# PHYTOSTEROL ANTIOXIDANT ACTIVITY AND EFFECTS ON SHELF LIFE OF FLUID MILK AND YOGHURT QUALITY

By

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A Thesis

Submitted to the Faculty of Graduate Studies of

The University of Manitoba

In Partial Fulfillment of the Requirements For the Degree of

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Department of Food Science

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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## ABSTRACT

Phytosterols play a major role in the area of functional foods. Their ability to reduce serum cholesterol in humans has been widely proven and they are now being added to various foods world-wide. Several studies have also reported on their antioxidant activity, and as such phytochemicals may have the potential to impart other benefits to food. The present study investigated the antimicrobial activity of several phytosterol mixtures produced by Forbes Medi-Tech Inc. (Vancouver, BC) against milk microflora including *Pseudomonas* as well as yoghurt starter cultures and the antioxidant activity of various free and esterified sterols in oils. The effect of phytosterols on the rheology of yoghurt was also investigated.

As indicated by the results, a commercial phytosterol preparation (CPP) containing ß-sitosterol, campesterol, sitostanol and campestanol, exhibited no antimicrobial activity at a concentration of 1.8% (w/v) towards the SPC (standard plate count) or psychrotroph population in milk stored at 4°C. A challenge study employing various *Pseudomonas* spps in milk at 4°C also confirmed that the CPP was not antimicrobial. Homogenization of the milk subsequent to CPP addition, in order to increase dispersal, did not increase its antimicrobial effect. However, the addition of dispersible forms of phytosterols designated ADP (dispersible phytosterol mixture A) and BDP (dispersible phytosterol mixture B), which contained sodium stearoyl lactylate (SSL, 0.03%; w/v) appeared to produce an antimicrobial effect against SPC and psychrotrophic bacteria in refrigerated milk. The ADP and BDP (0.72% w/v) did not, however, inhibit the growth of *Pseudomonas*. An investigation into the antimicrobial

activity of SSL alone revealed that it did not inhibit the growth of SPC in milk, indicating that it did not posses any intrinsic antimicrobial activity that added to that of phytosterol.

The CPP, as well as several other phytosterol mixtures had no effect on the growth of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* during yoghurt production at 33°C. The phytosterols also had no effect on acid development as evidenced by pH values. Acid and starter culture levels were similar to those of the controls during storage at 4°C for 30 d. This is seen as a beneficial feature since growth of and acid development by these organisms are crucial for yoghurt quality. *Saccharomyces cerevisiae* and *Aspergillus ochraceus,* inoculated into yoghurt samples to simulate spoilage by yeast and mold, were not inhibited by the inclusion of the CPP either during production or storage at 4°C for 30 d. Rheology results indicated characteristic flow and viscoelastic properties for both control and CPP containing yoghurts. Overall, the results indicated that the CPP did not contribute to an increase in shelf life, however, it also exhibited no negative effects on yoghurt quality.

Investigation into the antioxidant activity of phytosterols revealed that free phytosterols (3.4% w/w) exhibited greater activity than phytosterol esters (5.44% w/w) in MCT (medium-chain triglyceride) and canola oils. Also, only free phytosterols were shown to reduce lipid oxidation in oils stored for 12 months. The phytosterol esters offered no protection against lipid oxidation for oils heated to 100 and 190°C but, instead, appeared to increase the propensity to lipid oxidation. Free phytosterols reduced the thermal degradation of canola oil when held at 190°C for 7-12 h and reduced lipid oxidation in oils heated to 100°C. The loss of linolenic but not linoleic acid was also reduced by addition of free phytosterols to the oil. The increased activity of the free

versus esterified phytosterols was likely due to the availability of a terminal hydroxyl group in the former compounds, for interaction with peroxide radicals.

