood pressure, made possible due to the liberation of peptides with ACE-I inhibition properties by LAB proteolytic activities (Vermeirssen *et al*., 2002; Fuglsang *et al*., 2003; Ijäs *et al*., 2004). Many peptides with antihypertensive action have been characterized upon fermentation of milk with different microorganisms or by the action of pure proteinases on milk proteins (Tauzin *et al*., 2002; Hernandez-Ledesma *et al*., 2004). Interestingly, the yogurt capacity to inhibit ACE-I activities may be enhanced when milk was fermented in the presence of water extract from medicinal herbs such as *Azadirachta indica* (Shori and Baba, 2011) or *Mentha piperita*, *Anethum graveolence* and *Ocimum basilicum* (Amirdivani and Baba, 2011) and after a period of refrigerated storage. However, manipulation of bacterial fermentation of milk play crucial role in increasing yogurt anti- ACE-I activity. It cannot be overlooked that a number of compounds from plants including hydrolysable tannins, phenylpropanes, proanthocyanidins, flavonoids, xanthones, terpenoids, fatty acids, alkaloids oligosaccharides and peptide amino acids (Park *et al*., 2003) may directly be responsible for ACE-I inhibition as well. For instance, *A. sativum* and *C. verum* beneficial effects on blood pressure may be attributed to their effects to ease the spasm of the small arteries, slow the pulse and modify the heart rhythm (Harauma and Moriguchi, 2006; Preuss *et al.*, 2006). Therefore, the objectives of this study were: (i) to investigate the proteolytic activity of *A. sativum*- or *C. verum*- yogurt after fermentation and during 21 days of refrigerated storage and (ii) to evaluate the ability of herbal yogurt to inhibit ACE activity *in vitro* after fermentation and during storage.

8.2 MATERIALS AND METHODS

8.2.1 Substrates and chemicals

Cupric sulphate, sodium carbonate (Na_2CO_3), sodium hydroxide pellets (NaOH), Folin-Ciocalteu phenol reagent, bovine serum albumin (BSA), hydrochloric acid (HCl), sodium chloride (NaCl), Tris solution ($C_4H_{11}NO_3$), rabbit lung acetone powder and 2-furanacryloyl-1-phenylalanylglycylglycine (FAPGG) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All the chemicals used in O-phthaldialdehyde (OPA) assay were as described in Section 3.2.1.

8.2.2 Plant water extraction procedure

The water extract of plants was performed according to the method described in Section 3.2.3.

8.2.3 Yogurt manufacturing process

8.2.3.1 Starter culture and yogurt preparation

The preparation of starter culture was carried out using the method described in Section 3.2.4.1. The two types of bio-yogurt made from cow and camel milk both in the presence and absence of *A. sativum* or *C. verum* water extract were prepared as described in Section 3.2.4.2.

8.2.4 Sample preparation (yogurt water extract)

The preparation of yogurt water extract was carried out using the method described in Section 3.2.7.1.

8.2.5 Determination of proteolytic activity

Proteolytic activities of yogurt was assessed after fermentation and during refrigerated storage by measuring (i) liberated free amino groups using the OPA method and (ii) the quantity of total soluble protein using Lowry protein assay.

8.2.5.1 O-phthaldialdehyde (OPA) assay

The o-phthaldialdehyde (OPA) based spectrophotometric method was carried out according to Church, *et al.* (1983) see Section 3.2.6.

8.2.5.2 Determination of total soluble protein (TSP)

The total soluble protein was assessed by Lowry protein assay including the modifications suggested by Markwell *et al*., (1978). The alkaline copper reagent was prepared using a mixture of copper sulphate reagent (100 mg cupric sulphate and 200 mg of sodium tartrate dissolved in 50 ml of dH_2O and the mixture added slowly to sodium carbonate (10 g) dissolved in 50 m dH_2O), 5% (w/v) SDS solution and sodium hydroxide solution 3.2% (w/v) in the ratio of 1:2:1. The standard solution of BSA or yogurt-water extract (1ml) was added to 1ml of alkaline copper reagent and the mixture was mixed thoroughly using a vortex machine. The mixture was allowed to stand at room temperature (25°C) for 10 minutes prior to the addition of 0.5ml of the diluted Folin-Ciocalteu's phenol reagent 20% (w/v). The mixture was briefly vortexed and then left at room temperature for 30 minutes followed by absorbance readings at 750nm (Shimadzu spectrophotometer UV Mini 1240). Standard solution with varying concentrations (0 – 200 µg/ml) of stock solution of bovine serum albumin (BSA; 100mg/100ml dH_2O) was prepared in water and it was run simultaneously each time, when yogurt was analysed for TSP. The absorbance of sample was converted to TSP using the standard curve (see Figure 8.1) with the following typical equation:

$$\text{Total soluble protein (µg/ml)} = \frac{A_{750} - 0.0513}{0.0041}$$

where A_{750} was the spectrophotometric absorbance reading at 750 nm.

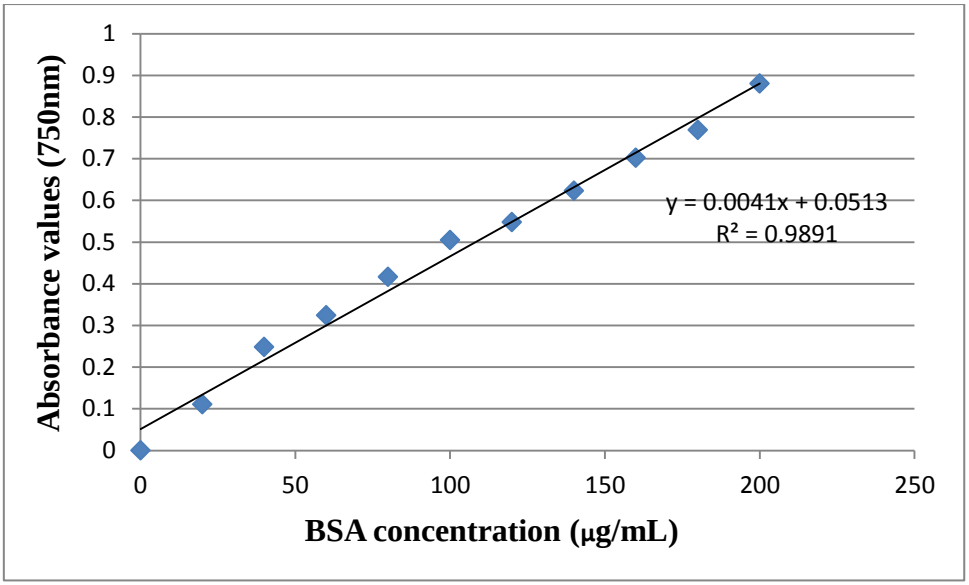


Figure 8.1 Typical calibration curve for total soluble protein.

8.2.6 ACE-I inhibition assay

8.2.6.1 Preparation of yogurt sample

Yogurt sample was prepared for ACE assay as described by Pripp, (2006). Yogurt (20 g) were mixed with 5 ml water and placed in a water bath at 45 °C for 5 min. The mixture was then homogenized by a homogenizer (Polytron PT2100) at maximum setting for 10 seconds and the pH was adjusted to 4.5 with 2 M HCl. The sample was further placed in a water bath at 45 °C for 1 h to distribute fat prior to centrifugation (6000 rpm for 20 min). The supernatant was harvested and kept at -20 °C for further analysis.

8.2.6.2 Preparation of rabbit lung acetone extract

The rabbit lung acetone extract was prepared by dissolving 1g rabbit lung acetone powder in 10 ml buffer (50 mmol/l Tris–HCl with 400 mmol/l NaCl, pH 8.3; Vermeirssen *et al*., 2002) followed by ultra-centrifugation (Eppendoft 5804; 2 hours at 20000 rpm, 4°C). The clear wine red supernatant contains high ACE-I activity and was aliquoted (1.0 ml) into ampoules and which were stored in -20^{o}C until required for analysis. The preparation was carried out at 4^{o}C (on ice condition)

in order to minimize the degradation of ACE. The supernatant was diluted 10 times prior to analysis using 50 mmol/l Tris–HCl in 400 mmol/l NaCl, pH 8.3.

8.2.6.3 Preparation of ACE reagent

The ACE reagent was prepared as described by Vermeirssen *et al.,* (2002). The following two solutions were prepared separately and each was ensured dissolved completely:-

1) Sodium chloride (NaCl: 2.34g) was dissolved in approximately 80ml of dH_2O and volume was made up to 100ml in a volumetric flask.

2) Tris solution was prepared by mixing 0.607g of Tris in 50ml of dH_2O, and the pH was adjusted to 8.3 and the final volume was brought up to 100ml.

Both NaCl and Tris solutions were mixed thoroughly together then 62.6ml of the mixed solution were added to 25mg Furanacryloyl-Phe-Gly-Gly (FAPGG). The dissolved FAPGG (ACE reagent) was aliquoted into ampoules of 500µl which were stored at -20°C until required for assay.

8.2.6.4 Measurement of anti-ACE-I inhibitory activity and IC_{50}

ACE-I inhibitory activity was measured spectrophotometrically as described by Vermeirssen *et al.,* (2002) with furanacryloyl-1-phenylalanylglycylglycine (FAPGG) as a substrate and extract from rabbit lung acetone powder as ACE source. ACE-I inhibitory activity was assayed by mixing 300µl of yogurt water extract or distilled water (control) and 500µl ACE reagent in a cuvette followed by incubation in a water bath (37°C) for 2 minutes. Afterward, diluted rabbit lung acetone extract (300 µl, 10 times dilution) was added and the mixture was mixed evenly prior to absorbance readings at 340 nm (Shimadzu spectrophotometer UV Mini 1240) which was recorded every 5 min for 20 min. The slope average over a linear interval between 0 and 20 min was taken as a measure of ACE-I activity. The ACE-I activity

was expressed as the slope of the decrease in absorbance at 340 nm. The ACE-I inhibition (%) was calculated according to the following formula:

ACE-I inhibition (%) = [1− ((*C*−*D*) / (*A*−*B*))] X 100

Where A is absorbance of ACE only, B is absorbance of blank, C is absorbance of ACE and the sample and D is absorbance of the sample only.

ACE-I inhibition activity was also expressed in terms of IC_{50}, defined as the protein concentration (µg /g) in the sample required to inhibit 50% of the ACE-I activity. The protein content of the samples was determined by Lowry protein assay using BSA as a standard (Figure 8.1). The IC_{50} value of yogurt extracts can be obtained through a graph of percentage of inhibition versus 3 different volumes of yogurt extracts (300 µl, 150 µl, and 75 µl). The IC_{50} was determined using graphical extrapolation by plotting ACE-I inhibition as a function of different protein concentrations against the 3 different volumes of yogurt water extracts.

8.2.7 Statistical analysis

Statistical analysis of all data obtained was performed as described in Section 3.2.8. In addition, the standard curves used to calculate free amino groups and TSP was plotted as described in Section 3.2.8 using Microsoft® Excel XP.

8.3 RESULTS

8.3.1 Proteolytic activity during refrigerated storage

8.3.1.1 The extent of proteolysis

LAB produced extracellular proteinases during fermentation that hydrolysed milk proteins resulting in an increase in the amount of free amino groups as quantified by the OPA method (Donkor *et al.*, 2007). The free amino groups value in cow milk was very low (12.8±0.01 µg/g) compared to that of camel milk (268.9±0.01 µg/g; Table 8.1). The presence of *A. sativum* water extract in cow- (166.5±0.02 µg/g) and

camel- (425.9±0.02 µg/g) milks showed higher ($p<0.05$) free amino groups than in presence of *C. verum* (19.2±0.01 µg/g and 294.6±0.01 µg/g respectively) .

The effect of *A. sativum* or *C. verum* water extract on the changes of proteolytic activities in yogurt made from cow or camel milk during 21 days of storage (4°C) are shown in Figures 8.2 and 8.3 respectively. The presence of *A. sativum* or *C. verum* water extract in fresh cow milk yogurt caused higher ($p<0.05$) protoelysis (262.6±1.8 µg/g and 172.9±3.1 µg/g respectively) than that in plain yogurt (80.1±3.1 µg/g; Figure 8.2). The extent of proteolysis continued during refrigerated storage in plain cow milk yogurt but not in *A. sativum*- or *C. verum*- yogurt. This can be seen in increasing peoteolysis for plain yogurt to 128.1±3.7 µg/g by day 21 of storage compared to those in *A. sativum*- and *C. verum*- yogurt (220.9 ± 2.5 µg/g and 169.7± 2.5 µg/g respectively). In contrast, the extent of proteolysis in fresh camel milk yogurt was 368.2±3.7 µg/g (Figure 8.3). The presence of *C. verum* water extract did not significantly change proteolysis in camel milk yogurt (397.1 ± 2.8 µg/g) whereas presence of *A. sativum* increased ($p<0.05$) proteolysis in yogurt to 470.7 ± 3.3 µg/g. Refrigerated storage for 21 days showed gradual increase ($p>0.05$) in proteolysis of *C. verum*- and *A. sativum*- yogurt to 477.1 ± 3.1 µg/g and 521.9 ± 3.2 µg/g respectively which was in contrast to the reduction ($p<0.05$) of proteolysis in plain-yogurt to 342.6 ± 3.4 µg/g.

Table 8.1 Proteolytic activity (µg/g) in cow and camel milk in the absence or presence of *A. sativum* and *C. verum* water extracts.

Sample	Proteolytic activity (µg/g)
Cow milk	12.8±0.01
AS+cow milk	166.5±0.02*
CV+cow milk	19.2±0.01
Camel milk	268.9±0.01
AS+camel milk	425.9±0.02*
CV+camel milk	294.6±0.01

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

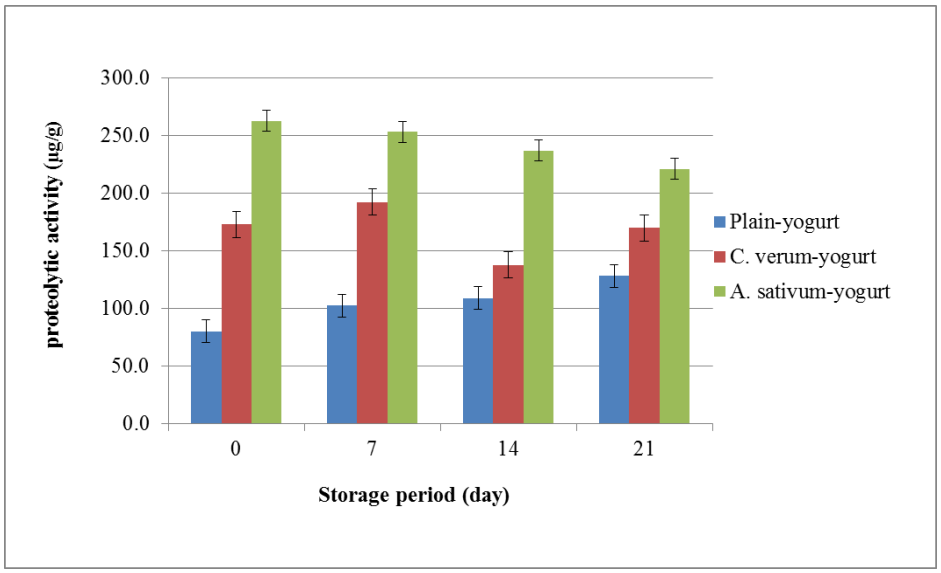


Figure 8.2 Proteolytic activity (µg/g) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

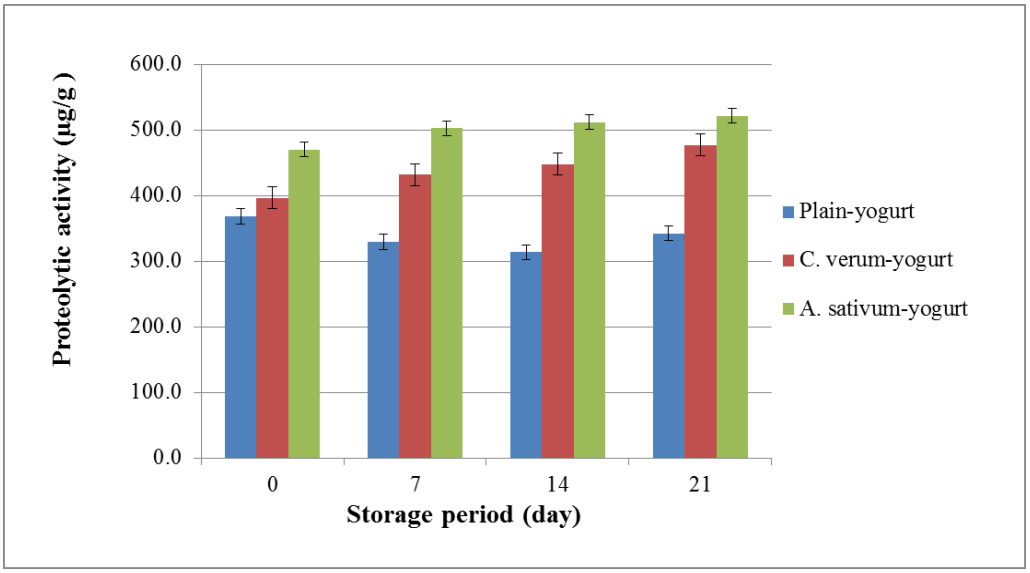


Figure 8.3 Proteolytic activity (µg/g) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

8.3.1.2 Total soluble proteins

Lowry assay is based on the reactions of copper ions with the peptide bonds and tyrosine residues of proteins present in the sample (Lindeboom and Wanasundara, 2007). The total soluble protein (TSP) in cow milk was 76.9±0.21 µg/g (Table 8.2). The mixing of *A. sativum* or *C. verum* water extract with cow milk increased TSP to 92.6±0.09 µg/g ($p<0.05$) and 88.3±0.08 µg/g respectively. Similarly, the mixing of *A. sativum* or *C. verum* with camel milk increased TSP (236.4±0.05 µg/g and 231.3±0.05 µg/g respectively) compared with plain camel milk (228.5±0.04 µg/g). Fermentation of cow milk in the presence of *A. sativum* or *C. verum* water extract resulted in higher TSP (129.9± 1.0 µg/g and 126.9± 2.2 µg/g respectively; $p<0.05$) as compared to plain yogurt (111.6± 2.2 µg/g; Figure 8.4). Refrigerated storage increased ($p>0.05$) TSP in both plain- and herbal- yogurt to similar extent throughout the 21 days of storage. However, *A. sativum*- yogurt showed higher TSP (172.9 ± 2.4 µg/g; $p<0.05$) than

plain-yogurt (141.9 ± 1.4 µg/g) at the end of storage (Figure 8.4). On the other hand, the addition of herbal extracts in fresh camel milk yogurt did not significantly enhanced TSP as compared to plain-yogurt (Figure 8.5). However, refrigerated storage showed increased ($p>0.05$) in TSP of plain-, *C. verum*- and *A. sativum*- yogurt to the highest values were shown on day 14 of storage (406.2 ± 2.8 µg/g; 418.4 ± 2.8 µg/g and 417.6 ± 1.4 µg/g respectively). Extended storage to 21 days resulted in significant decrease of TSP in plain-yogurt (367.9 ± 2.4 µg/g) but not in herbal-yogurt (Figure 8.5).

Table 8.2 Total soluble proteins (µg/g) in cow and camel milk in the absence and presence of *A. sativum* and *C. verum* water extracts.

Sample	TSP (µg/g)
AS	-
CV	-
Cow milk	76.9±0.21
AS+cow milk	92.6±0.09*
CV+cow milk	88.3±0.08
Camel milk	228.5±0.04
AS+camel milk	236.4±0.05
CV+camel milk	231.3±0.05

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as mean (n = 3) ± standard error. *$p < 0.05$ as compared to control.

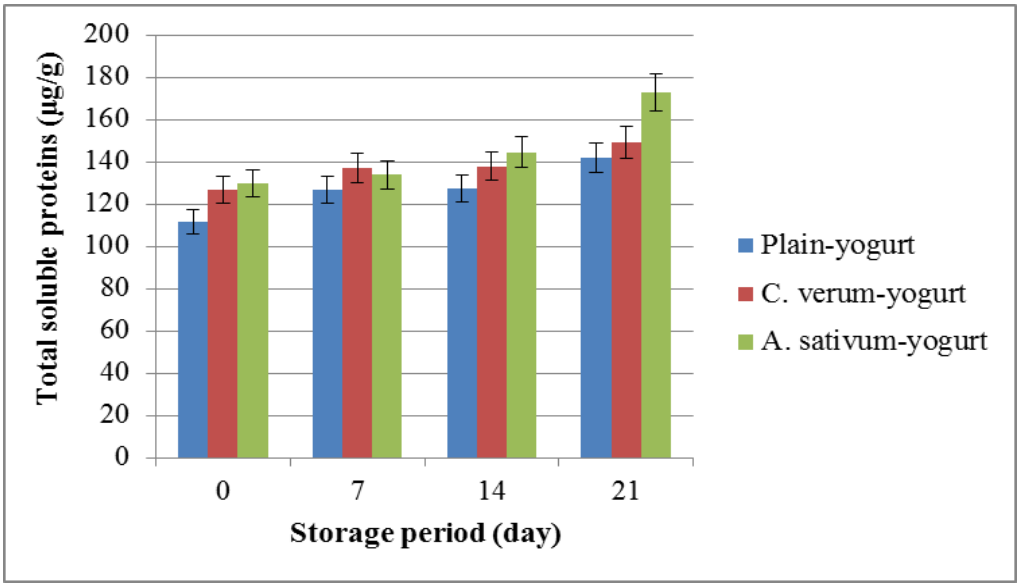


Figure 8.4 Total soluble proteins (µg/g) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

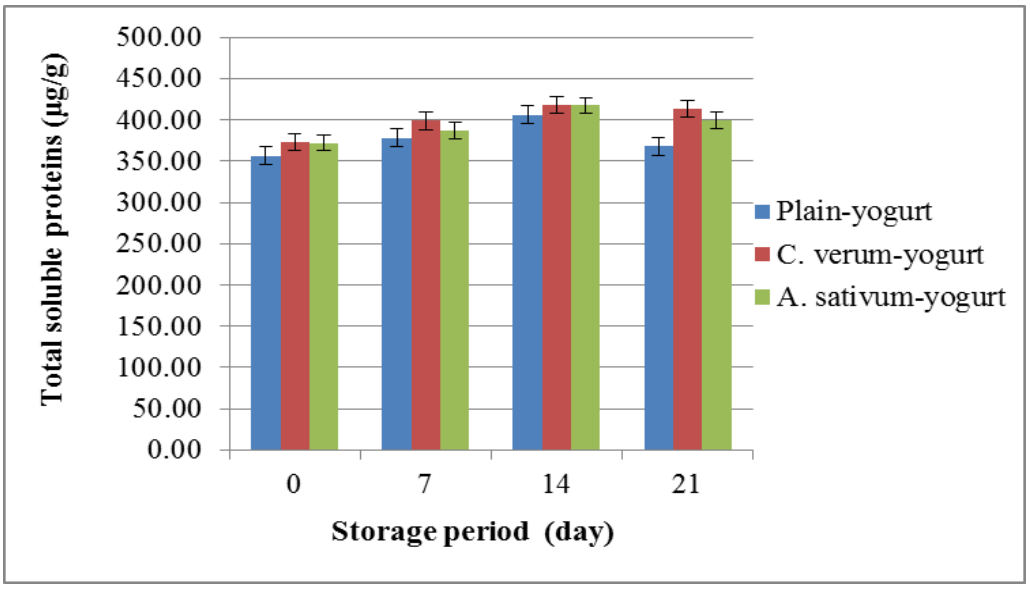


Figure 8.5 Total soluble proteins (µg/g) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

8.3.2 ACE-I inhibitory activity

A. sativum water extract showed higher (14.10± 2.1%; $p<0.05$) ACE-I inhibitory activity than *C. verum* water extract (4.3±1.9%; Table 8.3). The mixing of these herbal extracts with cow or camel milk increased ($p<0.05$) ACE-I inhibition which were more in presence of *A. sativum* ($p<0.05$) than *C. verum* as compared to that of milk alone (Table 8.4).

The presence of *A. sativum* water extract in yogurt made from cow milk increased ($p<0.05$) the inhibitory activity of ACE-I compared to fresh or stored plain yogurt both in fresh and storage conditions (Figure 8.6). In contrast, the presence of *C. verum* water extract had no significant effects on ACE-I inhibition except on day 7 of storage as compared to plain yogurt. All types of yogurt showed the highest inhibitory activity of ACE-I (46.6±0.02%, 70.2±0.1% and 56.3±0.03% for plain-, *A. sativum*- and *C. verum*-yogurt respectively) on day 7 of storage. Prolonged storage of yogurt to three weeks decreased ($p<0.05$) ACE-I inhibition activity to 39.1±0.04%, 53.89±0.02 and 43.9±0.01 for plain-, *A. sativum*- and *C. verum*-yogurt respectively.

Fresh plain camel milk yogurt showed about 30% of ACE-I inhibitory activity (Figure 8.7). The presence of *A. sativum* or *C. verum* water extract significantly increased the inhibitory activity of ACE-I to about 50% and 35% respectively. Refrigerated storage of herbal yogurt for up to 21 days caused significant increase in the inhibition of ACE-I activity as compared to plain yogurt (Figure 8.7). *C. verum*-yogurt showed the highest inhibition activity of ACE-I (56.2±0.04%) on day 7 of storage. However, the inhibition of ACE-I in both *A. sativum*- and *C. verum*-yogurt reduced ($p<0.05$) by day 14 and 21 of storage.

Table 8.3 ACE-I inhibitory activity (%) in *A. sativum* and *C. verum* water extracts.

Sample	ACE-I inhibition %
AS water extract	14.10± 2.1*
CV water extract	4.3±1.9

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts = 0.1g/ml. Results are shown as mean (n = 3) ± standard error. *p < 0.05.