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# 1. INTRODUCTION

A functional food is a widely used term and was defined by Health Canada in 1998 as "generally accepted to be foods similar in appearance to conventional foods and consumed as part of the regular diet. These foods have demonstrated physiological benefits, and/or reduce the risk of chronic disease beyond basic nutritional functions" (Moreau, 2004). Some of these foods can also be referred to as nutraceuticals, which specifically refers to "a product that has been isolated or purified from foods and generally sold in medical forms not usually associated with food" (Moreau, 2004). This definition of the term nutraceutical as a product category was abolished by Health Canada in 2001 and products falling under its definition were transferred to the natural health products (NHP) category (Moreau, 2004).

The first major developments in the area of modern functional foods occurred in Japan in the 1980's, and since then have spread world-wide. In 2000, the world market for functional foods was valued at $15 billion US (Gibson and Williams, 2000). These types of products appeal to health conscious consumers who are concerned about their dietary intake. Also, in many countries, there are concerns about managing the health of an increasing ageing population. New research linking diet and the prevention of chronic disease such as artherosclerosis has also increased awareness (Gibson and Williams, 2000).

Phytosterols are a group of compounds isolated from plants that have recently entered the functional food market. They have been added to various commercially available foods including fat spreads and yoghurt and their consumption has been proven

to lower serum cholesterol levels. As is the case with several phytochemicals, phytosterols also exhibit antioxidant activity (Yoshida and Niki, 2003). Since many phytochemicals displaying antioxidant activity, including extracts from spices, also possess antimicrobial activity (Dykes et al., 2003) it is possible that phytosterols can act as antimicrobials. If this were true it would provide an added benefit to food preservation since consumers would prefer products with all natural ingredients. Extracts of a primary phytosterol, ß-sitosterol, from several herbs has been shown to exhibit antimicrobial activity against the organisms *Bacillus subtilis* (Beltrame et al., 2002) and *Candida albicans* (Moshi et al., 2004).

The objectives of the present study were to: 1. investigate the antimicrobial activity of a phytosterol mixture against milk microflora and spoilage organisms 2. evaluate the effect of the same phytosterol mixture on the growth of starter culture organisms and spoilage organisms in yoghurt and 3. verify the antioxidant activity of the commercial phytosterol mixture.

To attain these objectives, the growth of aerobic mesophiles (standard plate count), psychrotrophs and pseudomonads in milk with and without phytosterol addition was investigated. Also, due to interest in adding sterols to commercial dairy products (Forbes Medi-Tech Inc., 2006), potential activity of phytosterols towards starter cultures and possible contaminants including yeast and mold in yoghurt (based on its popularity and the number of commercially available products containing nutraceuticals) was evaluated. The effect of phytosterols on yoghurt pH and rheology were included in this study as a means of assessing food quality. Finally, the antioxidant activity of the phytosterols was investigated as a means of correlating antimicrobial activity.

# 2. LITERATURE REVIEW

## 2.1. Phytosterols

### 2.1.1. History

Phytosterol is the name used to describe a group of naturally occurring sterol compounds found in plants that have a similar structure to cholesterol, differing only in their side-chain configuration or extra double bond (Baker et al., 1999). The chemical similarity between phytosterols and cholesterol is so striking that it is difficult to separate them by physical means, and techniques such as gas chromatography or high pressure liquid chromatography are often used to identify them (Ostlund, 2002). Their role in plants is similar to that of cholesterol in animals in that they play a vital role in maintaining membrane function (Ostlund, 2002).

Although phytosterols have been used to treat hypercholesterolemia since the 1950's (Miettinin, 2001), it is only in the last few decades that they have gained wide acceptance and availability to consumers in the form of functional foods. The ability to block absorption of cholesterol thereby lowering serum cholesterol levels is important, since it has been shown to decrease the risk of cardiovascular and coronary heart disease (Zawistowski and Kitts, 2004). The first phytosterol mixture produced was a pharmaceutical called Cytellin, which was marketed by Eli Lilly in 1957 (Moreau, 2004). In early studies, cholesterol reduction was studied by administering relatively large doses (25 g/day) of a phytosterol mixture in crystalline form (Institute of Food Science and Technology, 2000). Administering crystalline phytosterols, however, did not promote a significant decrease in serum cholesterol when compared to phytosterols dissolved in edible fat products (Institute of Food Science and Technology, 2000). The studies

concluded that the effect on serum cholesterol was not reliable because the crystalline phytosterols were not dispersed adequately (Moreau, 2004).