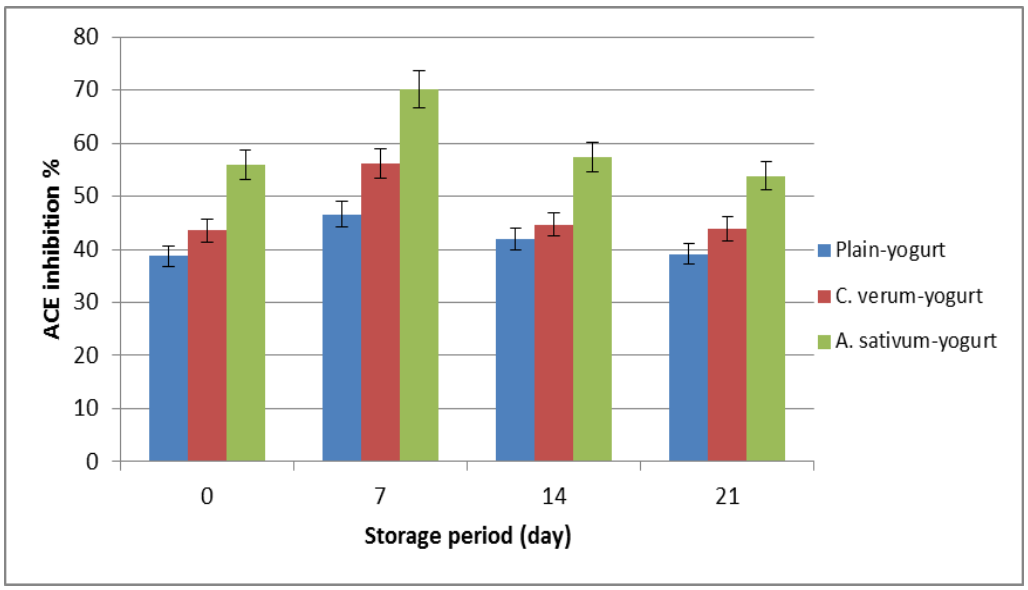


Figure 8.6 ACE-I inhibitory activity (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

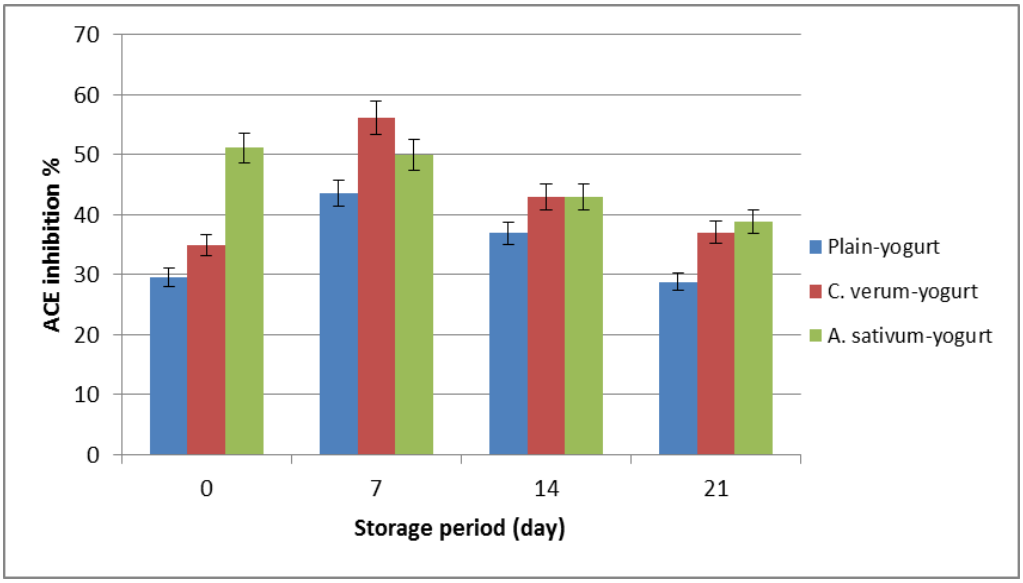


Figure 8.7 ACE-I inhibitory activity (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

8.3.2.1 ACE-I inhibitory activity (IC_{50})

The mixing of cow or camel milk with *A. sativum* or *C. verum* water extract showed higher ($p<0.05$) ACE-I inhibitory activity (IC_{50}) than respective milk alone (Table 8.4). Cow milk + *A. sativum* exhibited higher IC_{50} (0.45±0.04 µg/g) than camel milk + *A. sativum* (0.52±0.03 µg/g). No difference ($p>0.05$) was observed in IC_{50} between cow and camel milk in the presence of *C. verum* (Table 8.4).

IC_{50} values of herbal- cow or camel milk yogurt toward ACE-I inhibitory activities are presented in Table (8.5). The beneficial effect of *A. sativum*- or *C. verum*- yogurt made from both cow and camel milk toward ACE-I inhibition (IC_{50}) was higher than plain yogurt throughout the storage period. In addition, *A. sativum*-cow milk yogurt showed higher IC_{50} than *C. verum*- cow milk yogurt during 21 days of storage. *A. sativum*-camel milk yogurt showed higher IC_{50} (0.17±0.09 µg/g and 0.14±0.09 µg/g; $p<0.05$) than *C. verum*-camel milk yogurt (0.29±0.02 µg/g and 0.47±0.09 µg/g) on days 7 and 14 of storage respectively.

Table 8.4 ACE-I inhibitory activity (%) in cow and camel milk in the absence or presence of *A. sativum* and *C. verum* water extracts and IC_{50} values (µg/g) for ACE-I inhibition activity.

	Cow milk			**Camel milk**		
	Cow milk	Cow milk + AS	Cow milk + CV	Camel milk	Camel milk + AS	Camel milk + CV
ACE-I inhibition (%)	18.42±0.1	29.92±0.2*	23.04±0.1	18.2±0.1	30.3±0.1*	20.2±0.04
IC_{50} (µg/g)	0.61±0.032	0.45±0.04*	0.47±0.03*	0.91±0.04	0.52±0.03*	0.48±0.03*

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

Table 8.5 IC_{50} values for ACE-I inhibitory activity in *A. sativum*- and *C. verum*-yogurt made from cow or camel milk during 21 days of refrigerated storage.

Samples	**IC_{50} µg/g**			
	0 day	**7 day**	**14 day**	**21 day**
Plain-cow milk yogurt	0.54±0.06	0.39±0.08	0.42±0.09	0.59±0.08
AS-cow milk yogurt	0.34±0.02*	0.30±0.08*	0.35±0.03*	0.43±0.04*
CV-cow milk yogurt	0.40±0.02*	0.37±0.05	0.39±0.08	0.44±0.02*
Plain-camel milk yogurt	0.71±0.09	0.45±0.01	0.53±0.06	0.70±0.06
AS-camel milk yogurt	0.17±0.09*	0.39±0.06*	0.14±0.09*	0.59±0.03*
CV-camel milk yogurt	0.29±0.02*	0.39±0.07*	0.47±0.09	0.56±0.01*

AS= *A. sativum* and CV= *C. verum*. Plain-cow milk yogurt and plain-camel milk yogurt presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

8.4 DISCUSSION

8.4.1 Proteolytic activity

The growth of yogurt bacteria and subsequently developed acidity in yogurt are related to the proteolytic activities in yogurt (Donkor *et al*, 2007). Both *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* synthesize and release the primary enzymes (proteinase and peptidase respectively) responsible for proteolysis of milk proteins to yield the much needed amino acids (Shihata and Shah, 2000; Robinson *et al*., 2002). In the present study, the proteolytic activity of herbal yogurt was higher than plain yogurt in both cow- and camel- milk yogurt, suggesting that herbal water

extract from *A. sativum* and *C. verum* were facilitate proteolysis process of milk proteins. This is in agreement with El-Tanboly, (2007) who reported that proteolytic enzyme (proteinase) isolated from plant (*Artocarpus integrifolis*) can increase proteolysis in low fat yogurt during 15 days of storage. In addition, the proteins in *A. sativum* water extract (Suetsuna, 1998) could be degraded by the plant proteinase during herbal extract preparation resulting in higher amount of free amino acids which was also shown in the present study (Table 3.3). In this study, cow milk yogurt in the presence of herbal extracts showed 2-folds higher proteolytic activity than plain yogurt. Camel milk-yogurt on the other hand did not show comparable increased proteolyic activity in the presence of herbal extracts as cow milk yogurt, probably because the yogurt bacteria could get access to readily available peptides/amino acids in camel milk (Al-Alawi and Laleye, 2011; Table 8.1).

Camel milk contains higher TSP than cow milk (Table 8.2) and this may be explained by the higher protein content in the former (61,173.33 mg/l) than in the latter (44,888.00 mg/l; Al-Alawi and Laleye, 2011). The inclusion of *A. sativum*- or *C. verum* water extracts in both cow- and camel- milk causes increased in the TSP compared to control (Figures 8.4 and 8.5 respectively). This may suggest possible availability of peptides/ amino acids in the herbal water extracts resulting in TSP increase (Abdullah *et al.,* 1988; Augusti and Sheela, 1996; Al-Numair *et al*., 2007). However, the increase in TSP in herbal yogurt after fermentation and during refrigerated storage compared to plain yogurt could be due to higher viable cell counts of LAB in herbal yogurt (see Section 6.3.1). The high degradation of camel milk proteins may be explained by its higher content of low molecular masses polypeptides (proteins) than that in cow milk (Agrawal *et al.*, 2004; El-Said *et al*., 2010; Smits *et al*., 2011) caused the easier action of proteinase and greater extent of TSP in the

former than in the latter after fermentation and during refrigerated storage. The increase in TSP in both types of yogurt during refrigerated storage may be seen advantageous from nutritional point of view because milk proteins become more digestible (Adolfsson *et al*., 2004) thus ensuring higher intestinal availability of nitrogen.

8.4.2 ACE-I inhibitory activity

Fermented milk products such as yogurt are known to contain bioactive peptides acting as ACE inhibitors (Nakamura *et al*., 1995). These peptides can bind to the enzyme competitively and prevent the breakdown of substrate, furanacryloyl-Phe-Gly-Gly (FAPGG) to the product, furanacryloyl-Phe (FAP) and Gly-Gly (Shah, 2000). Bioactive peptides isolated from fermented milk in the form of valyl-prolyl-proline (Val-Pro-Pro), isoleucyl-prolyl-proline (Ile-Pro-Pro) and Tyr-Pro and Lys-Val-Leu-Pro-Val-Pro-Gln were found to reduce blood pressure in spontaneously hypertensive human and rat's model (Nakamura *et al.,* 1995). Previous studies reported that the presence of herbal extracts *Azadirachta indica* (Shori and Baba, 2011), *Mentha piperita*, *Anethum graveolence* or *Ocimum basilicum* (Amirdivani and Baba, 2011) in yogurt increased ACE-I enzyme inhibition. Both *A. sativum* and *C. verum* observed to contain polyphenols with anti-hypertensive properties (Harauma and Moriguchi, 2006; Preuss *et al.,* 2006). The present study supports this observation (Table 8.3) and provides further evidence that these herbs extracts also enhance yogurt anti-ACE- 1 activity. This study demonstrated that, the significant effect of *C. verum* water extract on ACE-I inhibitory activity of cow milk yogurt occurred after 7 days of storage. On the other hand, the presence of *C. verum* had significant impact on ACE-I inhibitory activity in camel milk yogurt throughout the 21 days storage period. The above observation could occur by the effect of *C. verum* to alter lactic acid

bacteria fermentation of milk by manipulating the proteolytic system of these bacteria (Figures 8.2 and 8.3) resulted in produce more anti-ACE-I peptides in yogurt. The presence of *A. sativum* water extract in cow- or camel- milk yogurt increased ($p<0.05$) ACE-I inhibitory activity. Again, this could be due to the increase in anti-ACE-I peptides during proteolysis of milk proteins associated with higher LAB viable cell counts (see Section 6.3.1). In addition, *A. sativum* water extract showed 14% of ACE-I inhibition (Table 8.3) thus could contribute to polyphenols with anti-ACE-1 activity. It was previously reported that certain phytochemicals such as mycerene and allicin found in *A. sativum* possess ACE inhibitory properties (Hosseini *et al.,* 2007). Furthermore, Suetsuna, (1998) isolated 7 bioactive dipeptides from garlic responsible for the lower blood pressure in spontaneously hypertensive rats after oral administration.

The findings from the present study supported the possibility of enhancing the anti-hypertensive properties of yogurt by inclusion of *A. sativum* or *C. verum* in milk during fermentation. Polyphenols derived from *A. sativum* and *C. verum* (Table 8.3) have low ACE-I inhibition activity in comparison to the inhibition caused by yogurt in the presence of these herbal extracts. However it is possible that the presence of *A. sativum* or *C. verum* water extract may enhance viability of yogurt bacteria (see Section 6.3.1). Thus this led to enhanced proteolysis (Figures 8.2 and 8.3) to such an extent that the liberation of anti-ACE-I peptides continued to take place at higher rate than that in plain-yogurt during storage. This hypothesis is supported by the fact that ACE-I inhibitory activity of cow or camel milk in the presence of *A. sativum* or *C. verum* water extract (BF) exhibited lower ($p<0.05$) ACE-I inhibitory activity (IC_{50}; Table 8.4) than fresh yogurt (IC_{50}; Table 8.5). The decrease in ACE-I inhibitory activity in yogurt during the last two weeks of storage could be related to degradation

of phenolic with anti- ACE-I resulted in further pH reduction (Perez-Vincente *et al*., 2002; Vallejo *et al*. 2004). In addition, the balance between the formation of bioactive peptides subsequent breakdown into inactive peptides and amino acids play decisive role in the inhibition of ACE activity (Gobbetti *et al*., 2004).

Lower ACE-I inhibitory activity (IC_{50}) in camel milk yogurt than cow milk yogurt during 7 and 21 days of storage may be explained by lower caseins (substrate for the extracellular LAB proteinases; Vermeirssen *et al*, 2003) in camel milk (14,632 mg/l) than cow milk (39,680.01 mg/l; Al-Alawi and Laleye, 2011).

8.5 CONCLUSIONS

A. sativum and *C. verum* water extracts increased proteolytic activity in both cow and camel milk yogurt. The proteolytic activity was more pronounced in cow- than camel-milk yogurt during refrigerated storage. Anti-ACE-I peptides present in yogurt are increased upon the inclusion of *A. sativum* water extract in both cow- and camel- milk yogurt. The presence of *C. verum* water extract increased ACE-I inhibitory activity of camel milk yogurt but not cow milk yogurt. Given the existing medicinal values of *A. sativum* and *C. verum* on patients with hypertension, it appears that the presence of these two herbal extracts in yogurt may further improve the nutritional and therapeutical values of yogurt by virtue of polyphenols compounds and formation of bioactive peptides with anti- ACE-I activities. Thus both types of herbal yogurt made from cow or camel milk have the potential to be further developed as a functional yogurt for consumers with hypertension.

9.0 Sustainability of ACE inhibitory activity of *Allium sativum*- and *Cinnamon verum*- yogurt made from cow, camel and goat milk under stimulated gastrointestinal digestion

9.1 INTRODUCTION

Chronic hypertension plays a key role in the development of cardiovascular diseases i.e. arteriosclerosis, stroke and myocardial infraction which lead to renal disease in the end-stage (Nejati *et al*., 2013). The high cost and side effects associated with hypertension drugs have encouraged the scientific community to look for alternatives (Miller *et al*., 2007). Other resources of anti- ACE peptides have been identified from animal (chicken muscle, sardine and tuna muscle) and plant proteins (water-soluble extracts of broccoli, mushroom, garlic, buckwheat and wine) as well as in protein hydrolysates of soybean, mung beans, sunflower, rice, corn, wheat, buckwheat and spinach (Guang and Phillips, 2009). The dietary approach is preferable because minimal side effects were experienced in comparison to synthetic drugs. The digestion of certain food can results in the formation of active peptides with anti-hypertension activity (Miguel *et al*., 2006; Quirós *et al*., 2008). These bioactive peptides are liberated from the native protein *in vivo* by digestive proteases or by enzymatic hydrolysis secreted by microorganism during fermentation (Pescuma *et al*. 2011). It must be borne in mind that the ingested bioactive peptides are subjected to further hydrolysis by digestive enzymes present in gastrointestinal tract such as pepsin, trypsin, chymotrypsin and peptidases into peptides with different lengths of amino acid sequences (Hernandez-Ledesma *et al*., 2004; Lignitto *et al*., 2010). The potency of yogurt derived bioactive peptides against ACE-1 activity has been studied in relation to exposure of these peptides to stomach and intestinal enzymes (Hernandez-Ledesma *et al*., 2004 and Lignitto *et al*., 2010). The enhanced anti-ACE-1 activities associated with herbal extracts or type of milk used as shown in chapter 8 may imply unique properties of *A. sativum* or *C. verum* and milk interactions towards preferential formation of bioactive peptides, some of which may

be resistant to digestive enzyme actions. Therefore, the aim of this work was to determine the effects of *A. sativum* or *C. verum* water extract on yogurt made from cow, camel and goat milks with respect to the extent of proteolysis and inhibition of ACE after being subjected to *in vitro* gastrointestinal digestion.

9.2 MATERIALS AND METHODS

9.2.1 Substrates and chemicals

All the substrates and chemicals used in this study are as described in Sections 7.2.1 and 8.2.1.

9.2.2 Experimental designs

This study examined the extent of milk proteins degradation and the ACE-1 inhibitory activity of the yogurt after being subjected to *in vitro* gastrointestinal digestion, in stomach (1 hour) and in intestine (1 and 2 hours). The design of samples used in the present study was performed as described in Section 7.2.2.

9.2.3 Plant water extraction procedure

The water extract of *A. sativum* or *C. verum* was obtained as described in Section 3.2.3.

9.2.4 Yogurt manufacturing process

9.2.4.1 Starter culture and yogurt preparation

The starter culture preparation was carried out using the method described in Section 3.2.4.1. The three types of bio-yogurt made from cow, camel or goat milks in the presence and absence of *A. sativum* or *C. verum* water extract were prepared as described in Section 3.2.4.2.

9.2.5 *In vitro* gastrointestinal model

9.2.5.1 Preparation of gastric and duodenum juices

The gastric and duodenum juices were prepared as described in section 7.2.5.1.

9.2.5.2 Simulation of gastrointestinal digestion (SGD)

The gastrointestinal digestion of yogurt was carried out as detailed in section 7.2.5.2. Treated samples at hourly digestion were collected for through 3 hours digestion period. Samples were centrifuged (10000 rpm; 10 min) to facilitate the removal of impurities and the supernatants were collected and used in further analysis.

9.2.6 Determination of proteolytic activity

The extent of proteolysis and total soluble protein (TSP) were determined by OPA and Lowry protein methods respectively as described in Sections 3.2.6 and 8.2.5.2.

9.2.7 ACE-I inhibition assay

ACE-I inhibition activity of plain and herbal yogurt following *in vitro* gastrointestinal digestion was performed as described in Section 8.2.6.

9.2.8 Statistical analysis

Statistical analysis of all obtained data was performed as described in Section 3.2.8.

9.3 RESULTS

9.3.1 The extent of proteolysis after SGD

A) Free amino groups in herbal water extract after SGD

Gastric digestion of *A. sativum* water extract showed higher ($p<0.05$) free amino groups (15.67±1.0 µg/g) than *C. verum* water extract (9.1±1.7 µg/g; Table 9.1). The intestinal digestion for 2 hours showed significant decrease in free amino groups for *A. sativum* but not for *C. verum* water extract treatments (Table 9.1).

Table 9.1 Extent of proteolysis after simulated gastrointestinal digestion (SGD) of *A. sativum* and *C. verum* water extracts.

Time (hrs)	AS water extract (µg/g)	CV water extract (µg/g)
1	15.67±1.0*	9.1±1.7
2	7.85±0.2	5.2±1.2
3	7.19±0.4	4.1±1.6

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts before digestion = 0.1g/ml. 1st hour represent *in vitro* gastric digestion, 2nd and 3rd hours represent 1 and 2 hours *in vitro* intestinal digestion respectively. Results are shown as mean (n = 3) ± standard error. *$p < 0.05$

B) Proteolysis of cow milk and cow milk- yogurt after SGD

Proteolysis of cow milk yield 367.7±2.5 µg/g after the 1st hour of gastric digestion (Figure 9.1). Proteolysis increased ($p<0.05$) in the presence of *A. sativum* water extract in milk (461.4±3.6 µg/g) but not in the presence of *C. verum* (379.34±2.4 µg/g). Intestinal digestion for 2 hours decreased proteolysis of cow milk in the absence and presence of *A. sativum* and *C. verum* water extracts (313.8±2.1 µg/g ($p<0.05$), 350.4±2.0 µg/g ($p<0.05$) and 365.2±1.6 µg/g respectively). Fresh yogurt showed almost similar proteolysis with those in milk after 1 hour gastric digestion. However, the proteolysis after the 1st and 2nd hours of intestinal digestion was significantly higher for all yogurt treatments when compared with corresponding milk treatments (Figure 9.1). Both plain and herbal yogurt showed similar extent of proteolysis after intestinal digestion compared to after gastric digestion. Seven days

old yogurt showed similar extent of proteolysis after gastric digestion (Figure 9.1). Intestinal digestion for one hour showed higher ($p<0.05$) extent of proteolysis for *A. sativum*- and *C. verum*-yogurt (527.2±1.3 µg/g and 470.2±1.9 µg/g respectively) than plain yogurt (398.2±1.5 µg/g). However, significant reduction in the extent of proteolysis was occurred by the 2nd hour of intestinal digestion in all treatments.

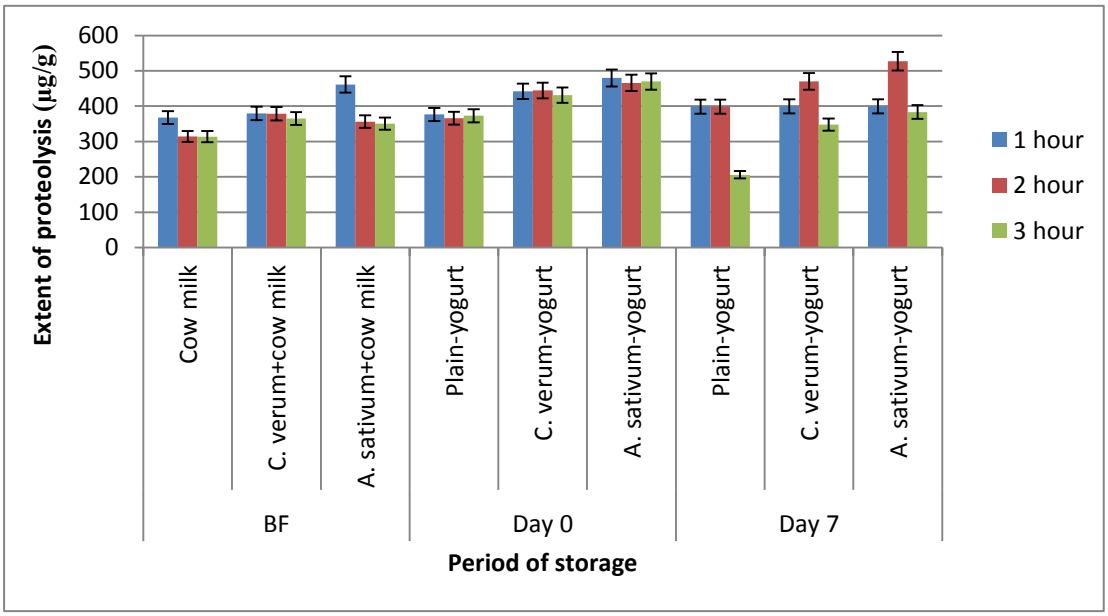


Figure 9.1 Extent of proteolysis (µg/g) of cow milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) Proteolysis of camel milk and camel milk- yogurt after SGD

The extent of proteolysis in gastric section after 1 hour *in vitro* digestion increased from 381.5± 2.6 µg/g for camel milk to 450.13± 2.3 µg/g for fresh yogurt but decreased to 347.8± 2.7 µg/g for 7 days old yogurt (Figure 9.2). The extent of proteolysis in milk was not affected by *A. sativum* or *C. verum* water extract after 1 hour gastric digestion but it tended to increase in fresh yogurt ($p<0.05$) by *C. verum* (552.5± 2.7 µg/g) and in 7 days old yogurt ($p<0.05$) by both *A. sativum* and *C. verum*

(399.6±2.2 µg/g and 395.6±2.3 µg/g respectively). The proteolysis was reduced in fresh *C. verum*- yogurt to 424.7±1.6 µg/g ($p<0.05$) after the 2nd hour of intestinal digestion and in all other treated samples after 2 hours of intestinal digestion ($p>0.05$).

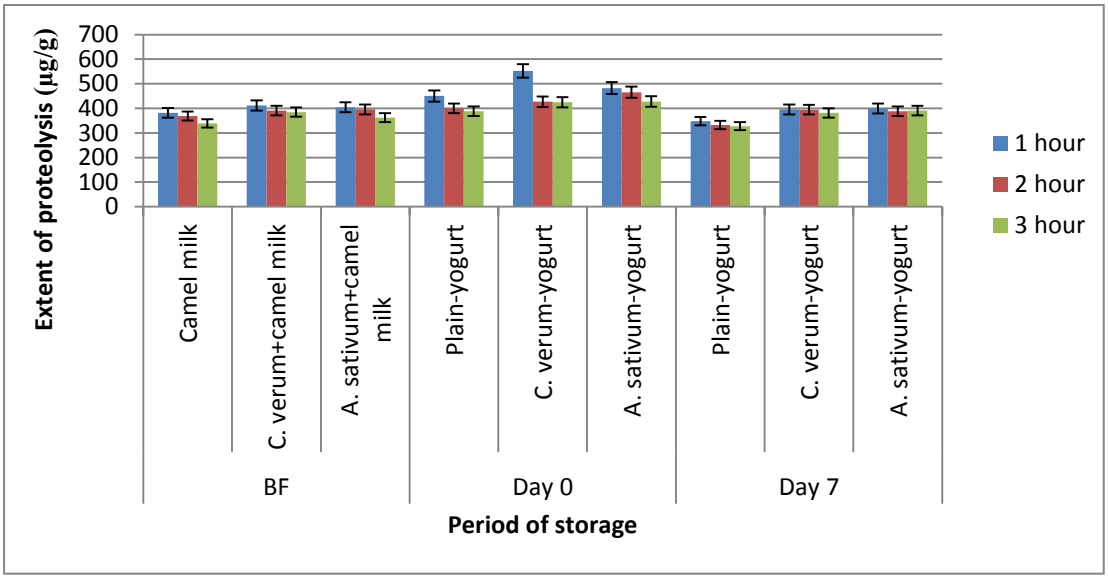


Figure 9.2 Extent of proteolysis (µg/g) of camel milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

D) Proteolysis of goat milk and goat milk- yogurt after SGD

The extent of proteolysis in goat milk both fresh and 7 days old yogurt yielded amount of liberated amino groups ranged from 551 - 627 µg/g after 1 hour gastric digestion (Figure 9.3). The addition of *A. sativum* or *C. verum* water extract tended to increase the proteolysis but this was significant ($p<0.05$) only for goat milk + *A. sativum* (645.2 ±2.9 µg/g) and fresh *A. sativum*- yogurt (661.5±1.2 µg/g). The proteolysis reduced further after intestinal digestion (1 and 2 hours) for milk and yogurt with 7 days old yogurt showing the lowest values (405 - 486 µg/g; Figure 9.3).

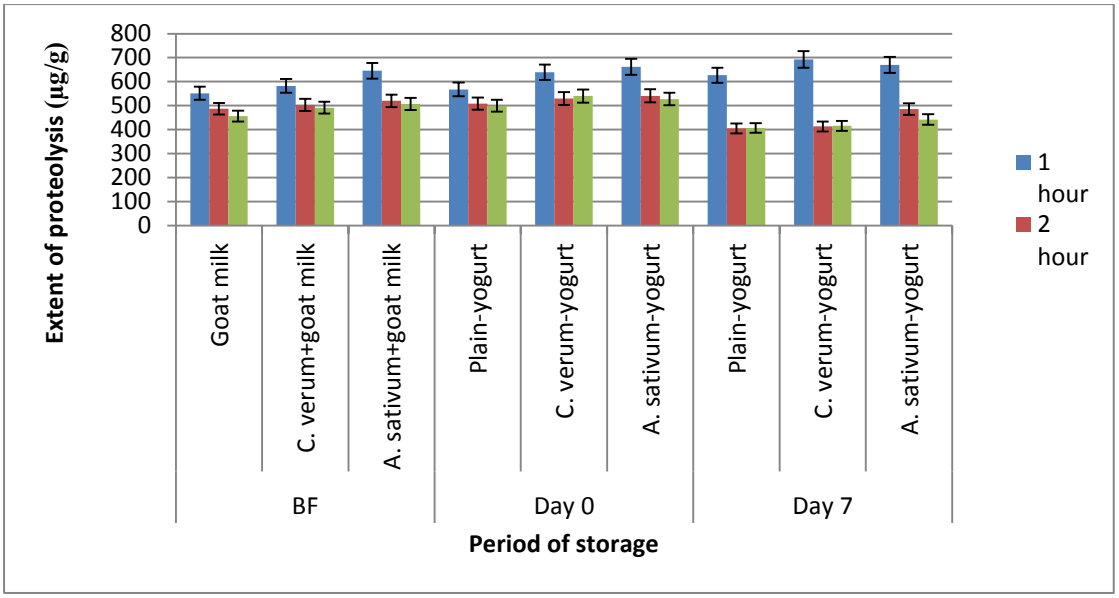


Figure 9.3 Extent of proteolysis (µg/g) of goat milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

9.3.2 Total soluble protein (TSP) after SGD

A) TSP of herbal water extract after SGD

TSP was higher for *A. sativum* water extract (6.5±1.5µg/g; $p<0.05$) than for *C. verum* water extract (4.44±1.1µg/g; Table 9.2) after 1 hour gastric digestion. Intestinal digestion increased TSP to 9.1±0.7 µg/g ($p<0.05$) and 5.08±0.6 µg/g for *A. sativum* and *C. verum* water extracts respectively at the 2nd hour of intestinal digestion (Table 9.2).

Table 9.2 Total soluble protein after simulated gastrointestinal digestion (SGD) of *A. sativum* and *C. verum* water extracts.

Time (hrs)	AS water extract (µg/g)	CV water extract (µg/g)
1	6.5±1.5*	4.44±1.1
2	7.9±1.2	4.49±0.6
3	9.1±0.7	5.08±0.6

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts before digestion = 0.1g/ml. 1st hour represent *in vitro* gastric digestion, 2nd and 3rd hours represent 1 and 2 hours *in vitro* intestinal digestion respectively. Results are shown as mean (n = 3) ± standard error. *p < 0.05

B) TSP of cow milk and cow milk yogurt after SGD

TSP in cow milk after 1 hour gastric digestion (86.0±2.4 µg/g) was not affected by the presence of *C. verum* (94.2±1.1 µg/g) or *A. sativum* (87.2±0.6 µg/g; Figure 9.4). TSP of fresh yogurt increased ($p<0.05$) to 130.9±2.6 µg/g after 1 hour gastric digestion. The presence of *A. sativum* and *C. verum* in yogurt did not affect TSP values (134.2±2.1 µg/g and 137.4±2.0 µg/g respectively). Seven days old yogurt showed TSP values in the same range as fresh milk ± herbal extracts. TSP in milk and yogurt after 1 and 2 hours intestinal digestion reduced ($p<0.05$) to 55 - 60 µg/g for milk and 7- days old yogurt (Figure 9.4). However, fresh yogurt had higher TSP (80 - 90 µg/g; $p<0.05$) after 1 and 2 hours intestinal digestion than 7 days old yogurt.

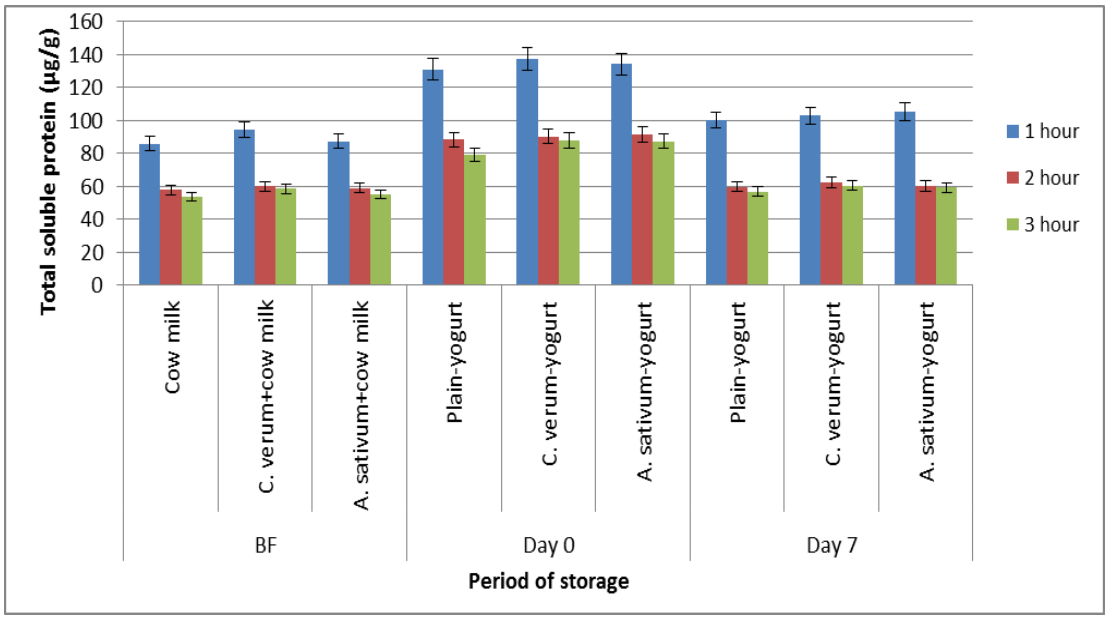


Figure 9.4 Total soluble protein (µg/g) of cow milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) TSP of camel milk and camel milk yogurt after SGD

TSP after 1 hour gastric digestion increased from 118.6±1.9 µg/g for camel milk to 145.9±1.2 µg/g for fresh yogurt (Figure 9.5). Seven days old yogurt showed similar range of TSP as in fresh milk. TSP values in milk and yogurt were not affected by the presence of *A. sativum* or *C. verum* water extract after gastric digestion. More than 50% reduction in TSP occurred as gastric digested milk/ yogurt moved to intestine (70 - 90 µg/g; Figure 9.5).

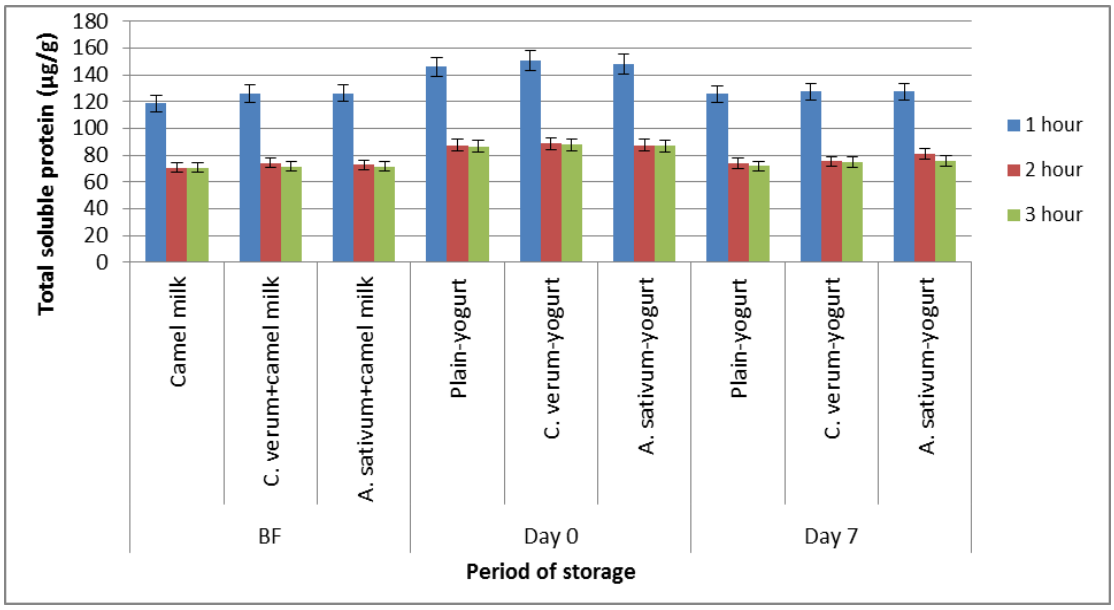


Figure 9.5 Total soluble protein (µg/g) of camel milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

D) TSP of goat milk and goat milk yogurt after SGD

There was no significant effect of *A. sativum* or *C. verum* on the TSP in milk subjected to SGD as compared to milk alone (Figure 9.6). However, fresh and 7 days old yogurt had higher TSP (127.4±1.4 µg/g and 118.63±1.3 µg/g respectively; $p<0.05$) than fresh milk after gastric digestion but the presence of both herbal extracts had no effect on TSP values ($p>0.05$). Intestinal digestion for 2 hours reduced TSP

content in all yogurt treatments with lower values recorded in fresh yogurt (45 - 50 µg/g; $p<0.05$) than in 7- days old yogurt (72 - 80 µg/g; Figure 9.6).

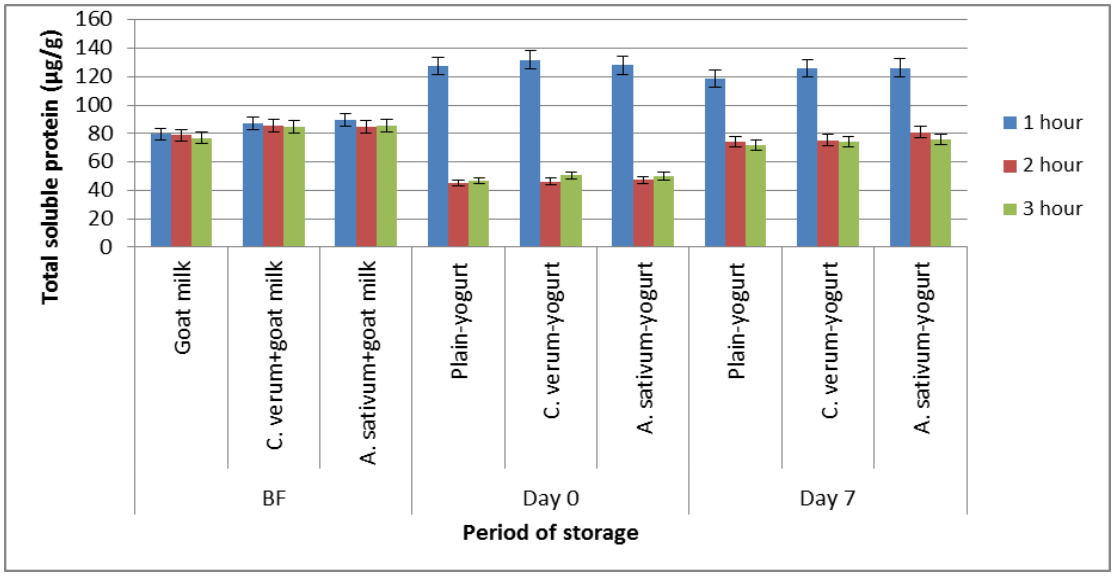


Figure 9.6 Total soluble protein (µg/g) of goat milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

9.3.3 ACE-I inhibitory activity after SGD

A) Anti- ACE activity of herbal water extract after SGD

There was no significant difference ($p>0.05$) in ACE-I inhibitory activity between *A. sativum* and *C. verum* water extracts (50.4±2.3 % and 46.6±2.1 % respectively) after being subjected to 1 hour gastric digestion (Table 9.3). However, ACE-I inhibitory activity decreased ($p>0.05$) for both herbal extracts after 2 hours intestinal digestion.

Table 9.3 ACE-I inhibitory activity after simulated gastrointestinal digestion (SGD) of *A. sativum* and *C. verum* water extracts.

Time (hrs)	AS water extract (%)	CV water extract (%)
1	50.4±2.3	46.6±2.1
2	48.1±2.2	42.1±2.5
3	44.1±1.7	38.6 ±2.1

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts before digestion = 0.1g/ml. 1st hour represent *in vitro* gastric digestion, 2nd and 3rd hours represent 1 and 2 hours *in vitro* intestinal digestion respectively. Results are shown as mean (n = 3) ± standard error. *p < 0.05

B) Anti- ACE activity of cow milk and cow milk yogurt after SGD

ACE-I inhibitory activity of cow milk was 13.2±1.7 % (Figure 9.7). The presence of *A. sativum* or *C. verum* water extract increased ($p<0.05$) ACE-I inhibition to 26.9±1.4 % and 15.7±1.5 % respectively after the 1st hour of gastric digestion. Intestinal digestion (2 hours) of cow milk showed higher (40%; $p<0.05$) ACE-I inhibition activity compared to gastric digestion. The presence of *C. verum* water extract in milk increased ACE-I inhibition to similar extent of milk after 2 hours intestinal digestion whereas the presence of *A. sativum* showed 46% ($p<0.05$) of ACE-I inhibition (Figure 9.7). Fresh *A. sativum*- and *C. verum*-yogurt showed no significant increase in ACE-I inhibitory activity (43% and 42% respectively) compared to control (38%) after the 1st hour of gastric digestion (Figure 9.7). Intestinal digestion increased the inhibition of ACE to 50% for all types of yogurt. Refrigerated storage of yogurt (7days) reduced ($p<0.05$) ACE-I inhibition after gastric digestion to about 24% for plain- and *C. verum*- yogurt compared to fresh yogurt. However, 7 days old *A. sativum* yogurt had higher ACE-I inhibition (32%; $p<0.05$) than plain yogurt after gastric digestion. Intestinal digestion of refrigerated storage yogurt increased ($p<0.05$) ACE-I inhibition activity from 30% to 40% for all types of yogurt (Figure 9.7).

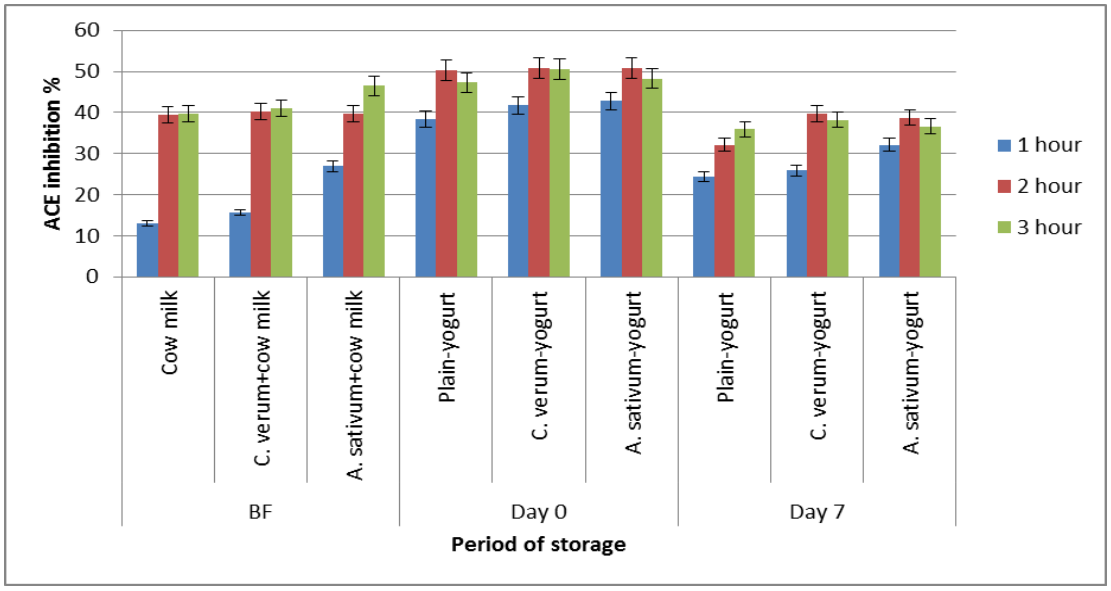


Figure 9.7 ACE-I inhibitory activity (inhibition %) of cow milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) Anti- ACE activity of camel milk and camel milk yogurt after SGD

Camel milk showed 20% inhibition in ACE-I activity after 1 hour gastric digestion. The presence of *A. sativum* water extract increased about 10% of the inhibition in milk after 1 hour gastric digestion whereas *C. verum* had no effect on the inhibition (Figure 9.8). Fresh and 7 days old yogurt had higher ACE-I inhibitory activity (~ 37%; $p<0.05$) than fresh milk after gastric digestion but the presence of both herbal extracts did not change ACE-I inhibition. Intestinal digestion showed small reduction ($p>0.05$) of ACE-I inhibitory activity compared to gastric digestion in all fresh milk samples and 7 days old yogurt (Figure 9.8). However, the inhibition activity of ACE-I increased in fresh yogurt (47% -50%; $p<0.05$) after two hours of intestinal digestion.

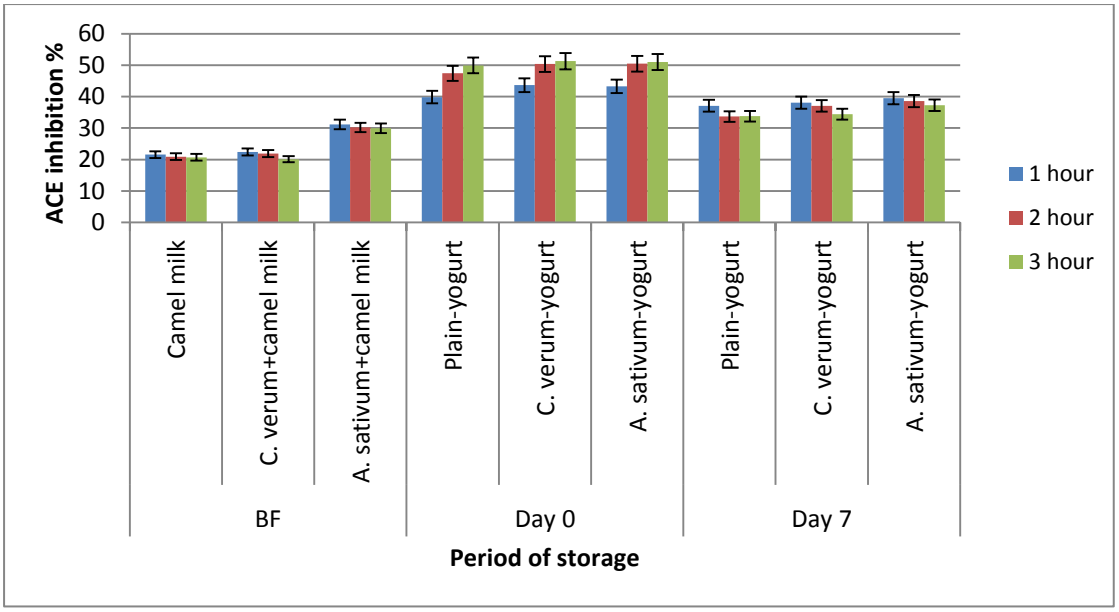


Figure 9.8 ACE-I inhibitory activity (inhibition %) of camel milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

D) Anti- ACE activity of goat milk and goat milk yogurt after SGD

Goat milk and yogurt after 1 hour gastric digestion showed ACE-I inhibitory activity ranged between 35% and 40% (Figure 9.9). ACE-I inhibitory activity in all treated samples after 1 hour gastric digestion was not affected by the presence of *A. sativum* or *C. verum* water extract. Intestinal digestion (1 and 2 hours) did not change the milk± herbal extracts and fresh yogurt inhibition of ACE-I activity but it tended to increase that in 7 days old yogurt (44%- 47%; $p<0.05$).

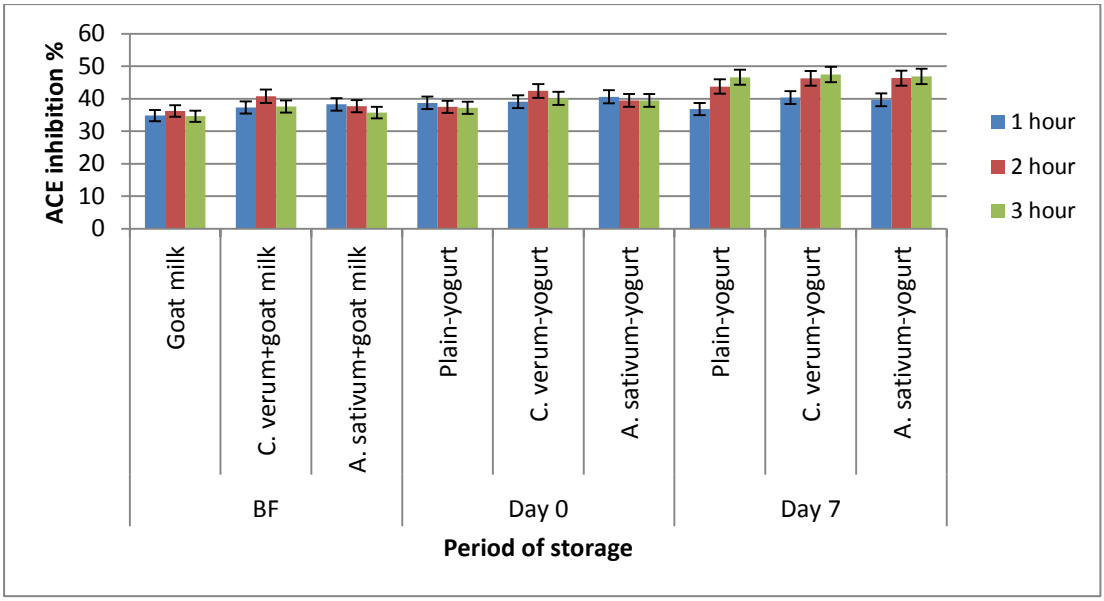


Figure 9.9 ACE-I inhibitory activity (inhibition %) of goat milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

9.4 DISCUSSION

9.4.1 The extent of proteolysis after SGD

Milk proteins are considered the most important source of bioactive peptides (Fitzgerald and Murray, 2006). Digestion of milk proteins liberates peptides with medium and low molecular weight (Korhonen and Pilanto, 2006; Korhonen, 2009). This was demonstrated *in vitro* whereby the hydrolysis of milk caseins and whey proteins with gastric and pancreatic enzymes causes the release of bioactive peptides (Vithana *et al*., 2012). These bioactive peptides can either be absorbed through the intestine to enter the blood system intact (Opatha Vithana *et al*., 2012) and exert physiological effects (Fujita *et al.*, 2003; Papadimitriou *et al*., 2007) or produce local effects in the gastrointestinal tract (Shimizu and Son, 2007). In the present study, the liberation of free amino groups in milk/ yogurt after SGD was demonstrated.

However, the presence of herbal extracts in cow, camel and goat milk/ yogurt showed some influence on the extent of proteolysis after SGD which was higher in presence of *A. sativum* than *C. verum*. This is in agreement with other studies which showed high content of amino acids in garlic (Abdullah *et al.*, 1988; Augusti and Sheela, 1996).

To simulate *in vivo* digestion, the present study has used *in vitro* digestion commercial pepsin and duodenum enzymes; trypsin and pancreatin. Pepsin is an aspartic protease which cleaves peptides at bonds with Phe, Tyr, Trp and Leu in position P1 or P1' (Fujimoto *et al*., 2004). Trypsin prefers to cleave peptides on the carboxyl side of the basic amino acids, arginine or lysine (Antal *et al*., 2001). Therefore the digestion profile with these enzymes will be different depending on the milk protein structures and this could lead to differences in digestibility subsequently different bioactivity of digestive products (Merin *et al*., 2001; Park *et al*., 2007). The present study showed that the proteins in goat milk/ yogurt were more digestible and produced more free amino groups than proteins from cow- and camel- milk yogurt. Natural homogenization of goat milk makes this milk easier to digest because of low molecular weight caseins (Park, 2007; Minervini *et al*., 2009). Park, (2007) reported that "goat milk have the same proteins as cow milk but their proportions and genetic polymorphs differ widely". The present study also showed both cow and camel milk yogurt have almost similar digestibility and liberated free amino groups to similar extent. Indigenous milk proteinases and viability of LAB used as starter cultures may result in formation of different peptides with different lengths (Park *et al*., 2007; Pescuma *et al*. 2011). The significant decrease of soluble protein in all three types of yogurt after intestinal digestion is in disagreement with Opatha Vithana *et al*. (2012) who reported rapid increase of peptide production in the simulated duodenum in deer

and cow milk. This could relate to different duodenum enzymes used in that study which are a mixture of trypsin, chymotrypsin and several amino acid and carboxypeptidases.