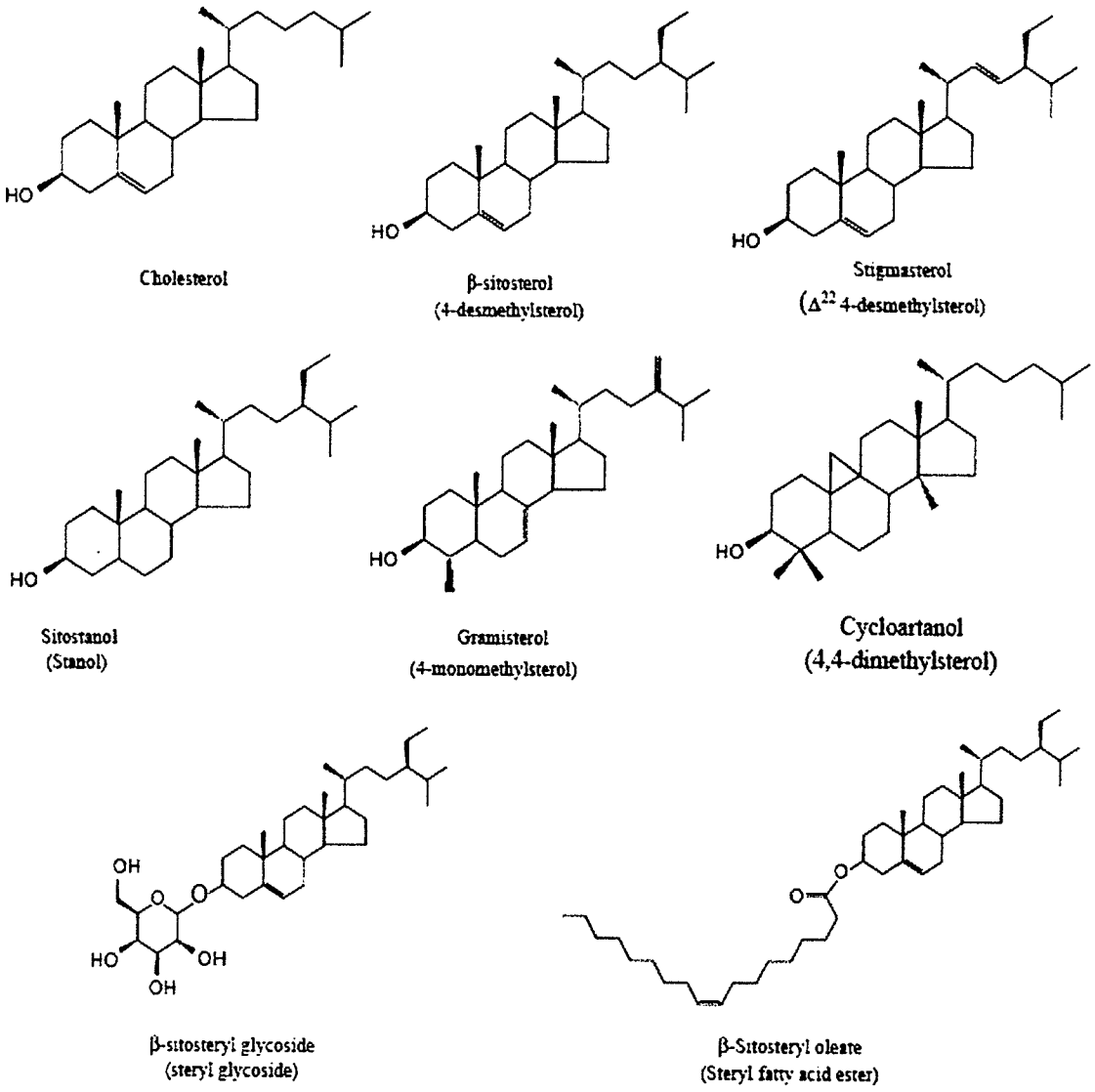
With the increasing popularity of functional foods, phytosterols are now being added to commercially available foods including fat spreads, salad dressing and yoghurt in order to help consumers lower their serum cholesterol levels. In the UK alone it is estimated that the use of phytosterol-enriched fat spreads have the capacity to save $150 million per year in healthcare costs (Zawistowski and Kitts, 2004).