9.4.2 ACE-I inhibitory activity after SGD

ACE inhibitors such as captopril, enalapril and lisinopril play an important in cardiovascular treatments by reducing the generation of angiotensin II which acts as vasoconstrictor, thus inhibiting increase in blood pressure. The use of these inhibitors is hampered by common side effects such as coughs, fever, exanthema eruption and leukopenia (Coates, 2003; Kang *et al.,* 2003). Milk proteins are a rich source of bioactive peptides that possess ACE inhibitory activity. These peptides are capable to inhibit ACE through binding interactions to specific receptors on target cells leading to induction of lowers blood pressure (Fitzgerald and Murray, 2006). Several peptides were isolated and identified from fermented milk such as αs1- , ß- and κ-CN fragments from yogurt (Yamamoto *et al*. 1999), *ß*-CN *f* (74–76, *f* (84–86), κ-CN *f* (108–111) from sour milk (Nakamura *et al*. 1995) and Ser-Lys-Val-Tyr-Pro from Dahi (Ashar and Chand, 2004). In addition, phytochemicals from plants were found to act as inhibitors against ACE (Pinto *et al*., 2008; Wu and Muir, 2008; Nileeka *et al*., 2011). In the present study *A. sativum* water extract was shown to have more anti- ACE-I activity than *C. verum* water extract after SGD (Table 9.3). However, this seems not to have an influence on ACE-I inhibition activity when these two herbal extracts were individually mixed with three types of milk/ yogurt. This could be due to the interactions between polyphenols compounds from the herbs and milk proteins as reported in previous studies (Alexandropoulou *et al*., 2006; Argyri *et al*., 2006; Cilla *et al*., 2009). On the other hand, the increase anti- ACE-I activity in milk from cow and camel subjected to SGD in the presence of *A. sativum* could be

related to some bioactive anti- ACE-I phenolic compounds released from *A. sativum*. Seven ACE-inhibitory peptides were isolated from garlic with ability to lower blood pressure in spontaneously hypertensive rats after oral administration (Suetsuna, 1998). Additionally, some phenolic compounds in *A. sativum* such as mycerene and allicin were found to act as inhibitors against ACE (Hosseini *et al.*, 2007).

The proteolytic activity of bacterial enzymes during milk fermentation can generate peptides with ACE-inhibitory activity (Gobbetti *et al*., 2004). The present result showed that after *in vitro* gastrointestinal digestion, ACE-I inhibitory activity from fresh *A. sativum*- and *C. verum*- yogurt made from cow and camel milk were higher than before fermentation (BF). This suggests the fermentation process increased the bioactive peptides derived from milk proteins with ACE-I inhibitory activity. This observation is also in agreement with our findings (Figures 8.6 and 8.7) that showed ACE-I inhibitory activity pre-digestion increased after fermentation (fresh yogurt) as compared to BF (Table 8.4). The significant decrease in ACE-I inhibitory activity of 7 days refrigerated *A. sativum*- and *C. verum*- yogurt made from cow and camel milks as compared to fresh yogurt after SGD was associated with decrease in the extent of proteolysis post-digestion (Figures 9.1 and 9.2).

In contrast, *A. sativum*- and *C. verum*-goat milk mixture (BF) had no significant difference in ACE-I inhibitory activity as compared to fresh yogurt after SGD. This is not surprising since the extent of proteolysis was almost similar in both treatments (Figure 9.3). Moreover, *A. sativum* or *C. verum* mixture with goat milk contained the highest ACE-I inhibitory activity among other treated milk after gastric digestion. This fiddling is again found to be associated with the extent of proteolysis demonstrated after SGD (Figure 9.3).

9.5 CONCLUSIONS

The presence of *A. sativum* or *C. verum* water extract in yogurt made from three types of milk (cow, camel and goat milk) influenced the extent of proteolysis after SGD and ACE-I inhibitory activity. In addition, *A. sativum* has more anti- ACE-I activity than *C. verum* which subsequently influence the yogurt capability to inhibit ACE-I activities. Daily consumption of fresh *A. sativum*- or *C. verum*- yogurt made from cow, camel or goat milk could provide ~50% of ACE-I inhibitory activity after gastrointestinal digestion and this may be a useful dietary approach to manage hypertension.

10.0 Effect of *Allium sativum* or *Cinnamomum verum* enriched yogurt on antioxidant activity and *in vitro* inhibition of α-amylase and α-glucosidase enzymes related to type 2 diabetes

10.1 INTRODUCTION

The enterocytes of the small intestine can only absorb carbohydrate in the form of monosaccharides such as glucose and fructose. Pancreatic α-amylase (E.C. 3.2.1.1) is a key enzyme in the digestive system and it catalyses the initial step in the hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose, and a number of α-(l-6) and α-(1 - 4) oligoglucans. This is followed by the action of α-glucosidase in the brush border of the small intestines to further break down the disaccharides into simpler sugars, readily available for the intestinal absorption (Krentz and Bailey, 2005). Digestion of this dietary starch proceeds rapidly and leads to elevated post-prandial hyperglycemia.

The increase in post-prandial blood glucose correlates with the activity of carbohydrate digestion enzymes in the small intestine (Gupta *et al*., 2003). Therefore retardation of starch digestion by inhibition of enzymes such as α-amylase and α-glucosidase play important role in the control of diabetes. Inhibitors of these enzymes help to delay carbohydrate digestion and prolong overall intestinal carbohydrate retention time, causing a reduction in the rate of glucose absorption and consequently suppressing rapid postprandial plasma glucose rise (Wild *et al*., 2004). This can be a promising strategy in the management of type-2 diabetes (Kwon *et al*., 2006) which forms the basis of the current clinical use of synthetic inhibitors (acarbose, miglitol which inhibit α-glucosidase and α-amylase and voglibose which inhibit α-glucosidase). These inhibitors delay carbohydrate digestion causing a reduction in the rate of glucose absorption and lowering the post-prandial serum glucose levels (Tarling *et al*., 2008). However, many of these synthetic hypoglycemic agents have their limitations, are non-specific, produce serious side effects and fail to eliminate diabetic complications (Sudha *et al*., 2011). The main side effects of these inhibitors

are gastrointestinal *viz*., bloating, abdominal discomfort, diarrhea and flatulence (Cheng and Fantus, 2005). Hence, attention has focused on natural substances that show potent inhibitory activity against α-amylase and α-glucosidase and have fewer side effects (McCue and Shetty, 2004; Kim *et al*., 2006; Ranilla *et al*., 2010). Much of the work related to these enzymes inhibitions has involved the use of plants extracts and some traditional foods (Fujita *et al.,* 2003; Ranilla *et al*., 2010).

The oxidative damage of cell components such as proteins, lipids, and nucleic acids one of the important factors associated with diabetes mellitus (Rahimi *et al*., 2005). This occurs as a result of imbalance between the generations of oxygen derived radicals and the organism's antioxidant potential (Rahimi *et al*., 2005). Natural antioxidants from plant ingredients can be used to control the increase formation of free radicals and decrease in antioxidant capacity in diabetes patients and to replace synthetic antioxidant activity with side effects such as liver damage and carcinogenesis (Meenakshi *et al*., 2009).

Cinnamon (*Cinnamomum verum*) and garlic (*Allium sativum*) which are rich in phenolics compounds with highly antioxidant activity play a mediating role in the inhibition of α-amylase and α-glucosidase activities and thus could be used to manage type 2 diabetes (Broadhurst *et al*., 2000; Jarvill-Taylor *et al*., 2001; Qin *et al.,* 2003). In the fermented dairy products, milk proteins serve as an important source of a range of bioactive peptides encrypted within the sequence of the native proteins and can thus be released during proteolytic activity (Singh and Rakesh Roshan Sharma, 2009). Some of these bioactive peptides are inhibitors of α-glucosidase and α-amylase enzymes, which have a central role in the regulation of blood glucose (Apostolidis *et al*., 2006; Yadav *et al*., 2007). The aims of the current study were to investigate the possibilities of enhancing natural yogurt antioxidant capacity and its ability to inhibit

diabetic enzymes (α-amylase and α-glucosidase) by adding *A. sativum* or *C. verum* as functional ingredients in yogurt making.

10.2 MATERIALS AND METHODS

10.2.1 Substrates and chemicals

Gallic acid, 95% ethanol, 1,1-Diphenyl-2-Picrylhydrazyl (DPPH), porcine pancreatic alpha-amylase, (A3176) type VI-B, 3,5-dinitrosalicyclic acid, potassium-sodium tartrate-4-hydrate, starch soluble, sodium di-hydrogen phosphate (NaH_2PO_4), di-sodium hydrogen phosphate (Na_2HPO_4), sodium hydroxide pellets (NaOH), α-glucosidase (EC 3.2.1.20), *p*-nitrophenyl-α-D-glucopyranoside, di-potassium hydrogen phosphate (K_2HPO_4) and other chemicals were purchased from Sigma Chemical Company (St Louis, MO USA). Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), sodium chloride (NaCl) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Merck (Santa Ana, CA, USA).

10.2.2 Experimental design

This chapter reports the inhibitory activity of α-amylase, α-glucosidase and phenolic-linked antioxidant activity of set bio-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract. Two groups of set bio-yogurt were prepared from cow and camel milk. Each group contained 3 treatments consisting of *A. sativum*-, *C. verum*- and plain- yogurt. The yogurt was used to evaluate *in vitro* inhibition of α-amylase and α-glucosidase activities and IC_{50}, total phenolic content (TPC) and antioxidant activity of yogurt after fermentation and during 21 days of refrigerated storage.

10.2.3 Plant water extraction procedure

The water extraction of *A. sativum* or *C. verum* was performed according to the method described in Section 3.2.3.

10.2.4 Yogurt manufacturing process

10.2.4.1 Starter culture and yogurt preparation

The starter culture preparation was carried out using the method described in Section 3.2.4.1.The two groups of bio-yogurt made from cow or camel milk both in the presence and absence of *A. sativum* or *C. verum* water extract were prepared as described in Section 3.2.4.2.

10.2.5 Sample preparation (yogurt water extract)

The yogurt water extract was performed as described in Section 3.2.7.1.

10.2.6 α-Amylase inhibition assay

The α-amylase inhibition assay was adapted from Apostolidis *et al*. (2006). Yogurt water extract (500µl) and 500µl of 0.02M sodium phosphate buffer, pH6.9 with 0.006M sodium chloride containing 0.5mg/ml α-amylase solution were pre-incubated at 37°C for 10 minutes. This was followed by the addition of 500µl of 1% starch solution in 0.02M sodium phosphate buffer, pH6.9 with 0.006M sodium chloride to each test tube at pre-determined time intervals. The reaction mixtures were then re-incubated at 37°C for 10 minutes. The reaction was stopped with 1.0ml of dinitrosalicyclic acid (DNSA, 1 g dissolved in 100 ml NaOH) color reagent. The test tubes were then incubated in a boiling water bath for 7 minutes followed by cooling to room temperature (25°C) after which 1.0ml of 18.2% tartrate solution was added to each tube. Distilled water (10ml) was then added to dilute the reaction mixture followed by absorbance reading at 540nm (Spectrophotometer, Shimadzu UV Mini 1240). The readings were compared to control, which had 500µl of buffer solution instead of the water extract of yogurt. The enzyme inhibition was calculated as follows:

$$\text{Inhibition percentage} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100 \quad (9.1)$$

10.2.6.1 Determination of inhibitory concentration (IC_{50}) of yogurt water extracts toward α-amylase activity

IC_{50} refers to the concentration of tested substance to inhibit 50% of enzymes activity. This value may be used to compare the effectiveness of *A. sativum*- and *C. verum*- yogurt as inhibitors of α-amylase enzyme activity compared to plain-yogurt (control). In the present study, in addition to 500µl of yogurt water extract (see Section 10.2.6), 2 other volumes (250 µl and 125µl of yogurt water extracts were mixed with 250 µl and 375 µl buffer solution respectively) were also tested. By assuming that the activity of the blank is 100%, IC_{50} can be obtained by linear regression of plots (plotting a graph of percentage of inhibition against the 3 different volumes of yogurt water extracts).

10.2.7 α-Glucosidase inhibition assay and IC_{50}

The α-glucosidase inhibition assay was performed as described by Apostolidis *et al*., (2006). Yogurt water extract (500 µl) and 1000 µl of 0.1 M potassium phosphate buffer (pH 6.90) containing α-glucosidase solution (1.0 U/ml) was incubated in the water bath (37°C) for 10 minutes. This is followed by the addition of 500µl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M potassium phosphate buffer (pH 6.90) to each tube at predetermined time intervals. The reaction mixtures were re-incubated at 37°C for 5 minutes. Absorbance was read at 405 nm (Spectrophotometer, Shimadzu UV Mini 1240) and the the readings were compared to the control treatment which had 500µl of buffer solution instead of the yogurt water extract. The α-glucosidase inhibitory activity was expressed as inhibition percentage using equation (9.1) in Section 10.2.6 and IC_{50} was calculated as described for α-amylase (Section 10.2.6.1).

10.2.8 Total phenolic assay

The total phenolic content in yogurt water extract was determined using Folin–Ciocalteu method as described by Shetty *et al*. (2008). One millilitre of standard solution or yogurt water extract was transferred into a test tube and this was mixed with 1ml of 95% ethanol and 5ml of dH_2O. Folin–Ciocalteu reagent (0.5ml of 50% v/v) was added to each test tube followed by a thorough mixing. After 5 min, 1ml of 5% Na_2CO_3 was added and the reaction mixture was allowed to stand for 60 min at room temperature (25°C). The absorbance of the resulting blue color was measured at 725 nm (Spectrophotometer, Shimadzu UV Mini 1240). The absorbance values were converted to total phenolics (expressed in micrograms equivalents of gallic acid per gram; µg GAE/g) from standard curve constructed using various concentrations of gallic acid (10 - 60 µg/g) in 95% ethanol and run each time assay was carried out. Typical equation of the standard curve (Figure 10.1) is as follows:

$$\text{Total phenolic content (µg GAE/g)} = \frac{A_{725} - 0.0017}{0.0083}$$

where A_{725} was the spectrophotometric absorbance at 725 nm

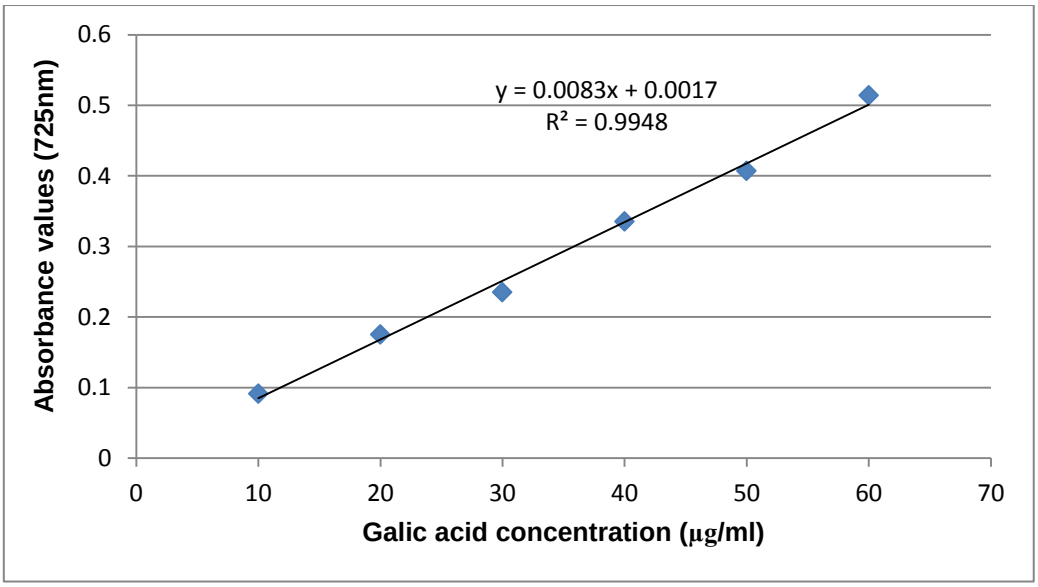


Figure 10.1 Typical calibration curve for total phenolic content.

10.2.9 Antioxidant activity by 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) inhibition assay

The antioxidant activity of yogurt water extract was determined using 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) inhibition assay as described by Shetty *et al*. (2008). An aliquot of the yogurt water extract (250µl) was added to 3ml of DPPH (60 µM in 95% ethanol). The mixture was shaken vigorously and allowed to stand at room temperature (25°C) for several minutes. The absorbance was then measured at 517 nm (Spectrophotometer, Shimadzu UV Mini 1240) against controls, which contained 250µl of 95% ethanol instead of the extract. The inhibition percentage of DPPH oxidation by yogurt water extract was calculated with the same equation (9.1) as for inhibition percentage in the α-amylase inhibition assay (Section 10.2.6).

10.2.10 Statistical analysis

Statistical analyses were performed as described in Section 3.2.8. The gallic acid standard curve used to calculate TPC was plotted as described in Section 3.2.8 using Microsoft® Excel XP.

10.3 RESULTS

10.3.1 *In vitro* α-amylase inhibitory activity

A. sativum water extract had lower (25.4±1.5%) α-amylase inhibitory activities than *C. verum* water extract (73.7±2.1%; Table 10.1). Cow milk showed 15.8±0.8% inhibition activity of α-amylase while the inclusion of *A. sativum* or *C. verum* water extract resulted in α-amylase inhibition of 32.1±1.7% and 24.9±0.8% respectively ($p<0.05$; Table 10.2). On the other hand, camel milk has higher α-amylase inhibition activity (23.2±2.1%) than cow milk. The mixture of *A. sativum* or *C. verum* with camel milk showed higher α-amylase inhibitory activities (34.3±2.4% and 32.2±2.2% respectively; $p<0.05$) compared to plain milk (Table 10.2).

Figure 10.2 shows the α-amylase inhibition activity by yogurt made from cow milk during 0, 7, 14 and 21 days refrigerated storage at 4°C. Inhibition of α-amylase by fresh yogurt (0 day) was 26.4 ± 1.5%. The addition of *A. sativum* or *C. verum* water extract in yogurt increased ($p<0.05$) the inhibition of α-amylase to 34.3 ± 3.2% and 55.8 ± 3.8% respectively. Refrigerated storage for 21 days decreased plain-yogurt inhibition to between 15-20%. In contrast to *C. verum* yogurt which underwent reduction in α-amylase inhibition (38.4 ± 2.0%, 21 days), *A. sativum*- yogurt had a transient increased capacity to inhibit α-amylase on the 7th day of storage (48.1 ± 1.9%) before settling to lower inhibition capacity of 38.7 ± 3.3% and 33.1 ± 1.2% on day 14 and 21 of storage respectively.

Inhibition of α-amylase activity by fresh camel milk yogurt was 33.2 ± 1.4% (Figure 10.3). This value was unchanged after 7 days of refrigerated storage but increased to 48.8 ± 1.2% on the 14th day of storage prior to reduction to 32.8 ± 2.7% on the 21st day of storage. Both fresh *A. sativum*- and *C. verum*- yogurt made from camel milk showed higher inhibition of α-amylase (56.4 ± 1.3% and 58.5 ± 2.6% respectively; $p<0.05$) than plain-yogurt (Figure 10.3). The capacity to inhibit α-amylase by *C. verum*-yogurt did not change as a result of storage up to 21 days although there was a transient decrease in the inhibitory value on the 7th day of storage (48.6± 1.0%). *A. sativum*-yogurt showed almost similar inhibition of α-amylase as *C. verum*-yogurt due to storage, except that the inhibition on day 21 of storage (41.8 ± 1.9%) was lower than in *C. verum* yogurt (54.1± 2.2%).

Table 10.1 Inhibition of α-amylase and IC_{50} in *A. sativum* and *C. verum* water extracts.

Samples	Inhibition (%)	IC_{50} (µg/g)
AS	25.4±1.5	0.18±0.005
CV	73.7±2.1*	0.06±0.002*

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts = 0.1g/ml. Results are shown as mean (n = 3) ± standard error. *p < 0.05

Table 10.2 Inhibition of α -amylase in cow and camel milk in the absence and presence of *A. sativum* or *C. verum* water extract.

Samples	Inhibition (%)
Cow milk	15.8±0.8
AS+cow milk	32.1±1.7*
CV+cow milk	24.9±0.8*
Camel milk	23.2±2.1
AS+camel milk	34.3±2.4*
CV+camel milk	32.2±2.2*

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

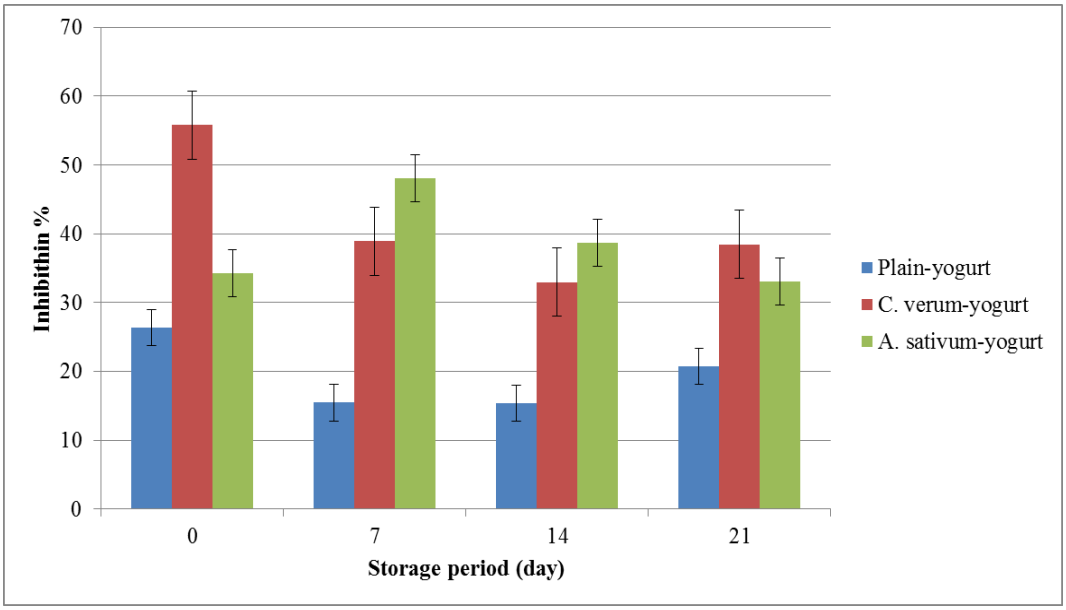


Figure 10.2 α-Amylase inhibitory activities (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

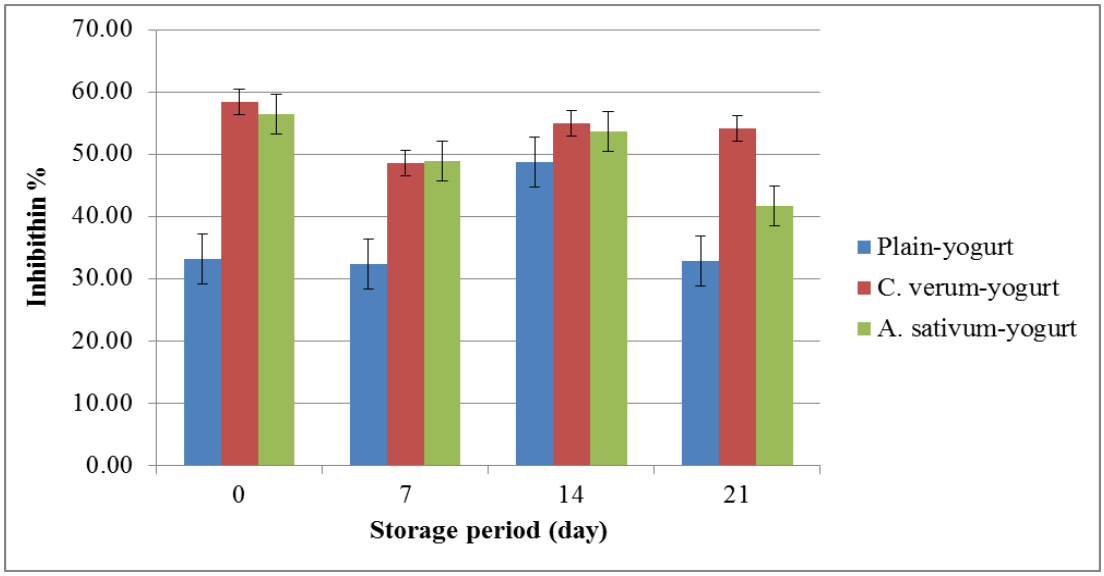


Figure 10.3 α-Amylase inhibitory activities (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

10.3.1.1 α-Amylase inhibitory activity (IC_{50})

C. verum water extract (0.06±0.002 µg/g; IC_{50}) was more potent than *A. sativum* water extract (0.18±0.005 µg/g) to inhibit α-amylase (Table 10.1). The addition of *A. sativum* or *C. verum* into cow and camel milk did not change the IC_{50} of α-amylase inhibition compared to plain milk (Table 10.3).

A. sativum-yogurt made from cow milk showed the highest inhibition activity on α-amylase (IC_{50}) followed by *C. verum*- and plain-yogurt during refrigerated storage (Table 10.4). The maximum effect of cow milk yogurt (IC_{50}) toward α-amylase inhibition was seen on day 7 of refrigerated storage (22.43±0.85 µg/g, 18.89±0.79 µg/g and 19.93±0.70 µg/g) for plain-, *A. sativum*- and *C. verum*-yogurt respectively. Both fresh *A. sativum*-and *C. verum*- yogurt made from camel milk showed higher ($p<0.05$) inhibition on α-amylase activity (20.23±0.39 µg/g and 21.49±0.18 µg/g respectively; IC_{50}) than plain- yogurt (26.24±0.01 µg/g; Table 10.4). α-Amylase inhibition (IC_{50}) was increased ($p<0.05$) on day 7 (16.92±0.56 µg/g) and

14 (16.90±0.68 µg/g) of refrigerated storage for *A. sativum*- and *C. verum*- yogurt respectively.

Table 10.3 IC_{50} values for α-amylase inhibitors in cow and camel milk in the absence and presence of *A. sativum* or *C. verum* water extract.

Samples	IC_{50} µg/g
Cow milk	8.57±0.11
AS+cow milk	8.49±0.65
CV+cow milk	8.55±0.84
Camel milk	7.27±0.35
AS+camel milk	7.01±0.21
CV+camel milk	7.25±0.07

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as a mean (n = 3) ± standard error. *p < 0.05 as compared to control.

Table 10.4 IC_{50} values for α-amylase inhibitors in *A. sativum*- and *C. verum*- yogurt made from cow or camel milk during 21 days of refrigerated storage.

Samples	IC_{50} (µg/g)			
	0 day	**7 day**	**14 day**	**21 day**
Plain-cow milk yogurt	35.31±0.17	22.43±0.85	34.66±0.63	35.76±0.89
AS-cow milk yogurt	28.86±0.32*	18.89±0.79	24.63±0.93*	26.28±0.29*
CV-cow milk yogurt	31.58±0.55	19.93±0.70	28.43±0.36*	28.23±0.39*
Plain-camel milk yogurt	26.24±0.01	24.08±0.67	22.20±0.75	27.09±0.96
AS-camel milk yogurt	20.23±0.39*	16.92±0.56*	19.80±0.35	22.38±0.63*
CV-camel milk yogurt	21.49±0.18*	21.33±0.23	16.90±0.68*	17.93±0.24*

AS= *A. sativum* and CV= *C. verum*. Plain-cow milk yogurt and plain-camel milk yogurt presented as controls. Results are shown as mean (n = 3) ± standard error. *p < 0.05 as compared to control.

10.3.2 *In vitro* α-glucosidase inhibitory activity

α-Glucosidase inhibition by *A. sativum* or *C. verum* water extract was 2.6±0.01% and 3.9±0.0% respectively (Table 10.5). Cow and camel milk showed 9.1±0.5% and 5.7±0.7% inhibition of α-glucosidase activity respectively (Table 10.6). The mixture of *A. sativum* or *C. verum* water extract with cow or camel milk increased ($p>0.05$) the inhibition of α-glucosidase activity as compared to respective controls (Table 10.6).

Fresh cow milk yogurt inhibited 11.3 ± 0.4% of α-glucosidase activity (Figure 10.4). Fresh *A. sativum*- or *C. verum*-yogurt increased ($p<0.05$) the inhibition of α-glucosidase to similar value (15%). Refrigerated storage for 21 days decreased the inhibition of α-glucosidase by plain-yogurt to 5.5 ± 0.2% whereas *A. sativum*- and *C. verum*-yogurt showed slower reduction in α-glucosidase inhibition to 12.8 ± 0.4% and 9.0 ± 0.5% respectively (Figure 10.4).

Inhibition of α-glucosidase by fresh camel milk yogurt was 8.4 ± 0.2% (Figure 10.5). Enzyme inhibition was higher ($p<0.05$) by both *A. sativum*- and *C. verum*-yogurt at similar inhibition value (11.70%). Refrigerated storage gradually increased the ability of yogurt to inhibit α-glucosidase to 13.7 ± 0.7%, 17.0 ± 0.6% and 18.8 ± 0.5% by day 21 of refrigerated storage for plain-, *C. verum*- and *A. sativum*-yogurt respectively (Figure 10.5).

Table 10.5 Inhibition of α-glucosidase and IC_{50} in *A. sativum* and *C. verum* water extracts.

Samples	Inhibition (%) 500 µl	IC_{50} (µg/g)
AS	2.6±0.01	0.15±0.032
CV	3.9±0.0	0.04±0.006

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts = 0.1g/ml. Results are shown as mean (n = 3) ± standard error. *p < 0.05

Table 10.6 Inhibition of α - glucosidase by cow and camel milk in the absence and presence of *A. sativum* or *C. verum* water extract.

Samples	Inhibition (%)
Cow milk	9.1±0.5
AS+cow milk	10.5±0.4
CV+cow milk	12.5±0.8
Camel milk	5.7±0.7
AS+camel milk	6.7±0.2
CV+camel milk	7.0±0.2

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as a mean (n = 3) ± standard error. *p < 0.05 as compared to control.

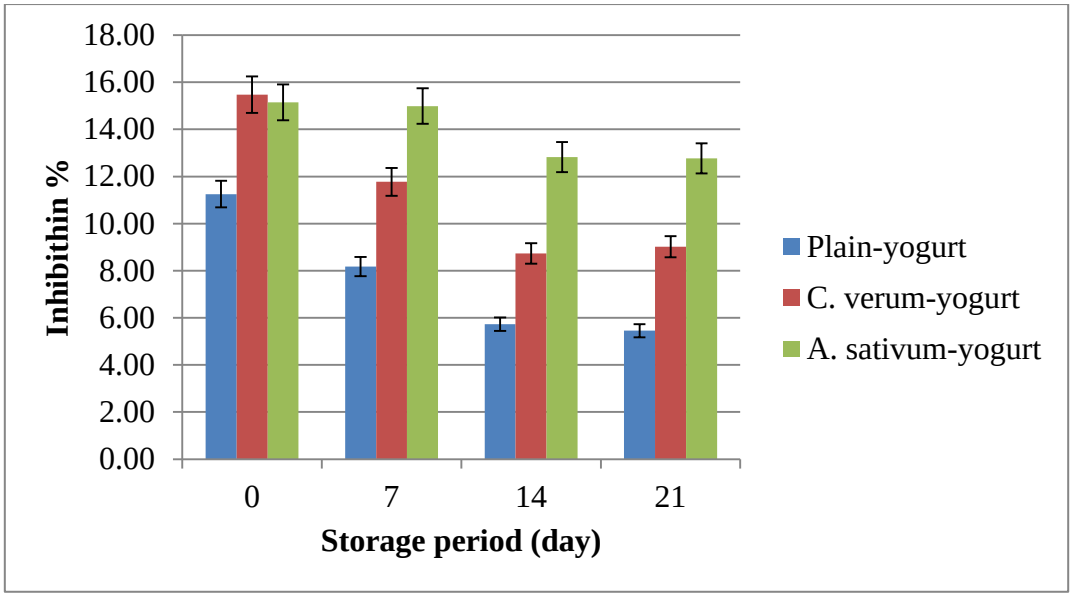


Figure 10.4 α-Glucosidase inhibitory activities (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

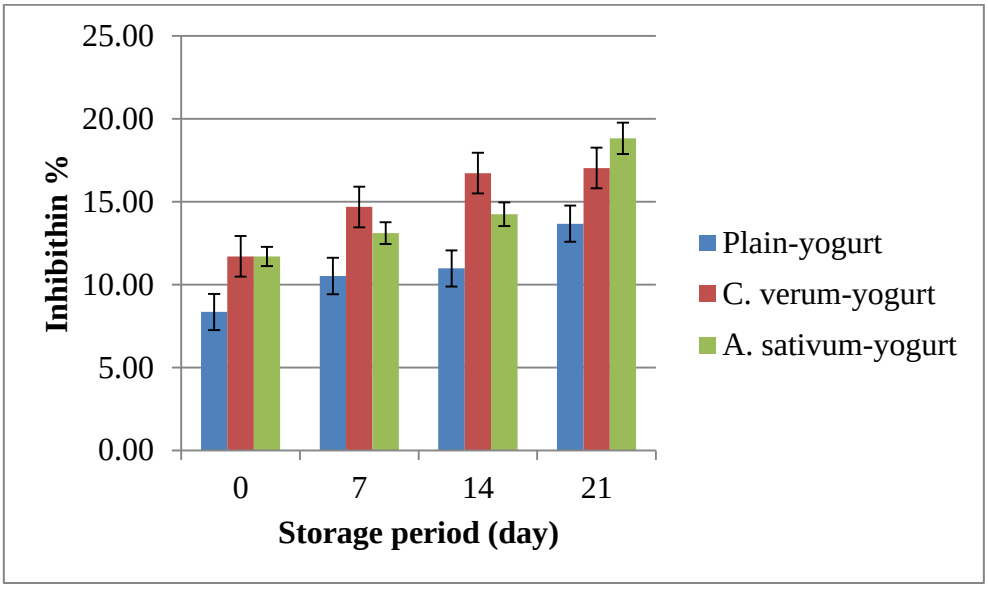


Figure 10.5 α-Glucosidase inhibitory activities (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

10.3.2.1 α-Glucosidase inhibitory activity (IC_{50})

A. sativum and *C. verum* water extracts had IC_{50} of α-glucosidase inhibitory activities < 1 μg/g (Table 10.5). The mixture of *A. sativum* or *C. verum* water extract with cow milk appeared to have no significant effect on IC_{50} of α-glucosidase inhibitory activity as compared to milk alone (Table 10.7). On the other hand, camel milk in the presence of *C. verum* or *A. sativum* water extract showed lower IC_{50} values (6.13±0.26 μg/g ($p<0.05$) and 7.35±0.29 μg/g respectively) than in the absence (9.13±0.19 μg/g; Table 10.7).

Fresh *C. verum*- cow milk yogurt (0 day) showed the highest potency in inhibiting α-glucosidase activity (8.4±0.55 μg/g; Table 10.8). Refrigerated storage to 7 days transiently increased *A. sativum*- cow milk yogurt inhibition potency on α-glucosidase activity (IC_{50}) from 20.7±0.63 μg/g to 12.0±0.89 μg/g (IC_{50}). *C. verum*-cow milk yogurt showed gradual increased in IC_{50} values of α-glucosidase inhibition activity from day 0 to day 21 of storage (IC_{50} 8.4±0.55 μg/g to 14.8±0.96 μg/g). Amongst fresh camel milk-yogurt, *C. verum*-yogurt showed the most potent inhibition on α-glucosidase activity (IC_{50} 9.3±0.45 μg/g) followed by *A. sativum*-and plain-camel milk yogurt (IC_{50} 27.6±0.14 and 39.0±0.13 μg/g respectively; Table 10.8). Both herbal- camel milk yogurt maintained their potency inhibiting α-glucosidase during the three weeks of storage with lower IC_{50} values compared to plain-yogurt.

Table 10.7 IC_{50} values for α-glucosidase inhibitors in cow and camel milk in the absence and presence of *A. sativum* or *C. verum* water extract.

Samples	IC_{50} µg/g
Cow milk	10.97±0.17
AS+cow milk	10.70±0.25
CV+cow milk	10.66±0.15
Camel milk	9.13±0.19
AS+camel milk	7.35±0.29
CV+camel milk	6.13±0.26*

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as a mean (n = 3) ± standard error. *p < 0.05 as compared to control.

Table 10.8 IC_{50} values for α-glucosidase inhibitors in *A. sativum*- and *C. verum*-yogurt made from cow or camel milk during 21 days of refrigerated storage.

Samples	IC_{50} µg/g			
	0 day	**7 day**	**14 day**	**21 day**
Plain-cow milk yogurt	30.8±0.11	24.1±0.24	32.2±0.66	37.1±0.28
AS-cow milk yogurt	20.7±0.63*	12.0±0.89*	28.3±0.25	29.0±0.79*
CV-cow milk yogurt	8.4±0.55*	9.9±0.06*	10.1±0.59*	14.8±0.96*
Plain-camel milk yogurt	39.0±0.13	37.4±0.48	36.5±0.81	54.1±0.11
AS-camel milk yogurt	27.6±0.14*	20.1±0.13*	27.5±0.67*	35.6±0.33*
CV-camel milk yogurt	9.3±0.45*	19.6±0.97*	21.8±0.45*	25.5±0.30*

AS= *A. sativum* and CV= *C. verum*. Plain-cow milk yogurt and plain-camel milk yogurt presented as controls. Results are shown as a mean (n = 3) ± standard error. *p < 0.05 as compared to control.

10.3.3 Total phenolic content (TPC)

C. verum water extract showed higher TPC (68.5±0.02 µg GAE/ml; $p<0.05$) than *A. sativum* water extract (49.8±1.0 µg GAE/ml; Table 10.9). The mixture of *A. sativum* and *C. verum* extracts with cow or camel milk increased ($p>0.05$) TPC compared to their respective milk alone (Table 10.10).

The changes in TPC due to *A. sativum* or *C. verum* water extract in cow- and camel-milk yogurt during refrigerated storage are as shown in Figures 10.6 and 10.7 respectively. TPC in fresh cow milk yogurt was lower (31.12±1.41 µg GAE/g; $p<0.05$) than in fresh *A. sativum*- or *C. verum*- yogurt (39.55 µg GAE/g). Refrigerated storage had little effect on TPC content of plain- and herbal- yogurt during the first 14

days. However, extended storage to 21 days resulted in reduction ($p<0.05$) of TPC in *A. sativum*- or *C. verum*- yogurt (37.15 ± 1.9 µg GAE/g and 39.55 ± 1.3 µg GAE/g respectively) but not in plain-yogurt (32.33± 1.2 µg GAE/g; Figure 10.6).

TPC in fresh camel milk yogurt (60.04±1.39 µg GAE/g) increased ($p<0.05$) in the presence of *A. sativum* or *C. verum* water extract (72.08±0.69 µg GAE/g and 67.27± 0.7 µg GAE/g respectively; Figure 10.7). TPC content was unchanged in all types of yogurt during the first 7 days of storage. However, *C. verum*- yogurt had increased TPC (75.69± 2.6 µg GAE/g; $p<0.05$) on day 14 of storage whereas *A. sativum*- yogurt had increased TPC (78.11± 2.3 µg GAE/g; $p<0.05$) on day 21 of storage (Figure 10.7).

Table 10.9 Total phenolic content (TPC) in *A. sativum* and *C. verum* water extracts.

Sample	TPC (µg GAE/ml)
AS	49.8±1.0
CV	68.5±0.02*

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts = 0.1g/ml. Results are shown as mean (n = 3) ± standard error. *p < 0.05

Table 10.10 Total phenolic content (TPC) in cow and camel milk in the absence and presence of *A. sativum* or *C. verum* water extract.

Sample	TPC (µg GAE/ml)
Cow milk	11.8±0.02
AS+cow milk	25.1±0.01
CV+cow milk	20.3±0.02
Camel milk	45.6±0.01
AS+camel milk	50.4±0.01
CV+camel milk	52.8±0.02

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as a mean (n = 3) ± standard error. *p < 0.05 as compared to control.

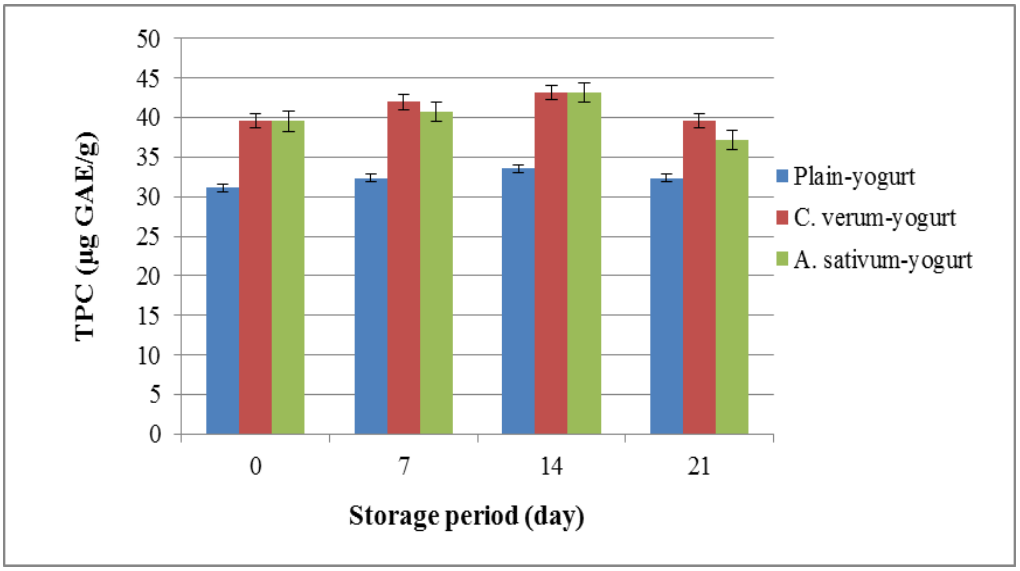


Figure 10.6 Total phenolic content (µg GAE/ml) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

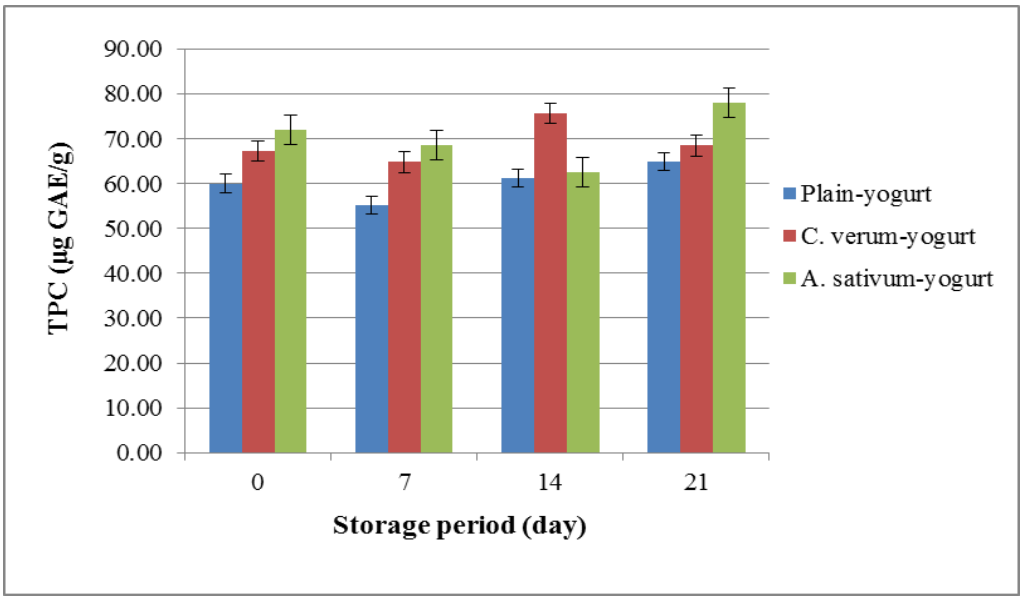


Figure 10.7 Total phenolic content (µg GAE/ml) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

10.3.4 Antioxidant activity by DPPH Assay

The antioxidant activities of *A. sativum* or *C. verum* water extract in milk before fermentation and in yogurt during refrigerated storage was monitored using DPPH radical inhibition assay. No significant differences were observed in antioxidant activities between *C. verum* water extract (13.8±3.1%) and *A. sativum* water extract (12.5±1.1; Table 10.11). The mixture of *C. verum* or *A. sativum* water extract with milk increased antioxidant activity ($p<0.05$) both in cow and camel milk compared to respective plain milk (Table 10.12).

The antioxidant activity in fresh cow milk yogurt was 26.4±0.7% (Figure 10.8). The presence of *A. sativum* or *C. verum* water extract increased the antioxidant activities in yogurt (37.9 ±0.8 % and 35.3±1.0 % respectively; $p<0.05$). The antioxidant activity decreased ($p<0.05$) in *A. sativum*- yogurt during the 3 weeks of refrigerated storage (24.3±1.6 %). However, *C. verum*- yogurt showed the highest ($p<0.05$) antioxidant activity on day 14 of storage (52.8±1.4 %) followed by small reduction to 48.04±1.5 % ($p>0.05$) on day 21 of storage.

The presence of *A. sativum* or *C. verum* water extract increased the antioxidant activities ($p<0.05$) in fresh camel milk yogurt (26.1±0.8 % and 27.1±1.1 % respectively) compared to fresh plain-yogurt (15.44 ±1.2 %; Figure 10.9). Refrigerated storage for two weeks increased ($p<0.05$) antioxidant activity in *C. verum*- and *A. sativum*- yogurt to 64.3±0.7 % and 65.1±1.2 % respectively compared to fresh yogurt. Prolonged refrigerated storage to 21 days resulted in reduction in antioxidant activity for herbal-yogurt ($p>0.05$; Figure 10.9). The correlations between TPC and DPPH scavenging activity in all treated yogurt made from both cow and camel milk were very low ($r^2 \leq 0.2$; Table 10.13).

Table 10.11 DPPH scavenging activity in *A. sativum* and *C. verum* water extracts.

Sample	Inhibition (%)
AS	12.5±1.1
CV	13.8±3.1

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts = 0.1g/ml. Results are shown as mean (n = 3) ± standard error. *p < 0.05

Table 10.12 DPPH inhibition activity in cow and camel milk in the absence and presence of *A. sativum* or *C. verum* water extract.

Sample	Inhibition (%)
Cow milk	10.1±0.01
AS+cow milk	17.5±0.01*
CV+cow milk	23.9±0.01*
Camel milk	7.3±0.01
AS+camel milk	13.4±0.01*
CV+camel milk	17.8±0.01*

AS= *A. sativum* and CV= *C. verum*. Cow milk and camel milk presented as controls. Results are shown as a mean (n = 3) ± standard error. *p < 0.05 as compared to control.

Table 10.13 Regression analysis (correlation) between TPC and DPPH inhibition activity.

Cow milk yogurt	Regression equation	r^2	Camel milk yogurt	Regression equation	r^2
Plain-yogurt	y = 0.0947x + 29.822	0.1569	**Plain-yogurt**	y = 0.0063x + 60.072	0.0009
CV- yogurt	y = 0.0229x + 40.125	0.0231	**CV- yogurt**	y = 0.0874x + 64.409	0.1087
AS- yogurt	y = -0.0064x + 40.342	0.0003	**AS- yogurt**	y = -0.1059x + 75.983	0.0908

CV= *C. verum* and AS= *A. sativum*

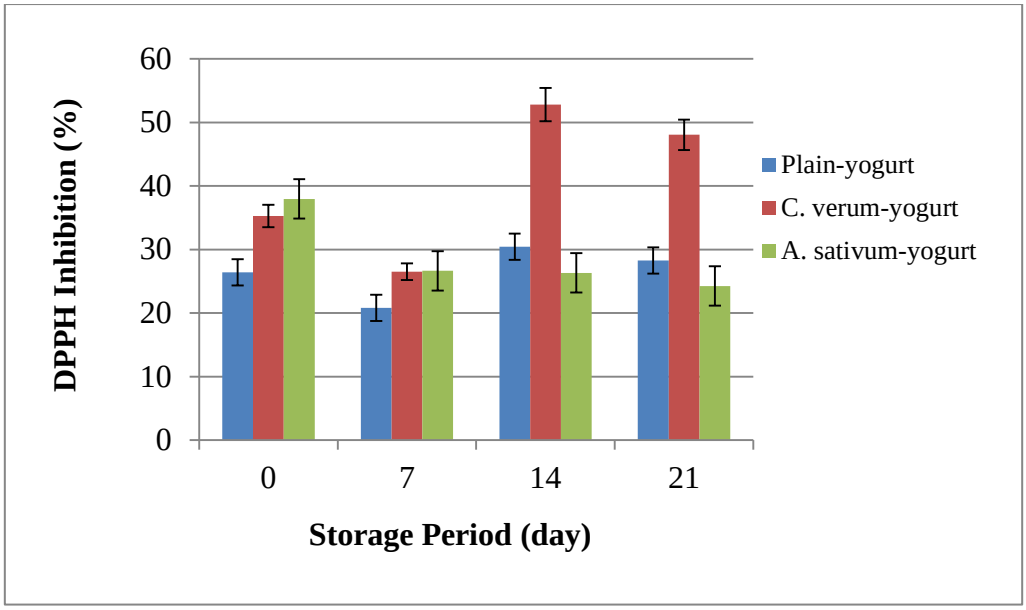


Figure 10.8 DPPH scavenging activity (%) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

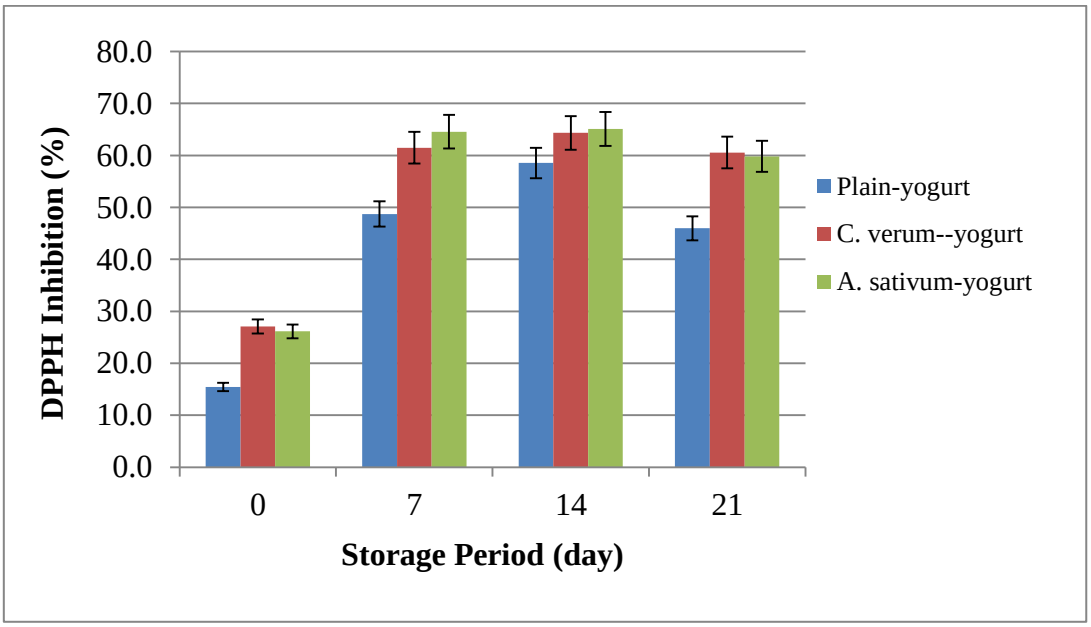


Figure 10.9 DPPH scavenging activity (%) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days refrigerated storage (4°C). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

10.4 DISCUSSION

10.4.1 α-Amylase inhibitory activity

α-amylase is an enzyme that present in saliva as well as in pancreatic juice. This enzyme breaks down starch into smaller molecules so that it can be easily absorbed by the digestive wall. By inhibit this enzyme, the breakdown of carbohydrate is slowed down and this slows the rate of glucose absorption and subsequently decreases the blood glucose level. In the present study, the inhibition of α-amylase activity increased in *A. sativum*- and *C. verum*-yogurt compared to plain-yogurt. This finding is of practical importance since yogurt low glycaemic index (GI) associated with its ability to inhibit α-amylase activity (Östman *et al*., 2001) can now be further increased using *A. sativum* or *C. verum* water extract. The addition of these two herbal extracts could increase yogurt α-amylase activity by a) intrinsic *A. sativum* and *C. verum* ability to inhibit enzyme activity (Grover *et al*., 2002; Eidia *et al*., 2006; Gupta *et al*., 2008), and/or b) alteration of yogurt fermentation products which have inhibitory effects on enzymes activities.

Intrinsic activity of herbal water extracts under study on the inhibition of α-amylase were previously reported by Nickavar and Yousefian (2009) for *A. sativum* (10-55%) and Ponnusamy *et al*., (2011) for *C. verum* (≥ 50%). This finding was reconfirmed in the present study with ~75% inhibition by *C. verum* water extract and 25% inhibition by *A. sativum* water extract (Table 10.1) which is partially explain the higher inhibition of α-amylase activity in the presence of *C. verum* or *A. sativum* water extract than in the absence during refrigerated storage.

The increase in inhibitory effects of α-amylase in yogurt (Figures 10.2 and 10.3) can be attributed to enhancing effects of phytochemical in *A. sativum* or *C. verum* water extract on yogurt fermentation related to their intrinsic capacity to inhibit

α-amylase (Nickavar and Yousefian, 2009; Ponnusamy *et al*., 2011). Protein content, organic acids and some phytochemical compounds such as polyphenols, flavonoids and antioxidant compounds do contain some α-amylase inhibitor (Vosloo, 2005). The inclusion of *A. sativum*, *C. verum* water extracts or perhaps any other plant (Shori and Baba, 2011) in yogurt may be good source of phytochemical compounds that increase the inhibition of α-amylase activity of yogurt. The exact mechanisms on how these phytochemical compounds inhibit α-amylase are not fully known but binding to the reactive sites of enzymes thus altering its catalytic activity have been proposed (McCue and Shetty, 2004; Payan, 2004; Dewanjee *et al*., 2011).