### 2.1.2. Nomenclature and structures

There are several types of phytosterols found in nature. Over 250 compounds are included in this category. The main compounds are campesterol, stigmasterol and β-sitosterol, with ß-sitosterol being the most prevalent (Quilez et al., 2003). The structures of these compounds are shown in Figure 2.1. Phytosterols are synthesized in plants through a series of steps initially involving acetate subunits through squalene leading to the production of cycloartenol (Ostlund, 2002). A variety of final sterol products are produced, with each plant species possessing a distinct distribution of sterols that can be used as a type of fingerprint to identify them (Ostlund, 2002).

Phytosterols are divided into two groups. The first group is termed sterols or Δ-5 sterols and includes compounds such as campesterol, ß-sitosterol and stigmasterol that have a double bond at the 5 carbon position. The second group is termed stanols and contain a 5α-reduction of the double bond (Ostlund, 2002). Stanols are the saturated form of sterols, meaning they contain no carbon-carbon double bonds (Moreau, 2004). For example, sitostanol is the saturated form of sitosterol.

**Figure 2.1.** Chemical structure of cholesterol and primary group of phytosterols




adapted from Quílez et al., 2003

### 2.1.3. Dietary sources

Phytosterols are naturally present in foods (such as vegetables) that are regularly consumed in the human diet, but levels vary widely and each plant species contains specific phytosterols in fixed amounts. Although levels can change slightly by variations in cultivation method and genetic factors (Quilez et al., 2003), they are not high enough to achieve the daily 2 g phytosterol intake necessary to cause a significant decrease in blood cholesterol. In the USA, daily intake of phytosterols as part of a healthy diet is estimated at 180 mg; in Japan it may be as much as 400 mg (Moghadasian and Frohlic, 1999). As previously mentioned, phytosterols are found in vegetables and in the oil fractions of plants. Corn oil, for example, contains nearly 8 g/kg while palm oil contains 0.5 g/kg (Quilez et al., 2003). Phytosterols are also present in pulses and dried fruits (Quilez et al., 2003). The phytosterols most commonly found in these foods are ß-sitosterol, campesterol and stigmasterol (Miettinin et al., 1995). The phytosterol levels of several foods are listed in Table 2.1.

**Table 2.1.** Phytosterol content of several cereals, vegetables and fruits (mg $kg^{-1}$ fw[1])

| Sample | Campesterol | Sitosterol | Stigmasterol | Avenasterols | Stanols | Total |
|---|---|---|---|---|---|---|
| Barley | 150-192 | 437-484 | 24-36 | 56-69 | 17-19 | 720-801 |
| Millet | 112 | 371 | 18 | 87 | N.D.[2] | 770 |
| Corn | -[4] | - | - | - | - | 662-1205 |
| Rice | 146 | 375 | 104 | 20 | 32 | 723 |
| Broccoli | 67-69 | 285-310 | 8-11 | 2 | 18 | 367-390 |
| Carrot | 10 | 70 | 30 | - | - | 120 |
| Lettuce | 10 | 50 | 40 | - | - | 100 |
| Tomato | 10 | 30 | 30-35 | - | - | 70 |
| Apple | 3.6-9 | 130-157 | 1 | 7 | 8 | 130-185 |
| Banana | 20 | 110 | 30 | - | - | 160 |
| Raspberry | 9 | 233 | Tr[3] | 10 | 2 | 274 |