Apart from adding plant extracts increasing the inhibition of α-amylase may be achieved by the use of certain milk. The higher α-amylase inhibitory activity (IC_{50}) in camel milk yogurt than in cow milk yogurt (both in presence and absence of *A. sativum* and *C. verum*; Table 10.4) may be partially explained by the differences in chemical composition in both types of milk. The inorganic metal ion such as Fe^{2+} is known to inhibit amylases at high concentrations (Muralikrishna and Nirmala, 2005) and this metal ions are present at about 10 times higher in camel milk than in cow milk (Al-awadi and Srikuma, 2001). Moreover, lactoferrin which is an iron containing milk protein is also higher in camel milk than cow milk (El Agamy, 2000).

The presence of *C. verum* or *A. sativum* water extract enhanced the yogurt inhibition of α-amylase activity *in vitro* with camel milk yogurt showing more pronounced effects than cow milk yogurt. The sustained enhanced inhibition on α-amylase activities through the storage period suggest that *C. verum*- and *A. sativum*-yogurt should be considered in further studies as effective functional food to regulate carbohydrate digestion.

10.4.2 α-Glucosidase inhibitory activity

α-glucosidase is an enzyme located at brush border of microvillus in the small intestine of digestion system. This enzyme is a regulatory enzyme where it catalyzes the final step of carbohydrate metabolism whereby the oligosaccharides is degraded into monosaccharide. An inhibitor of this enzyme, acarbose which is commonly prescribed, prolong the breakdown of oligosaccharides to monosaccharides, thus reduce the amount of glucose entering the circulation system (Dicarli *et al.*, 2003). Besides, α-glucosidase enzyme catalyzes the cleavage of glycosidic bond and consequently releases glucose from the non-reducing end of oligosaccharides chain (De Melo *et al.*, 2006). The present study has discovered the possibility of enhancing natural yogurt ability to inhibit diabetic enzymes by adding *A. sativum* or *C. verum* water extract. Such an effect is not only attributed to these herbs natural ability to inhibit α-glucosidase activity (Pham *et al*., 2007; Gupta *et al*., 2008; Ranilla *et al.*, 2010) but also possibly from these herbs- induced increase in inhibitors during fermentation of milk.

The inherent ability to inhibit α-glucosidase may be due to sallyl cysteine sulfoxide (alliin) identified from *A. sativum* extract (Augusti and Sheela, 1996; Ashraf *et al*., 2011) and naphthalene, 1, 2, 3, 4-tetrahydro- 1, 1, 6-trimethyl, eugenol and 4 acetoxycinnamic acid identified from *C. verum* extract (Maridass and GhanthiKumar, 2008). Fermented milk products such as yogurt for instance have α-glucosidase inhibitory activities (Ramchandran and Shah, 2008) and this may explain why the consumption of yogurt was recommended to help reduce post-prandial hyperglycemia.

Although the addition of *A. sativum* or *C. verum* water extract may explain the increase in α-glucosidase inhibitory activity in cow- and camel- milk yogurt, the

effects seen were synergistic rather than additive. In fact the synergistic effects of herbal extracts addition to yogurt has been previously reported for *Azadirachta indica*, peppermint, dill and basil (Shori and Baba, 2011, Shabboo and Baba, 2011) or fruit such as strawberry, blueberry, and peach (Apostolidis *et al*., 2006). Secondary metabolites products from plants may also responsible for the α-glucosidase inhibition include polyphenols and flavonoids (Kim *et al*., 2000, Jung *et al*., 2006), tannins (Tang *et al*., 2006) and terpenoids (Ojewole, 2002). These compounds have been suggested capable to inhibit the enzyme by forming complexes with proteins (Nickavar and Yousefian *et al*., 2009). The observation from the present study also supports the possible roles of compounds derived during fermentation such as organic acids (Hansawasdi *et al*. 2000), phenolic components (Kim *et al*., 2000; McCue and Shetty, 2004), free amino acids and soluble proteins to inhibit the α-glucosidase (Appendix 2). These are demonstrated by the lower α-glucosidase inhibitory activity in the mixture of milk with herbal extracts (before fermentation) than those after fermentation thus implicating the possible roles of components produced from the activity of yogurt culture. Apostolidis *et al*. (2007) found that fermented soy milk with *L. bulgaricus* or *L. acidophilus* show around 12% of α-glucosidase inhibitory activity after 24 hour of fermentation. This is supported by the present study in which both fresh *A. sativum*- and *C. verum*- yogurt made from cow or camel milk showed 15% and 12% respectively α-glucosidase inhibition after 4-5 hours of fermentation. *A. sativum*- or *C. verum* - yogurt made from cow or camel milk could therefore delay the digestion of dietary carbohydrates resulting in the slowing down of postprandial hyperglycemia.

10.4.3 TPC and antioxidant activity

Based on the present result, higher TPC in *A. sativum*- and *C. verum*- yogurt than plain-yogurt made from either cow or camel milk may be related to phenolic compounds available in *A. sativum* and *C. verum* extracts. Despite high TPC present in *A. sativum* and *C. verum* water extracts (49.8±1.0 µg GAE/ml and 68.5±0.02 µg GAE/ml respectively; Table 10.9) both herbal cow- and camel- milk yogurt did not contain appreciable amount of TPC compared to respective controls. This could possibly happen as a result of the binding-interaction between phenolic compounds and milk proteins (casein) as demonstrated by the interaction of milk protein with reactive phenolic compounds present in green tea, red wine and fruit beverage (Alexandropoulou *et al*., 2006; Argyri *et al*., 2006; Cilla *et al*., 2009).

Fermentation of milk resulted in an increase in TPC in both types of yogurt as compared to before fermentation (Table 10.10). This could be explained by the degradation of milk proteins during protoelytic activity of yogurt bacteria resulting in the release of some phenolic compounds attached to protein (McCue and Shetty, 2005). The degradation of milk proteins itself resulted in the release of phenolic amino acids and non-phenolic compounds such as sugars and proteins which may interfere during total phenolic evaluation (Ainsworth and Gillespie, 2007).

The consumption of high dietary antioxidant capacity has strong correlation with glycaemic index and prevention of the development of diabetes (Psaltopoulou *et al*., 2011). In the present study, higher antioxidant activity was found in the presence of *A. sativum* or *C. verum* water extract in cow- and camel- milk yogurt than in the absence. The antioxidant activates in these two plants can be attributed to the presence of phytochemical compounds. The DPPH radical scavenging activities in *C. verum* is possibly associated with polymeric phenolics and flavonoid such as quercetin,

quercetrin and kaempferol (Singh *et al*., 2002; Prasad *et al*., 2005; Prasad *et al.*, 2009; Roussel *et al.*, 2009; Ranilla *et al.*, 2010). On the other hand, the ability of *A. sativum* extract to scavenge different radicals can be attributed to organosulfur, phenolics, flavonoids and terpenoid compounds (Bhagyalakshmi *et al*., 2005; Pedraza-Chaverri *et al.*, 2006; Wilson and Adams, 2007; Bozin *et al.*, 2008).

Prolonged refrigerated storage to 14 and 21 days was associated with higher antioxidant activity in *C. verum*-cow milk yogurt (Figure 10.8) but this was not correlated with higher TPC. Such a low correlation (Table 10.13) is in agreement with study in soy yogurt (Apostolidis *et al*., 2007). Thus other elements in yogurt such as nitrogenous compounds from protein breakdown (volatile and nonvolatile compounds) with antioxidant character (Virgili *et al*., 2006) may provide alternative explanation to the low association between TPC and antioxidant activity. In fact a high correlation between antioxidant activity and OPA values (see Appendix 2) indicate that the increase in proteolytic products from cow or camel milk proteins degradation may contribute to the total increase in DPPH inhibition during storage.

In general, the antioxidant activity in camel milk yogurt (with/without herbal extracts) was higher than in cow milk yogurt. This could be partially explained by the higher content of antioxidant components such as vitamins (vitamin C), minerals and volatile acids (linoleic acid and polyunsaturated acids) which are present in higher concentrations in camel milk than in cow milk (Wernery *et al*., 2005; Kumar *et al*., 2009).

Antioxidants in dietary products were found to be positively correlated with anti-diabetic properties (Shetty *et al.*, 2008). Therefore, the highly consumption of antioxidant activity present in *A. sativum*- and *C. verum*- yogurt may be expected to play a role in the management of type 2 diabetes.

10.5 CONCLUSIONS

The presence of *A. sativum* and *C. verum* water extracts in both cow and camel milk yogurt increased α-amylase and α-glucosidase inhibitory activities compared to the absence during period of storage. In addition, treated camel milk- yogurt had higher α-amylase and α-glucosidase inhibition activities than treated cow milk- yogurt during 21 days of storage. *A. sativum*- and *C. verum*- camel milk yogurt have higher TPC than that in cow milk- yogurt. The antioxidant activity of cow- and camel- milk yogurt increased in the presence of *A. sativum* or *C. verum*. Refrigerated storage significantly increased the antioxidant activity in camel milk-yogurt whereas in cow milk-yogurt the antioxidant activity enhanced only in *C. verum*-yogurt during the last two weeks of storage. The enhanced antioxidant activity in yogurt has not correlated with TPC. The addition of *A. sativum* or *C. verum* can enhance yogurt functional properties with regard to profound inhibition on α-amylase and α-glucosidase activities. These herbal- yogurts have the potential to provide additional benefits to human nutrition and product shelf-life stability by virtue of the increased antioxidant capacity.

11.0 Anti-diabetic enzymes and antioxidant properties of *Allium sativum*- and *Cinnamomum verum*- yogurt after simulated gastrointestinal digestion

11.1 INTRODUCTION

Chronic hyperglycaemia of type 2 diabetes is associated with long-term damage, dysfunction and failure of various organs such as eyes, kidneys, nerves, heart and blood vessels (Ranade *et al*., 2001). α-Amylase is an enzyme produced by the pancreas and salivary glands required in the digestion of carbohydrate such as starch, dextrin and glycogen by cleaving the α-1, 4-glycosidic linkages of polysaccharides to yield smaller molecules such as glucose, maltose and the limit dextrin. α-Glucosidase is an enzyme produced by the intestinal walls. This enzyme works by catalyzing the cleavage of glycosidic bond and release glucose from the non-reducing end of oligo- or poly-saccharides chain (De Melo *et al.*, 2006). By inhibiting these two enzymes, the rate of glucose that will be absorbed from ingested food can be reduced, thus prevent the sharp rise in blood sugar levels which is particularly useful for people with type 2 diabetes (De Melo *et al.*, 2006). There is an urgent need for safe agents that can regulate blood level without any adverse side effects. Anti-hyperglycemic drug such as metformin, acarbose, and orlistat are commonly presented to treat type 2 diabetes mellitus (Sudha *et al*., 2011). The side effects of these drugs i.e. flatulence and diarrhea (Cheng and Fantus, 2005) are well documented. Natural ways to inhibit these two enzymes include the use of certain plant extracts and some traditional foods (Fujita *et al*., 2003; Djomeni *et al*., 2006). Fermented protein-based food such as yogurt, milk and soymilk are known to inhibit the activity of α-glucosidase and α-amylase (Apostolidis *et al*., 2006; Yadav *et al*., 2007) by virtue of bioactive peptides derived from enzymatic hydrolysis and/or microbial fermentation (Apostolidis *et al*., 2006; Darmawan, 2010). Some of these peptides also possess high oxidative inhibitory capacity due to their ability to scavenge free radicals (Elias *et al*., 2008)

which provide added advantage to reduce oxidative damage of cell components associated with diabetes mellitus (Rahimi *et al*., 2005).

Foods enriched with phenolic compounds are widely produced in processed food industry. This is because these compounds provide antioxidant activities and acclaimed health benefits such as anti-diabetes, anti-hypertension, anti-inflammatory and anti-carcinogenic (Perez-Vicente *et al*., 2002; Gumienna *et al*., 2009; Kunyanga *et al*., 2012; Chan *et al*., 2012). Medicinal herbs such as *Allium sativum* and *Cinnamomum verum* have been studied extensively for their inhibitory activity towards α-glucosidase and α-amylase activity *in vitro* (Augusti and Sheela, 1996; Maridass and GhanthiKumar, 2008; Nickavar and Yousefian, 2009; Ponnusamy *et al*., 2011). These two herbs are also rich in antioxidants and phenolic compounds (Bozin et al., 2008; Gumienna *et al*., 2009; Prasad *et al.*, 2009; Ranilla *et al.*, 2010). The presence of *A. sativum* and *C. verum* in yogurt inhibited diabetes enzymes (α-amylase and α-alucosidase) during refrigerated storage (see Sections 10.3.1 and 10.3.2). However, it is important to demonstrate that these effects are retained even after exposure to acidic and alkaline digestive tract environments which may interfere with phenolic and other bioactive compounds with functional properties. Therefore, the current study has investigated the effects of exposure to simulated gastrointestinal digestion on the changes in the inhibition of α-amylase and α-glucosidase activities and contents of phenolic and antioxidant of *A. sativum*- and *C. verum*- yogurt made from cow, camel and goat milk.

11.2 MATERIALS AND METHODS

11.2.1 Substrates and chemicals

All the substrates and chemicals used in this study are as described in Sections 7.2.1 and 10.2.1.

11.2.2 Experimental design

The experimental design is as described in Section 9.2.2.

11.2.3 Plant water extraction procedure

The water extraction of plant was performed according to the method described in Section 3.2.3.

11.2.4 Yogurt manufacturing process

11.2.4.1 Starter culture and yogurt preparation

The starter culture preparation was carried out using the method described in Section 3.2.4.1. The three groups of bio-yogurt made from cow, camel or goat milk both in the presence and absence of *A. sativum* or *C. verum* water extract were prepared as described in Section 3.2.4.2.

11.2.5 *In vitro* gastrointestinal model

11.2.5.1 Preparation of gastric and duodenum juices

Preparation of gastric and duodenum solutions were performed according to the method described in Section 7.2.5.1.

11.2.5.2 Simulation of gastrointestinal digestion (SGD)

Simulation of gastrointestinal digestion was assessed according to the procedure described in Sections 7.2.5.2 and 9.2.5.2.

11.2.6 α-Amylase inhibition assay

α-Amylase inhibition assay was as described in Section 10.2.6.

11.2.7 α -Glucosidase inhibition assay

α -Glucosidase inhibition assay was as described in Section 10.2.7.

11.2.8 Total phenolic assay

Total phenolic assay was as described in Section 10.2.8.

11.2.9 DPPH inhibition assay

DPPH inhibition assay was as described in Section 10.2.9.

11.2.10 Statistical analysis

Statistical analysis of data was as described in Section 3.2.8.

11.3 RESULTS

11.3.1 α-Amylase inhibitory activity after SGD

A) In herbal water extract

Gastric digestion of *A. sativum* or *C. verum* water extract showed α-amylase inhibitory activity of 64.6±1.1% and 52.5±1.8% respectively (Table 11.1). α-Amylase inhibitory activity after 2 hours intestinal digestion increased ($p>0.05$) for *C. verum* to 58.88±1.7% but decreased ($p>0.05$) for *A. sativum* to 61.29±0.5%.

Table 11.1 Effects of *in vitro* gastrointestinal digestions of *A. sativum* and *C. verum* water extracts on α-amylase inhibition activity.

Incubation time (hour)	α-Amylase inhibition activity (%)	
	AS water extract	CV water extract
1	64.59±1.1	52.45±1.8
2	61.49±0.8	57.7±2.0
3	61.29±0.5	58.88±1.7

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts before digestion = 0.1g/ml. 1st hour represent *in vitro* gastric digestion, 2nd and 3rd hours represent 1 and 2 hours *in vitro* intestinal digestion respectively. Results are shown as mean (n = 3) ± standard error. *$p < 0.05$

B) In cow milk and cow milk yogurt

The inhibition activity of α-amylase in cow milk was 40.16±2.6% after the 1st hour of gastric digestion (Figure 11.1). The mixture of milk with *A. sativum* or *C. verum* water extract increased ($p<0.05$) α-amylase inhibitory activity to similar value (47%) after gastric digestion. Intestinal digestion for 2 hours decreased ($p<0.05$) α-amylase inhibitory activity to 29.99±2.0%, 33.23±2.4% and 31.81±2.1% for milk, milk + *A. sativum* and milk + *C. verum* respectively.

Gastric digestion of fresh yogurt showed higher ($p<0.05$) α-amylase inhibitory activity (59.26±2.4%, 60.87±2.4% and 60.79±2.4% for plain-, *A. sativum*- and *C. verum*- yogurt respectively) compared to those before fermentation (Figure 11.1). Fresh plain- and herbal- yogurt showed slight reduction in α-amylase inhibitory activity ($p>0.05$) after intestinal digestion. Gastric digestion of 7 days old yogurt had higher ($p<0.05$) α-amylase inhibitory activity (69.58±0.8%, 74.87±1.5% and 72.04±1.1% for plain-, *A. sativum*- and *C. verum*-yogurt respectively) compared to fresh yogurt (Figure 11.1). α-Amylase inhibitory activity decreased ($p<0.05$) to 55.41±2.0 %, 58.93±2.1 % and 58.16±2.2 % for plain-, *A. sativum*- and *C. verum*-yogurt respectively after the 1st hour of intestinal digestion. Prolonged intestinal digestion to another one hour had little effects on further reduction of α-amylase inhibitory activities.

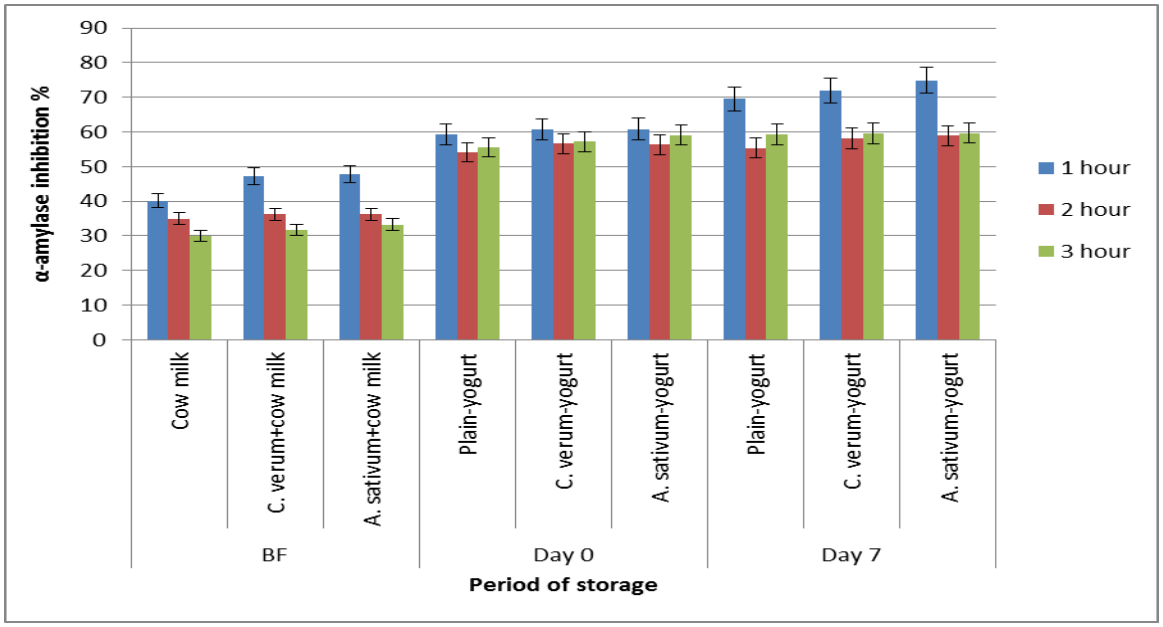


Figure 11.1 α-Amylase inhibitory activities (%) of cow milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) In camel milk and camel milk- yogurt

The mixture of camel milk with *A. sativum* or *C. verum* water extract showed higher ($p<0.05$) α-amylase inhibitory activity (39.76±2.5 % and 38.94±2.2 % respectively) than milk alone (32.95±2.4 %) after 1 hour gastric digestion (Figure 11.2). Intestinal digestion for one hour decreased ($p<0.05$) α-amylase inhibitory activity of milk with *A. sativum* or *C. verum* water extract (34.39±2.7 % and 33.38±2.9 % respectively) but not that of milk alone (32.78±2.3 %). The inhibition of α-amylase activity was significantly reduced in milk and milk + *A. sativum* (16.58±2.8 % and 21.53±2.1 % respectively) after the 2nd hour of intestinal digestion.

The α-amylase inhibitory activities of fresh *A. sativum*- and *C. verum*- yogurt (71.53±1.0 % and 70.48±1.0 % respectively) were not significantly different from plain yogurt (68.06±2.4 %) after 1 hour gastric digestion (Figure 11.2). The inhibitory

activity toward α-amylase decreased (51.31±2.0 %, 52.71±2.3 % and 55.41±2.1 % for plain-, *A. sativum*- and *C. verum*-yogurt respectively; $p<0.05$) after the 1st hour of intestinal digestion. No further decrease in α-amylase inhibitory activity of plain- and herbal-yogurt occurred after the 2nd hour of intestinal digestion (Figure 11.2). Refrigerated storage (7 days) showed no significant differences in α-amylase inhibitory activity of all treated yogurt as compared to fresh yogurt after both gastric and intestinal digestions (Figure 11.2).

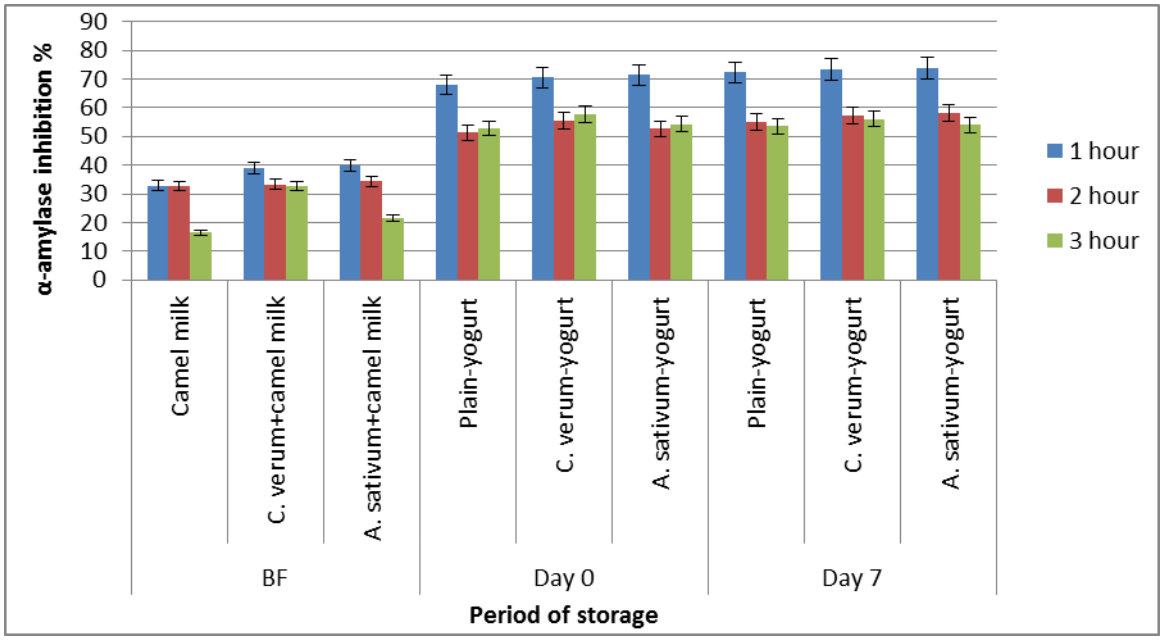


Figure 11.2 α-Amylase inhibitory activities (%) of camel milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

D) In goat milk and goat milk- yogurt

Gastric digestion of goat milk showed α-amylase inhibitory activity of 20.75±0.3% whereas *A. sativum* or *C. verum* water extract mixed with goat milk (23%; $p>0.05$) was not different compared to milk alone. One hour intestinal digestion of the three different milk treatments had no effects on α-amylase inhibitory activity as compared to gastric digestion (Figure 11.3). However, α-amylase inhibitory activity in milk alone reduced ($p<0.05$) to 18.19±0.2 % after the second hour of intestinal digestion.

Fresh *C. verum*-yogurt had higher ($p<0.05$) α-amylase inhibitory activity (28.64±0.5 %) than that of fresh plain yogurt (24.07±0.9 %) after gastric digestion whereas fresh *A. sativum*-yogurt (24.24±0.3 %) was not different compared to plain-yogurt (Figure 11.3). α-Amylase inhibitory activity for all treatments increased (35.62±2.3 %, 37.82±0.4 % and 36.84±2.5 % for plain-, *A. sativum*- and *C. verum*-yogurt respectively; $p<0.05$) after the second hour of intestinal digestion. Seven days old yogurt showed higher ($p<0.05$) α-amylase inhibitory activity than fresh yogurt after 1 hour gastric digestion with highest inhibition was seen by *C. verum*-yogurt (37.94±0.8 %) followed by *A. sativum*-yogurt (33.42±1.3 %) and plain- yogurt (31.89±2.1 %; Figure 11.3). Intestinal digestion (1 and 2 hours) increased α-amylase inhibitory activity to similar levels (41.75±1.7 %, 43.86±1.2 % and 44.2±1.1 % for plain-, *A. sativum*- and *C. verum*-yogurt respectively; $p<0.05$).