[1] Fresh weight

[2] Not determined

[3] Traces

[4] Not reported

adapted from Piironen and Lampi, 2004

### 2.1.4. Commercial sources

Several sources are used for the commercial production of phytosterols. One major source is from the deodorization of vegetable oils during the refining process (Quilez et al., 2003). Other sources include fibre oils such as maize, tall-oil which is the fat-soluble fraction of the hydrolysate obtained during wood (conifer) pulping process in paper production (Quilez et al., 2003) and sunflower oil processing (Baker et al., 1999). Tall-oil yields a higher proportion of stanols than vegetable oils, with β-sitostanol being the primary stanol compound (Forbes Medi-Tech Inc., 2006). Stanols are frequently added to foods fortified with phytosterols (Davidson et al., 2001) and are believed by some researchers to be more effective at lowering blood cholesterol than sterols (Miettinin et al., 1995). It is estimated that 2500 tonnes of vegetable oil yields 1 tonne of plant sterols during refining, while 2500 tonnes of pine oil is required to produce 1 tonne of plant sterols through the pulping process (Forbes Medi-Tech Inc., 2006).

### 2.1.5. Cholesterol lowering mechanisms

Several human trial studies have confirmed that phytosterols reduce total and low density lipoprotein (LDL) serum cholesterol, which is primarily attributed to their structural similarity with dietary and endogenously secreted cholesterol. Phytosterols become incorporated into fat micelles in the intestinal lumen, displacing both endogenous and biliary cholesterol and inhibiting their absorption in the small intestine (Quilez et al., 2003). In particular, it has been shown that phytosterols compete with cholesterol and displace it from bile salt/phospholipid micelles (Ostlund, 2002). Studies have also shown

that ß-sitosterol has an increased affinity for biliary micelles compared to cholesterol and therefore its uptake is favored (Ostlund, 2002). Unabsorbed cholesterol is then excreted in the feces. Overall, phytosterols remain relatively unabsorbed (Quilez et al., 2003) due to their micellar solubility, mucosal esterification and their slower rate of transfer from the cell surface to the intracellular site compared to cholesterol (Moghadasian and Frohlic, 1999). During consumption, approximately 56% of cholesterol was absorbed while 90% of sitosterol was excreted in the feces (Ostlund, 2002). Data using more recent analytical methods to determine phytosterol absorption suggest that 1.9% campesterol, 0.51% ß-sitosterol, 0.04% sitostanol and 0.16% campestanol are absorbed (Ostlund, 2002).

Phytosterols may also lower blood cholesterol by other means. For example, phytosterols remaining in the intestinal epithelium after ingestion have been shown to displace cholesterol within intestinal enterocytes as well as at intra luminal sites (Ostlund, 2002).

According to clinical trials, reductions in serum cholesterol of approximately 10% can be achieved when soluble phytosterol doses of 2 g per day are administered (Ostlund, 2002). Daily intakes consisting of 2-3 g of plant-based stanols have been shown to reduce LDL cholesterol levels in people who are hyper- as well as non-hypercholesterolemic by 10-14% (Mensink et al., 2002). Sitostanol has proven to be the most effective phytosterol at lowering serum cholesterol levels, but further study has shown that a mixture of plant sterols and stanols can be as effective as stanols alone (Moghadasian and Frohlic, 1999).

### 2.1.6. Addition to low-fat foods

Although many studies have explored the effect of phytosterols and stanols on lowering serum cholesterol in humans consuming high fat diets, relatively few studies have been conducted on populations consuming low fat diets (Moghadasian and Frohlic, 1999). Most studies have utilized phytosterols dissolved in oil or egg fat, emulsified in an aqueous solution with triglycerol monooleate, or reduced to a particle size of a few microns and mixed with fatty foods when evaluating their effect on blood cholesterol (Ostlund, 2002). These preparations increase the bioavailability of phytosterols. As previously mentioned, many studies have been conducted with respect to the health benefits of incorporating phytosterols in fat spreads. However, studies focusing on addition of phytosterols to low-fat or non-fat aqueous phase products such as milk and low fat yoghurt are fairly new (Noakes at al., 2005). Although the addition of phytosterols to these products is being explored as a means of broadening their utility, it is recognized that health conscious consumers may not ingest enough fat spreads (sometimes as much as 16 g) to achieve the recommended 2 g/day intake of phytosterols necessary to achieve the desired decrease in blood cholesterol levels (Noakes at al., 2005). Since low-fat dairy products are already seen as health foods, it is expected that they would be readily acceptable to health conscious consumers. In this respect it has been shown that the consumption of a low-fat (0.7%) yoghurt containing 3 g/day plant stanols reduced LDL cholesterol by 13.7% (Noakes at al., 2005).

### 2.1.7. Incorporation into food

The incorporation of phytosterols into certain foods can be difficult. Phytosterols have a lipophilic nature, which aids in their incorporation into high fat foods, but they are also somewhat waxy, which can hinder their incorporation. Phytosterol preparations have a very slow dissolution rate; they also exhibit low aqueous and organic solubility (Ostlund, 2002). Purified phytosterols that are used commercially are in a highly stable, crystallized form that can take days or weeks to dissolve in bile salt solutions (Ostlund, 2002). The solubility of free phytosterols in triglycerides is also low (1-2%), however, esterification with long chain fatty acids increases their solubility (Noakes at al., 2005) to levels as high as 10-20% (Ostlund, 2002). These phytosterol esters are hydrolyzed in the intestine to free sterols (Institute of Food Science and Technology, 2000). In addition to esterification, products containing free phytosterols can be dispersed in a protein, lecithin, or diacylglycerol matrix. For example, a product manufactured by Kraft Foods Inc. uses free phytosterols dispersed with emulsifiers (Moreau, 2004).

### 2.1.8. Safety

Studies have examined the safety of phytosterols, in particular how they affect levels of fat-soluble vitamins. However, a study by Davidson et al. (2001) reported that there were no adverse health effects (gastrointestinal, skin, urinary, nervous system, psychiatric, vascular or respiratory problems) related to the ingestion of esterified phytosterols and on levels of fat-soluble vitamins. The study did show a decreased level of serum α-tocopherol, however, this was expected because most α-tocopherol is carried in LDL particles, which are reduced by phytosterol ingestion. Studies have also shown

that serum levels of α- and β-carotene decreased by 11-22% following phytosterol ingestion (Ostlund, 2002). Again, this is not surprising as there is a correlation between reductions in cholesterol absorption and reductions in carotene and lycopene levels (Ostlund, 2002). Reductions of this magnitude are not dangerous to healthy individuals, but may be problematic to certain individuals, such as those with vitamin deficiencies or pregnant women.

A study was also conducted by Westrate et al. (1999) to determine the effects of phytosterol ingestion on fecal bile acid levels and neutral sterol excretion. These compounds are deemed important because they have been shown to play a vital role in the development of colon cancer (Westrate et al., 1999). The study demonstrated that phytosterol esters had no effect on neutral sterols while secondary bile acid levels in feces were reduced by phytosterol ingestion. This was likely accomplished by the inhibition of microbial 7-dehydroxylation activity, which is responsible for the conversion of primary to secondary bile acids (Westrate et al., 1999). Results of this study were in agreement with those conducted on mice, in which it was found that phytosterols provided protection against colon cancer caused by chemical carcinogens. This protection was likely due to the reduction of bile acid-induced colonic cell proliferation (Westrate et al., 1999).

### 2.1.9. Market

Phytosterols have been added to fat spreads, yoghurt, yoghurt drinks, chocolate, salad dressings, cheese, juice and soy drinks. Phytosterol-enriched foods are especially popular in the European market, which is very focused on functional foods and non-

GMO (genetically modified) foods (Forbes Medi-Tech Inc., 2006). In 1995, the first phytosterol-enriched fat spread was introduced by a Finnish company called Raisio Oy (Zawistowski and Kitts, 2004). In the UK alone the annual value of phytosterol-enriched spreads, yoghurt and yoghurt drink sales in 2005 was $300 million (Forbes Medi-Tech Inc., 2006).