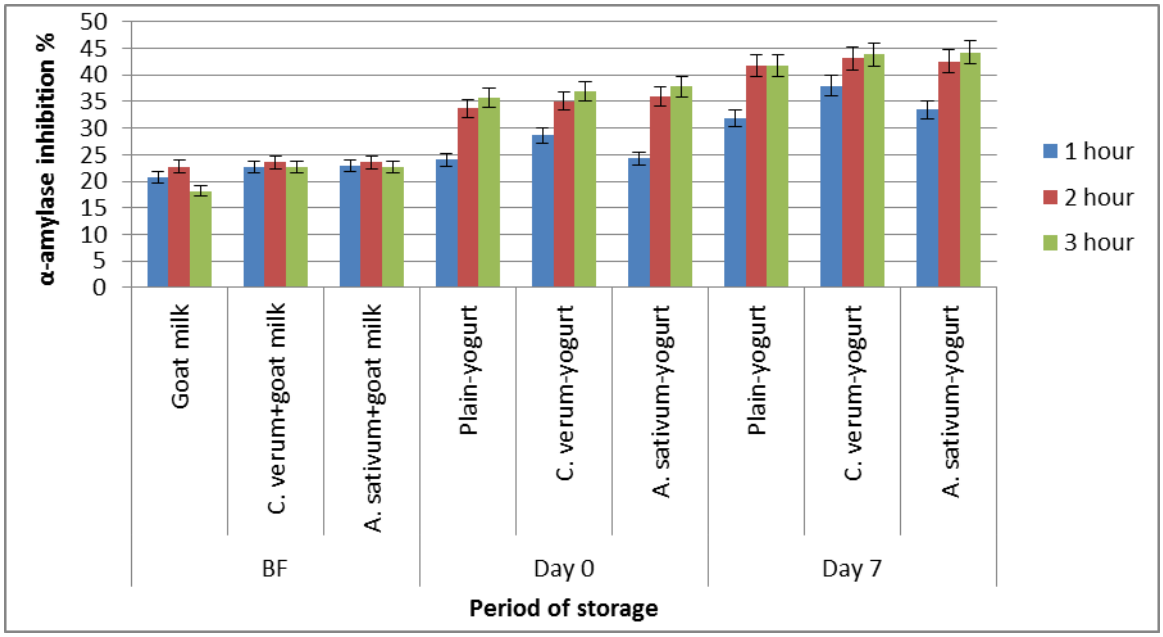


Figure 11.3 α-Amylase inhibitory activities (%) of goat milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

11.3.2 α-Glucosidase inhibitory activity after SGD

A) In herbal water extract

A. sativum and *C. verum* water extracts inhibited 65.0±0.2% and 66.4±1.4% respectively of α-glucosidase activities after gastric digestion (Table 11.2). This inhibition increased ($p<0.05$) after 2 hours intestinal digestion to 97.9±0.1% and 96.9±0.3% for *A. sativum* and *C. verum* water extracts respectively.

Table 11.2 Effects of *in vitro* gastrointestinal digestions of *A. sativum* and *C. verum* water extracts on α-Glucosidase inhibition activity.

Incubation time (hour)	α-Glucosidase inhibition activity (%)	
	AS water extract	CV water extract
1	65.0±0.2	66.4±1.4
2	97.9±0.1	96.9±0.3
3	97.9±0.9	96.3±0.6

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts before digestion = 0.1g/ml. 1st hour represent *in vitro* gastric digestion, 2nd and 3rd hours represent 1 and 2 hours *in vitro* intestinal digestion respectively. Results are shown as mean (n = 3) ± standard error. *p < 0.05

B) In cow milk and cow milk- yogurt

Gastric digestion of cow milk inhibited 34.47±2.6% of α-glucosidase activity (Figure 11.4). The mixture of *A. sativum* or *C. verum* water extract with the milk had no effect on α-glucosidase inhibitory activity (34.57 ±0.8% and 37.81±2.9% respectively) after gastric digestion. The 2 hours intestinal digestion increased ($p<0.05$) α-glucosidase inhibitory activity for all milk treatments to about 92%.

The presence of *A. sativum* or *C. verum* water extract in fresh yogurt showed higher ($p<0.05$) α-glucosidase inhibitory activity (55.48±2.8% and 53.4 ±2.8% respectively) than plain-yogurt (41.91±2.2%) after gastric digestion (Figure 11.4). Intestinal digestion (2 hours) increased ($p<0.05$) α-glucosidase inhibitory activity to similar level for plain- (94.42±0.6%), *A. sativum*- (96.19±0.4%), and *C. verum*– (95.1±0.7%) yogurt. Gastric digestion of 7 days old yogurt showed higher α-glucosidase inhibitory activity (69.57±2.1%, 73.86±1.0% and 73.34±0.6% for plain-, *A. sativum*-, and *C. verum*- yogurt respectively) than fresh yogurt (Figure 11.4). Intestinal digestion for 2 hours increased ($p<0.05$) α-glucosidase inhibitory activity to similar level (96.99±0.4%, 97.17±0.4% and 97.24±0.3% for storage plain-, *A. sativum*- and *C. verum*- yogurt respectively).

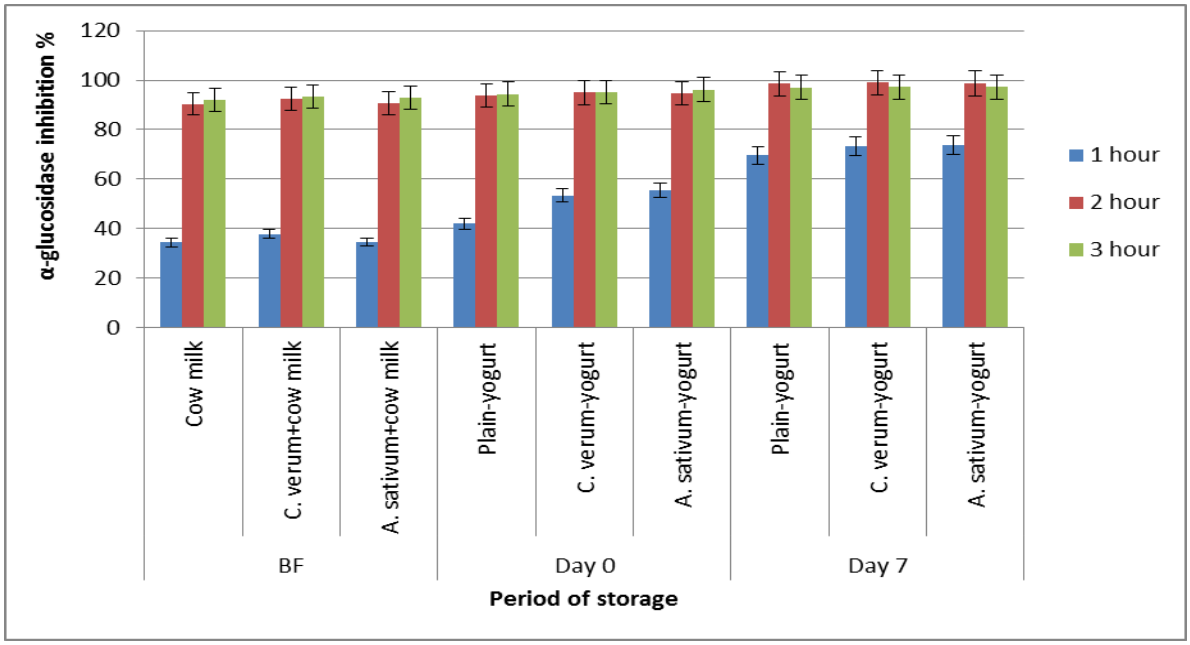


Figure 11.4 α-Glucosidase inhibitory activities (%) of cow milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) In camel milk and camel milk- yogurt

A. sativum or *C. verum* water extract mixed with camel milk (37.32±1.8% and 35.67±1.3% respectively) had almost similar α-glucosidase inhibition activity with camel milk alone (33.97±0.9%) after gastric digestion (Figure 11.5). Intestinal digestion for two hours increased ($p<0.05$) α-glucosidase inhibitory activity to 95.49±1.8%, 96.56±1.1% and 95.52±0.9% for milk, milk + *A. sativum* and milk + *C. verum* respectively.

Fresh yogurt showed almost 2 times higher in α-glucosidase inhibitory activity (66.73±1.1%, 69.2±1.5% and 68.72±1.3% for plain-, *A. sativum*- and *C. verum*-yogurt respectively) compared to milk after gastric digestion (Figure 11.5). Intestinal digestion for 2 hours increased ($p<0.05$) α-glucosidase inhibitory activity to about 97% for all treatments. There was no difference ($p>0.05$) in α-glucosidase inhibitory

activity between storage and fresh yogurt whether after being subjected to *in vitro* gastric and intestinal digestions (Figure 11.5).

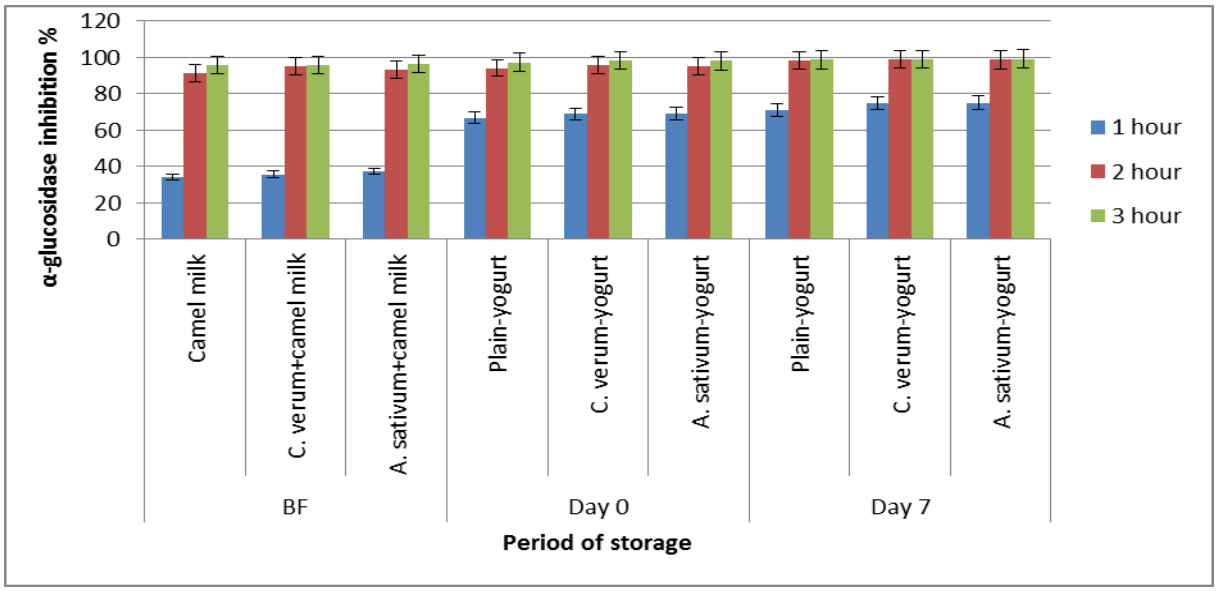


Figure 11.5 α-Glucosidase inhibitory activities (%) of camel milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

D) In goat milk and goat milk- yogurt

Goat milk with or without *A. sativum* or *C. verum* water extract showed α-glucosidase inhibitory activity ranged from 38- 41% after gastric digestion (Figure 11.6). α-lucosidase inhibitory activity increased ($p<0.05$) to 90.0±0.7%, 91.25±0.5% and 91.62±0.5% for milk alone, milk + *A. sativum* and milk + *C. verum* respectively after the 2nd hour of intestinal digestion. No difference between fresh and 7 days old plain- and herbal yogurt and milk alone in α-glucosidase inhibition after gastric and intestinal digestion (Figure 11.6).

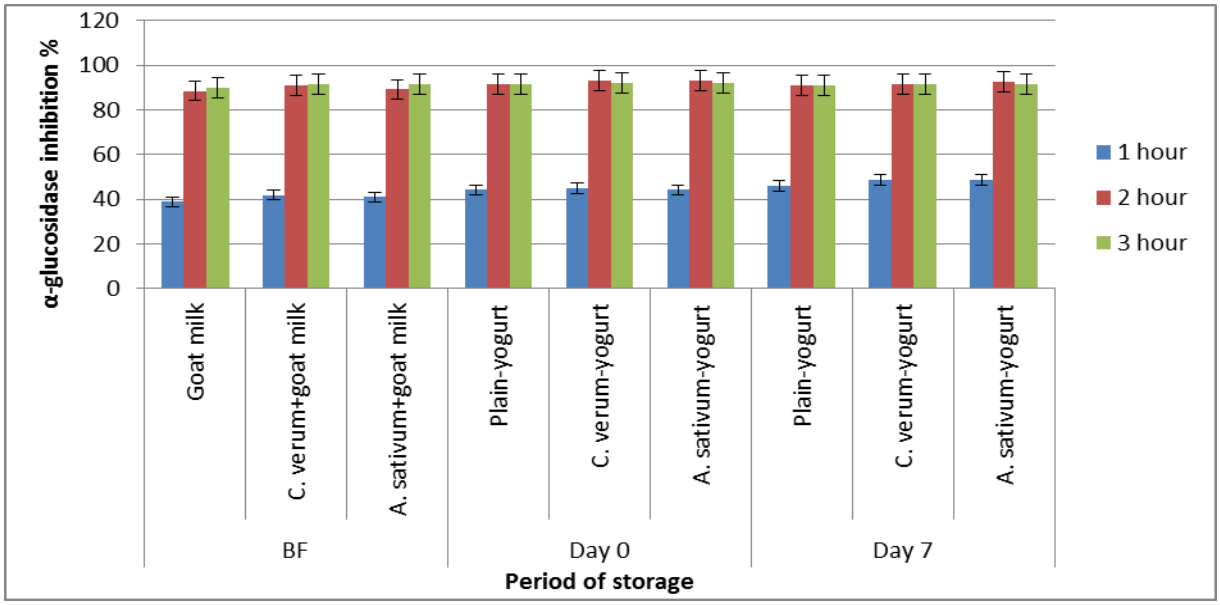


Figure 11.6 α-Glucosidase inhibitory activities (%) of goat milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

11.3.3 Total phenolic content after SGD

A) In herbal water extract

TPC of *A. sativum* and *C. verum* water extracts after gastric digestion was 57.75±1.6 µg GAE/g and 98.17±1.0 µg GAE/g respectively (Table 11.3). Intestinal digestion for 1 hour reduced ($p<0.05$) TPC of *A. sativum* and *C. verum* water extracts to 27.74±1.1 µg GAE/g and 35.86±0.6 µg GAE/g respectively. Prolonged intestinal digestion to 2nd hour showed no further changes in TPC (Table 11.3).

Table 11.3 Effects of *in vitro* gastrointestinal digestions of *A. sativum* and *C. verum* water extracts on total phenolic content.

Incubation time (hour)	TPC (µg GAE/g)	
	AS water extract	CV water extract
1	57.75±1.6	98.17±1.0*
2	27.74±1.1	35.86±0.6
3	29.95±1.5	33.61±0.7

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts before digestion = 0.1g/ml. 1st hour represent *in vitro* gastric digestion, 2nd and 3rd hours represent 1 and 2 hours *in vitro* intestinal digestion respectively. Results are shown as mean (n = 3) ± standard error. *p < 0.05

B) In cow milk and cow milk- yogurt

The mixture of *A. sativum* or *C. verum* water extract with milk showed similar TPC values (80.48 ±2.2 µg GAE/g and 85.06±2.3 µg GAE/g respectively) compared to milk alone (80.48±2.0 µg GAE/g) after 1 hour gastric digestion (Figure 11.7). Intestinal digestion decreased ($p<0.05$) TPC to similar level (~ 40 µg GAE/g) for all milk treatments.

TPC of fresh yogurt was 85.46±2.2 µg GAE/g after gastric digestion (Figure 11.7) which was not different from fresh *A. sativum*- and *C. verum*- yogurt (86.98±2.2 µg GAE/g and 90.80±2.3 µg GAE/g respectively). The TPC of all types of yogurt decreased to 43.09±1.1 µg GAE/g, 48.23±2.3 µg GAE/g and 50.28±2.5 µg GAE/g for plain-, *A. sativum*- and *C. verum*-yogurt respectively after 2 hours of intestinal digestion. Refrigerated yogurt (7 days) had higher ($p<0.05$) TPC (104.09±3.6 µg GAE/g, 110.96±3.1 µg GAE/g and 109.27±1.3 µg GAE/g for plain-, *A. sativum*- and *C. verum*-yogurt respectively) than fresh yogurt after 1 hour of gastric digestion (Figure 11.7). However, TPC decreased ($p<0.05$) after 1 and 2 hours of intestinal digestion ranging between 43 - 50 µg GAE/g.

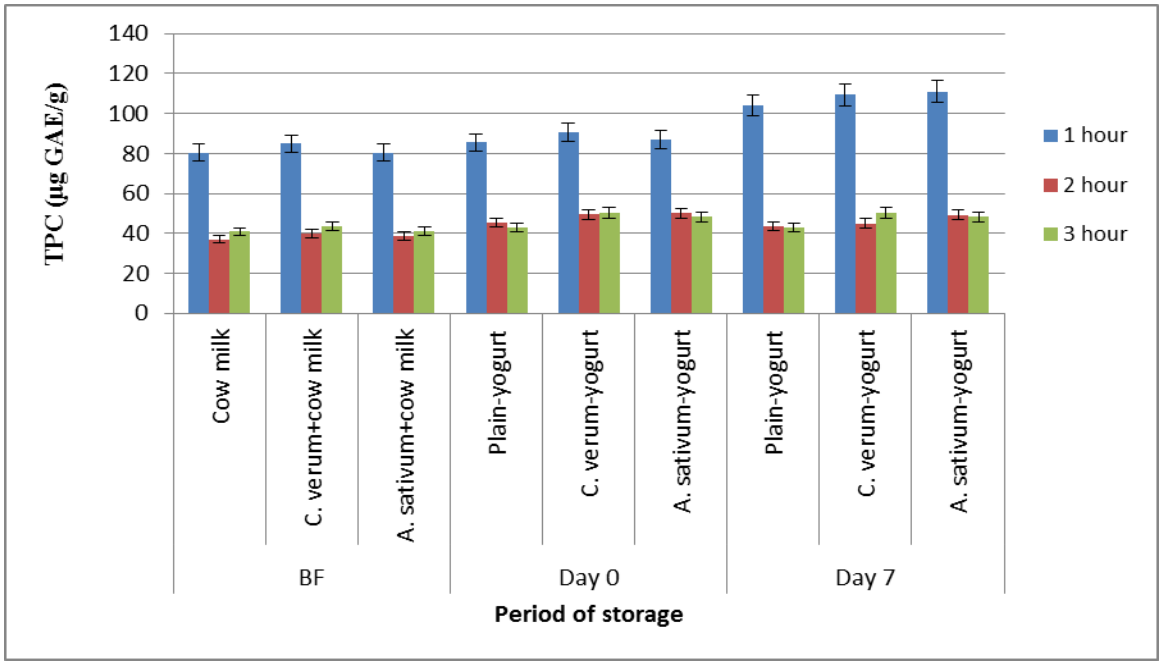


Figure 11.7 Total phenolic content (µg GAE/g) of cow milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) In camel milk and camel milk- yogurt

TPC in camel milk was 66.1±1.2 µg GAE/g after gastric digestion and the mixture of milk with *A. sativum* or *C. verum* water extract had approximately similar values of TPC (74.69±2.6 µg GAE/g and 72.77±2.0 µg GAE/g respectively; Figure 11.8). Intestinal digestion decreased ($p<0.05$) TPC to similar values for milk with or without herbal extracts (Figure 11.8). TPC after the 1st hour of gastric digestion of fresh *A. sativum*- and *C. verum*-yogurt was 78.14±2.1 µg GAE/g and 86.18±1.8 µg GAE/g respectively whereas plain-yogurt was 77.23±3.1 µg GAE/g (Figure 11.8). Intestinal digestion decreased ($p<0.05$) TPC to 43.29±1.0 µg GAE/g, 47.87±1.2 µg GAE/g and 47.58±3.0 µg GAE/g for plain-, *A. sativum*- and *C. verum*-yogurt respectively by the 2nd hour of intestinal digestion. *in vitro* gastrointestinal digestion

of 7 days old yogurt showed lower ($p>0.05$) TPC than fresh yogurt both in the presence and absence of herbal extracts (Figure 11.8).

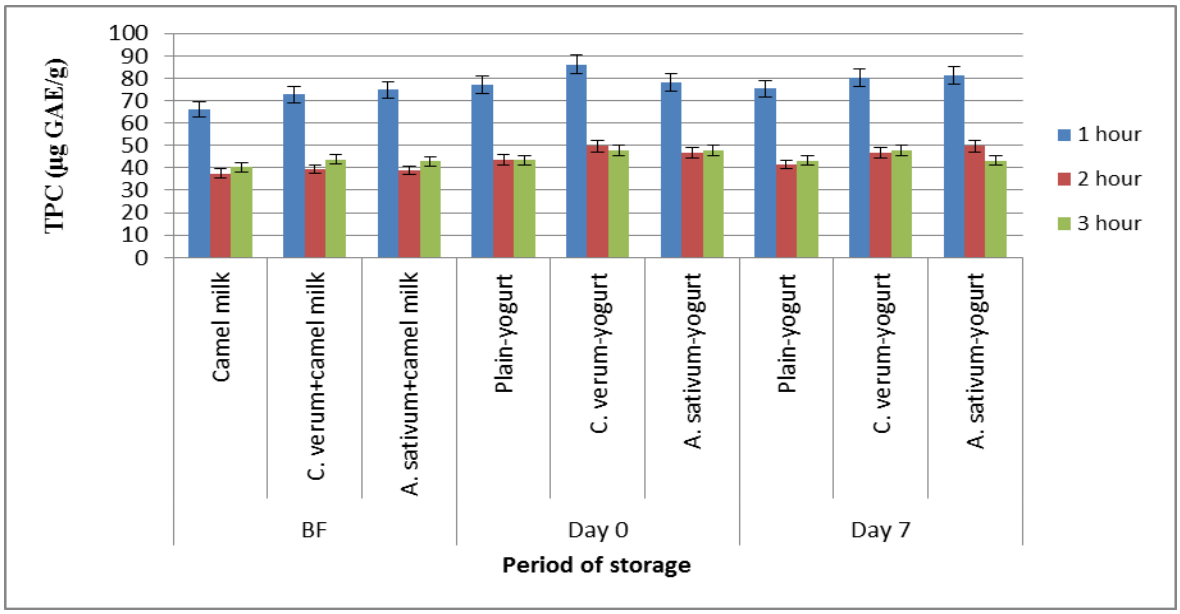


Figure 11.8 Total phenolic content (µg GAE/g) of camel milk before and after fermentation (0day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

D) In goat milk and goat milk- yogurt

The mixing of *A. sativum* or *C. verum* water extract with goat milk showed small difference in TPC (80.64±1.6 µg GAE/g and 82.53±2.0 µg GAE/g respectively) as compared to milk alone (76.26 ±1.8 µg GAE/g; Figure 11.9) after the 1st hour gastric digestion. TPC decreased ($p<0.05$) to 41.48±1.3 µg GAE/g, 42.32±1.2 µg GAE/g and 42.45±1.1 µg GAE/g for milk, milk+ *A. sativum* and milk+ *C. verum* respectively after the 1st hour of intestinal digestion. Prolonged intestinal digestion to another hour showed no significant changes in TPC of all treated samples (Figure 11.9).

The highest TPC of fresh- yogurt was shown in *A. sativum*-yogurt (104.26±2.6 µg GAE/g) followed by *C. verum*- (94.45±3.4 µg GAE/g) and plain- (92.17±1.7 µg

GAE/g) yogurt respectively after the 1st hour of digestion (Figure 11.9). Significant reduction of TPC occurred after the 1st hour of intestinal digestion with no further changes in TPC after prolonged intestinal digestion to another hour. Seven days old yogurt decreased ($p<0.05$) TPC after gastric digestion (79.68±3.2 µg GAE/g, 84.45±1.0 µg GAE/g and 84.86±3.4 µg GAE/g for plain-, *A. sativum*- and *C. verum*-yogurt respectively) compared to fresh yogurt. There were no significant differences in TPC between storage and fresh yogurt both in presence and absence of herbal extracts after 2 hours of intestinal digestion (Figure 11.9).

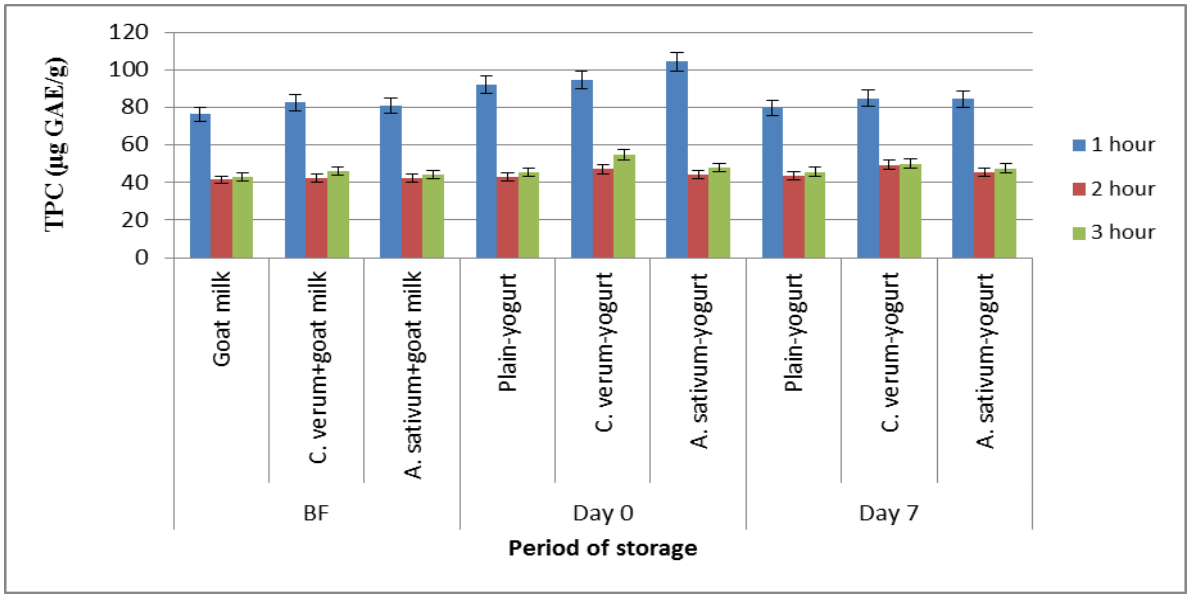


Figure 11.9 Total phenolic content (µg GAE/g) of goat milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

11.3.4 Antioxidant activity after SGD

A) In herbal water extract

C. verum water extract showed higher ($p<0.05$) antioxidant activity (95.23±1.6%) than *A. sativum* water extract (48.52±0.6%) after 1 hour gastric digestion (Table 11.4). Intestinal digestion for one hour showed reduction in antioxidant activity for both *A. sativum* and *C. verum* water extracts (9.08±0.3% and 60.37±0.6% respectively; $p<0.05$). An additional one hour digestion in intestinal section for both herbal extracts resulted in further increase (11.07±0.6%; $p<0.05$) for *A. sativum* and decrease for *C. verum* (55.46±1.2%) on antioxidant activity (Table 11.4).

Table 11.4 Effects of *in vitro* gastrointestinal digestions of *A. sativum* and *C. verum* water extracts on antioxidant activity.

Incubation time (hour)	DPPH inhibition (%)	
	AS water extract	CV water extract
1	48.52±0.6	95.23±1.6*
2	9.08±0.3	60.37±0.6
3	11.07±0.6	55.46±1.2

AS= *A. sativum* and CV= *C. verum*. The concentration of both herbal extracts before digestion = 0.1g/ml. 1st hour represent *in vitro* gastric digestion, 2nd and 3rd hours represent 1 and 2 hours *in vitro* intestinal digestion respectively. Results are shown as mean (n = 3) ± standard error. *p < 0.05

B) In cow milk and cow milk- yogurt

The antioxidant activity of cow milk was 23.01±1.2% after 1st hour gastric digestion (Figure 11.10). The antioxidant activity was increased in the mixture of milk with *C. verum* water extract (40.42±0.1%; $p<0.05$) but not with *A. sativum* water extract (24.72±1.6%) after gastric digestion. The antioxidant activity of cow milk with or without *A. sativum* water extract increased ($p<0.05$) after intestine digestion (1 and 2 hours) to about the same amount (40%; Figure 11.10).

Fresh *C. verum*-yogurt showed higher ($p<0.05$) antioxidant activity (42.4±0.4%) than plain- yogurt (31.0± 0.2%) after gastric digestion (Figure 11.10). In contrast, *A. sativum*-yogurt showed no difference (32.9± 1.1%; $p>0.05$) in antioxidant activity compared to plain- yogurt. Intestinal digestion of yogurt for one hour increased ($p<0.05$) antioxidant activities in plain- and *A. sativum*- yogurt to similar value (43%) but not in *C. verum*-yogurt (46.0±1.1%; Figure 11.10). There was only minimal increase ($p>0.05$) in antioxidant activity after additional 1 hour intestinal digestion for all treatments. Seven days old yogurt both in the presence and absence of herbal water extracts showed higher ($p<0.05$) antioxidant activities than fresh yogurt after gastric digestion (62.2±0.35, 61.5±0.9% and 67.1±1.5% for plain-, *A. sativum*- and *C. verum*- yogurt respectively). Intestinal digestion (1 and 2 hours) increased ($p<0.05$) antioxidant activity of all treated yogurt to similar values (~80%; Figure 11.10).

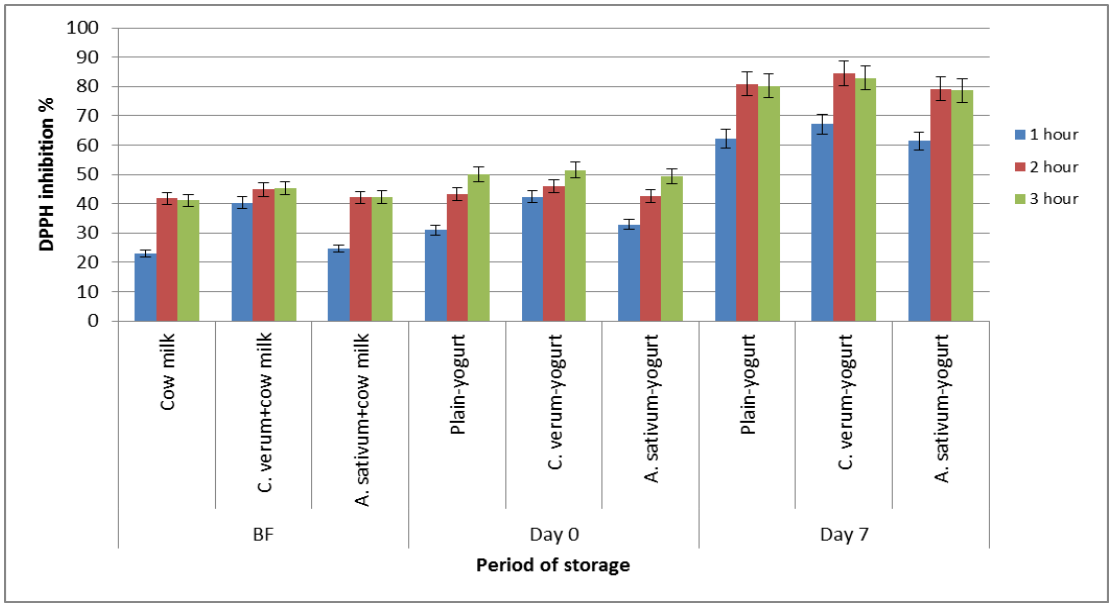


Figure 11.10 Antioxidant activities (DPPH inhibition %) of cow milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

C) In camel milk and camel milk- yogurt

Camel milk showed 28.10±1.6% antioxidant activity after 1 hour gastric digestion (Figure 11.11). The mixture of milk with *C. verum* or *A. sativum* water extract increased antioxidant activity (39.39±2.7% ($p<0.05$) and 30.28±0.4% respectively). Further digestion of milk mixtures with herbal extracts for 2 hours intestinal digestion showed no changes in antioxidant activity compared to control.

The antioxidant activity of fresh *C. verum*-yogurt (42.64 ± 1.2%) subjected to gastric digestion was higher than plain-yogurt (28.84 ± 1.5 %; $p<0.05$) which the latter was similar to *A. sativum*- yogurt (30.51±1.3 %). An hour intestinal digestion increased the antioxidant activity in plain- and *A. sativum*-yogurt to similar value (43%; $p<0.05$) but not in *C. verum*-yogurt (46.43± 1.6 %) as compared to respective gastric digestion (Figure 11.11). An additional hour of intestinal digestion showed only small increase in antioxidant activity to about 50% of all treatments. Gastrointestinal digestion of 7 days old yogurt increased ($p<0.05$) antioxidant activities compared to fresh yogurt both in the presence and absence of herbal water extracts. The antioxidant activities of 7 days old yogurt were 61.85± 1.6 %, 67.5± 1.6 % and 68.46± 0.5 % for plain-, *A. sativum*- and *C. verum*-yogurt respectively. Intestinal digestion of plain- and herbal- yogurt increased ($p<0.05$) antioxidant activity to almost same level compared to respective gastric digestion (Figure 11.11).

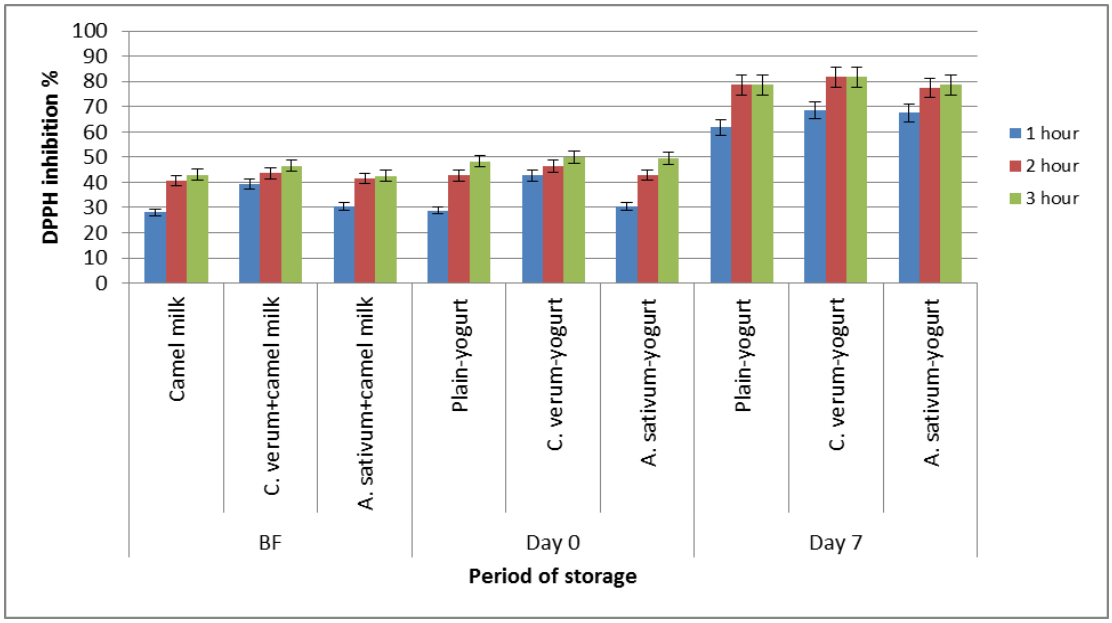


Figure 11.11 Antioxidant activities (DPPH inhibition %) of camel milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

D) In goat milk and goat milk- yogurt

The mixture of *C. verum* and goat milk showed higher ($p<0.05$) antioxidant activity (27.87±0.7%) than milk and milk with *A. sativum* (13.93±0.4% and 15.35±0.3% respectively) after 1 hour gastric digestion (Figure 11.12). Intestinal digestion for 2 hours decreased ($p<0.05$) antioxidant activity to 4.49±0.7%, 5.32±0.3% and 11.29±0.2% for milk, *A. sativum*+milk and *C. verum*+milk respectively.

The antioxidant activity of fresh goat milk yogurt was 19.63±1.0% (Figure 11.12) after 1 hour gastric digestion. The presence of *C. verum* or *A. sativum* water extract in yogurt increased the antioxidant activity to 34.49±0.8% ($p<0.05$) and 22.83±0.4% respectively. Intestinal digestion (2 hour) of yogurt showed a slight increase in antioxidant activities for plain- and *A. sativum*-yogurt but not for *C.*

verum-yogurt which had a reduction ($p<0.05$) in antioxidant activity to 24.21±0.6%. In comparison to fresh yogurt, 7 days refrigerated yogurt had increased ($p<0.05$) antioxidant activity (28.66±0.8%, 31.34±0.9% and 39.12±0.9% for plain-, *A. sativum*- and *C. verum*-yogurt respectively) after gastric digestion (Figure 11.12). However, the antioxidant activities decreased ($p<0.05$) to 20.46±0.5%, 21.90±0.4% and 28.24±0.5% for plain-, *A. sativum*- and *C. verum*-yogurt respectively after the 2nd hour of intestinal digestion.

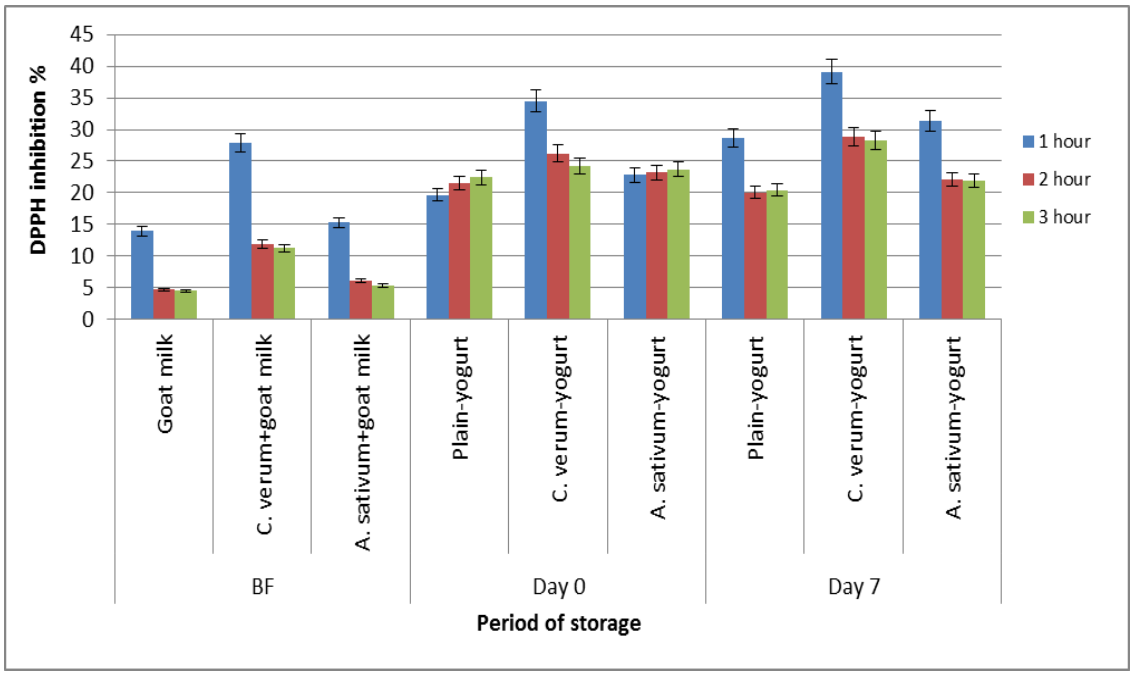


Figure 11.12 Antioxidant activities (DPPH inhibition %) of goat milk before and after fermentation (0 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 hours (1st hour represent gastric digestion, 2nd and 3rd hours represent 1 and 2 hours in intestinal digestion respectively). Error bars represent a pooled standard error of the mean (n=3). The level of significance was preset at p = 0.05 compared to plain-yogurt at the same storage period.

11.4 DISCUSSION

11.4.1 α-Amylase and α-glucosidase inhibition activities after SGD

The inhibition of these rate-limiting enzymes (α-amylase and α-glycosidase) in carbohydrate metabolism could serve as an approach to suppress post-prandial hyperglycemia, a condition commonly found in type-2 diabetes (Kwon *et al.,* 2006). Polyphenols in plants have ability to act as good inhibitors of key enzymes linked to type 2 diabetes (Ranilla *et al*., 2010; Cheplick *et al*., 2010; Adefegha and Oboh, 2012). Ingestion of the medicinal plants extracts can lead to structural modifications in the inhibitors because of low pH of the stomach (Simão *et al*., 2012). In the present study both *C. verum* and *A. sativum* water extracts showed ability to inhibit α-amylase (50% - 60%) and α-glycosidase (65% - 97%) post- SGD. Anson *et al*. (2009) reported that the bioavailability of polyphenols and their release after digestion is dependent on the food matrix. In the current study, the mixing of *C. verum* or *A. sativum* water extract with milk (BF) showed lower inhibition of α-amylase and α-glycosidase than herbal extracts alone. This could be due to interaction between phenolic and milk proteins (Alexandropoulou *et al*., 2006; Argyri *et al*., 2006; Cilla *et al*., 2009) which caused a decrease in the bioavailability of polyphenols in milk as α-amylase and α-glycosidase inhibitors. This reasoning is also in agreement with McDougall *et al*. (2005) who found reduction in phenol bioaccessibility of raspberries co-digested with ice cream. Similar observation could also occur in fresh and 7 days old yogurt in the presence of *C. verum* or *A. sativum*. Yogurt has the ability to inhibit α-amylase and α-glycosidase activities (Korhonen and Pihlanto, 2006) due to bioactive peptides released by the proteolytic activity of starter culture to hydrolyse milk proteins particularly caseins into peptides and amino acids (Fitzgerald and Murray, 2006). This could explain higher inhibition of diabetes enzymes in yogurt than milk. The lowest

inhibition of α-amylase and α-glycosidase activities seen in goat milk yogurt compared to cow- and camel- milk yogurt could be due to the difference in fermentation of bioactive peptides capable to inhibit these two enzymes.

High inhibitory effects toward α-glycosidase but not α-amylase were shown in all treated samples prepared from cow and camel milk post- intestinal digestion for one hour. This is indicated that both cow and camel milk have capacity to maximally inhibit α-glucosidase post- intestinal digestion. Similar observation was shown by Darmawan, (2010) who found high inhibition of α-glucosidase (~80%) in protein-based beverages after being subjected to pepsin pancreatin hydrolysates. This ability to inhibit α-glucosidase may explain why the consumption of yogurt is recommended to help reduce post-prandial hyperglycemia (Apostolidis *et al*., 2007; Ramchandran and Shah, 2008). However, the bioeffectiveness of theses peptides in goat milk treated samples was not limited only to the increase in the inhibitory activity of α-glycosidase (~ 90%) but also the extent of α-amylase inhibition activity (~ 40%) post- intestinal digestion for one hour. High stability of these bioactive peptides (slight changes) in all treated samples made from cow, camel and goat milk toward α-amylase and α-glycosidase inhibitory activities was shown even after the 2nd hour of intestinal digestion.

11.4.2 TPC and antioxidant activity in yogurt after SGD

There are limited studies on yogurt enriched with phenolic and antioxidant activity compounds and to the best of our knowledge little is known about the fate of these phenolics and antioxidant activity compounds after gastrointestinal digestion. Results obtained in this study indicate that both *A. sativum* and *C. verum* water extracts showed TPC about 55 µg GAE/g and 98 µg GAE/g respectively post- gastric digestion. However, the presence of *A. sativum* or *C. verum* in the three types of milk

before and after fermentation did not increase TPC post- SGD. This is in agreement with Gumienna *et al*. (2009) who showed no increase in TPC of pasta made from green lentil seeds containing garlic after 4 and 2 hours digestion in stomach and small intestine respectively. It is possible that milk proteins have formed a complex with polyphenols, reducing the bioavailability of the phenolic compounds (Keogh *et al*., 2007). Anson *et al*. (2009) reported that some polyphenols such as ferulic acid which is one of the most abundant polyphenols in cinnamon (Lv *et al*., 2012) and garlic (Beato *et al*., 2011) have a low bioavailability after digestion due to the difficulty being release from the food matrix. The release phenolic compounds are subjected to intestinal digestion (2 hours) thus the lower TPC observed in all treated samples. This in agreement with Gumienna *et al*. (2009) who found lower TPC in the green lentil seeds pasta containing garlic after 2 hours of digestion in intestine. TPC values in milk/yogurt reflect amino acid with side chain group similar to that in phenolic group which give rise to the reading in TPC (Shah, 2000).

Synthetic antioxidants such as 2,3-*tert*-butyl-4-methoxy phenol (BHA) and 2,6-di-*tert*-butyl-4-methyl phenol (BHT) are commonly used in the food industry. However, carcinogenic potential of these substances posed justified consumers concerns (Sarafian *et al*., 2002; Saito *et al*., 2003) thus the necessity to use natural food alternatives to replace synthetic additives. Polyphenols-derived antioxidants in plants have attracted the interests of food scientists, consumers, and manufacturers (Etcheverry *et al*., 2012). In the present study, both *A. sativum* and *C. verum* water extracts were showed to have high antioxidant activity post- SGD (Table 11.4). The presence of *A. sativum* in milk and yogurt did not influence on the changes of antioxidant activity. Similar observation was reported by Gumienna *et al*. (2009) who showed antioxidant activities of garlic water extracts enriched green lentil seeds pasta

are not significantly different from plain pasta over 21 hours of *in vitro* gastrointestinal digestion. In contrast, *C. verum*-yogurt made from all three types of milk showed increased DPPH inhibition compared to their respective plain-yogurt post- gastric digestion. The *C. verum*- goat milk yogurt still showed profound DPPH inhibition ($p<0.05$) even at post-intestinal digestion. This could be attributed directly to the high antioxidant activity of *C. verum* polyphenols as demonstrated in post-SGD (Table 11.4). Fresh plain- and herbal- yogurt made from goat milk contained much higher antioxidant activity than before fermentation whereas those made from cow and camel milk showed no significant differences. It is possible that more bioactive peptides which were being produced during goat milk fermentation exert strong antioxidant activity (Apostolidis, 2007; Elias *et al*., 2008; Darmawan *et al*., 2010).

The antioxidant activities of 7 days old plain- and herbal- yogurt were enhanced more in yogurt made from cow and camel milk ($p<0.05$) than that made from goat milk post- SGD. Antioxidant activity may be derived from milk proteins after proteolytic release of some amino acids/peptids (Arcan and Yemenicioglu, 2007) which may either donate protons to free radicals or because of their capacities to chelate metal cations (Elias *et al*., 2005). Goat milk-yogurt showed lower antioxidant activities compared to cow- and camel- milk yogurt post- intestinal digestion, thus indicting possible low formation of amino acids/peptids derived antioxidant activities.

11.5 CONCLUSIONS

Under simulated gastrointestinal digestion the presence of *A. sativum* or *C. verum* water extract had no significant effects on inhibitory activity against digestive enzymes related to diabetes and total phenolic content. However, the presence of *C. verum* in the three types of yogurt increased antioxidant activity post- SGD but not in the presence of *A. sativum*. The present study showed that all treated yogurt may offer anti-diabetic effects up to 99% inhibition of α-glucosidase activity and 75% of α-amylase activity after *in vitro* gastrointestinal digestion.

12.0 Overall conclusions and future research directions

12.1 Overall conclusions

This study has provided much valuable evidence on how yogurt production can be enhanced in the presence of medicinal plants such as *A. sativum* and *C. verum*. The LAB fermentation of milk has clearly been modified and the modified fermentated products appeared to exhibit functional properties likely to benefit people with reduced post-prandial glycaemia and blood pressure. The metabolic activities of LAB in herbal yogurt have some influences on the physicochemical properties, rheological and sensory characteristics. Changes in fermentation of milk due to *A. sativum* or *C. verum* did not contribute to post-acidification, ash, fat, solids-not-fat, lactose and carbohydrates contents but increased total solids and reduced moisture content in goat-milk yogurt. The presence of *A. sativum* or *C. verum* increased WHC and decreased STS in both cow- and camel- milk yogurt. Although the presence of these herbs in both cow- and camel- milk yogurt increased EPS production, these were not enough to improve the textural quality. The inclusion of *A. sativum* in yogurt reduced aromatic score for *A. sativum*-cow milk yogurt, the sensory properties as a whole was not affected.

The presence of *A. sativum* or *C. verum* stimulated LAB growth during fermentation of milk with better growth of *Lactobacillus* spp. being observed in camel milk whereas the growth of *S. thermophilus* and *B. bifidum* was enhanced in cow milk. Refrigerated storage of yogurt to 21 days had no effect on the survival of LAB and *B. bifidum*. The presence of *A. sativum* and *C. verum* enhanced milk proteolysis with an appreciable protoelytic activity in cow milk compare to camel and goat milk during fermentation. Furthermore, the proteolytic activities of LAB in herbal- cow milk yogurt have continued to show 2-folds higher rates than plain yogurt during 21 days of refrigerated storage. The increased proteolytic activities in herbal-yogurt and

the presence of polyphenolic compounds played a central role in the *in vitro* inhibition of ACE-I, α-amylase and α-glucosidase activities. These inhibitory activities however maybe compromised as a result of exposure to gastrointestinal enzymes. The free radical scavenging-linked antioxidant activity increased in the presence of herbs in both cow- and camel-milk yogurt. In contrast, the increased antioxidant activities attributed to herbal- (especially *C. verum*) yogurt made from three types of milk (cow, camel and goat milk) were further increased after simulated gastrointestinal digestion.

12.2 Future research directions relating to studies on herbal- yogurt

1) Characterization of organic acids/ amino acids produced as a result of alteration of yogurt fermentation in the presence of *A. sativum* or *C. verum* extract.

2) The isolation and characterization of bioactive peptides responsible for ACE-inhibitory activity in *A. sativum*- or *C. verum*- yogurt during refrigerated storage and after gastrointestinal digestion.

3) The phytochemicals screening in order to identify the bioactive constituents in *A. sativum* and *C. verum*, before and after the LAB fermentation of milk.

4) The kinetic studies to screen the mode of action of inhibitor on the diabetes enzymes as a step to gain a better understanding of the relationships between the structure and catalytic reactions of enzymes.

5) The *in vivo* studies to establish an optimal dose response and efficacy of *A. sativum*- and *C. verum*- yogurt which is safe for long term consumption.

6) The quality of the final product of goat milk yogurt in the presence of *A. sativum* and *C. verum*, viability of bacteria in yogurt and their metabolites associated with therapeutical values as anti-diabetes and antihypertensive effects during 21 days of storage (4°C).

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APPENDIX 1: Changes of pH in gastric content upon addition of yogurt

Changes of initial pH in gastric content (pH 3) after addition of fresh yogurt (0 day)

Type of yogurt	Plain-yogurt	*C. verum*-yogurt	*A. sativum*-yogurt
Cow milk-yogurt	3.71	3.82	3.75
Camel milk-yogurt	4.11	4.13	4.11
Goat milk-yogurt	3.77	3.86	3.84

Changes of initial pH in gastric content (pH 3) after addition of 7 days refrigerated yogurt.

Type of yogurt	Plain-yogurt	*C. verum*-yogurt	*A. sativum*-yogurt
Cow milk-yogurt	3.68	3.70	3.68
Camel milk-yogurt	4.07	4.1	4.09
Goat milk-yogurt	3.74	3.82	3.79

APPENDIX 2: Regression analysis (correlation)

Regression analyses (correlation %) between α-amylase inhibition activity and total acidity, TPC, DPPH inhibition activity, OPA values and TSP respectively.

	Regression analysis (correlation %)						
	Cow milk yogurt						
α-amylase inhibition activity	**Samples**	**TA**	**TPC**	**DPPH inhibition activity**	**OPA values**	**TSP**	**Total**
	Plain yogurt	20%	74%	-	23%	54%	171%
	AS yogurt	-	25%	-	13%	-	38%
	CV yogurt	59%	48%	22%	13%	85%	227%
	Camel milk yogurt						
	Samples	**TA**	**TPC**	**DPPH inhibition activity**	**OPA values**	**TSP**	**Total**
	Plain yogurt	-	-	31%	48%	79%	158%
	AS yogurt	72%	40%	30%	59%	-	201%
	CV yogurt	16%	14%	48%	12%	14%	104%

CV= *C. verum* and AS= *A. sativum*

Regression analyses (correlation %) between α-glucosidase inhibition activity and TA, TPC, DPPH inhibition activity, OPA values and TSP respectively.

	Regression analysis (correlation %)						
	Cow milk yogurt						
α-glucosidase inhibition activity	**Samples**	**TA**	**TPC**	**DPPH inhibition activity**	**OPA values**	**TSP**	**Total**
	Plain yogurt	80%	70%	20%	87%	87%	344%
	AS yogurt	82%	21%	48%	85%	66%	302%
	CV yogurt	85%	-	48%	25%	85%	243%
	Camel milk yogurt						
	Samples	**TA**	**TPC**	**DPPH inhibition activity**	**OPA values**	**TSP**	**Total**
	Plain yogurt	97%	34%	40%	17%	-	188%
	AS yogurt	97%	26%	25%	68%	25%	241%
	CV yogurt	98%	30%	83%	91%	98%	400%

CV= *C. verum* and AS= *A. sativum*

Regression analysis (correlation= r^2) between DPPH inhibition activity and OPA values.

Cow milk yogurt	**r^2**	**Camel milk yogurt**	**r^2**
Plain-yogurt	0.0201	**Plain-yogurt**	0.9322
CV- yogurt	0.7942	**CV- yogurt**	0.6538
AS- yogurt	0.6422	**AS- yogurt**	0.7952

CV= *C. verum* and AS= *A. sativum*