In the USA, the market for phytosterol-enriched foods is somewhat smaller, but is still growing. The first phytosterol-enriched spreads Benecol® and Take Control® entered the US market in 1999. In 2003, a phytosterol-enriched orange juice was launched by Minute Maid using phytosterols produced by Cargill Foods. In 2005, General Mills launched the first phytosterol-enriched yoghurt for the American market as Yoplait Healthy Heart® (Winter, 2005).

In Canada, there are no phytosterol-enriched foods available. Becel Pro-activ® (a phytosterol-enriched fat spread available in the USA) was available briefly in 2001, but it was removed when Health Canada advised consumers that it was not in compliance with Canadian Foods and Drugs Act Regulations (Moreau, 2004).

### 2.1.10. Legislation

Legislation concerning phytosterols differs around the world. As of 2000, it was determined that the use of phytosterols in vegetable oil spreads must not exceed a concentration of 20% (Institute of Food Science and Technology, 2000). The US Food and Drug Administration (FDA) has also cleared spreads containing up to 20% plant sterol esters and plant stanol esters as GRAS (generally recognized as safe) (Institute of Food Science and Technology, 2000).

In Europe, the development of phytosterol-enriched foods is hampered by tight regulations and many applications that must be filed as they are classified as a "novel foods" (Winter, 2005). The Scientific Committee on Food (SCF) of the European Union (Official Journal of the European Union, 2004) determined that since long term phytosterol intake can affect β-carotene levels, all foods containing phytosterols, phytosterol esters, phytostanols and phytostanol esters should have labeling indicating their presence. The SCF also determined that levels should not exceed 3 g/day, as there was no evidence to indicate that higher levels produced additional health benefits. Therefore, the Commission of European Communities decided, based on the results obtained by the SCF, that product labels should indicate serving or single portion size "containing either maximum 3 g or maximum 1 g of phytosterols/phytostanols", calculated as free phytosterols/phytostanols (Official Journal of the European Union, 2004). When this is not possible or suitable, there should be a clear indication as to the portion that equals one serving size in ml or g and the level of phytosterols contained in that portion. The Commission also requires that products containing phytosterols state that the product is intended for people who wish to lower their cholesterol and that people who are already on medication to lower cholesterol must only consume the product under medical supervision. There must also be a statement that the product may not be nutritionally suitable for pregnant or nursing mothers (Official Journal of the European Union, 2004).

In Japan, several phytosterol-enriched fat spreads and oils are approved as FOSHU (Foods for Specified Health Use). Australia and New Zealand also allow the sale of phytosterol-enriched foods. The Australian and New Zealand Food Authority

(ANZFA) has approved several fat spreads, which are required to inform consumers on their label that certain groups may be at risk by consuming the product (Moreau et al., 2004).

As mentioned previously, phytosterol-enriched foods are not available in Canada. Health Canada claims that phytosterols may pose a risk to specific groups like pregnant women, children and people who are on cholesterol-lowering medication. Because of these statements and a lack of regulations by Health Canada, the sale of phytosterol-enriched foods is not allowed under the Canadian Food and Drug Act and Regulations (Moreau et al., 2004).

## 2.2. Milk

Milk is a nutritious food product that is universally consumed in one form or another. The addition of phytosterols would make this medium an ideal functional food candidate.

### 2.2.1. Sources of spoilage organisms

Although the commercial production of milk has been enhanced by pasteurization and improved distribution methods, the premature spoilage of milk is still a problem in the dairy industry. The shelf life of pasteurized milk is approximately 14-17 days when maintained at 4-7°C (Dairy Management Inc., 2006). Spoilage is due to a variety of microorganisms originating from various environmental sources. Although the majority of spoilage microorganisms in raw milk are destroyed during the pasteurization process, gram-positive spore-forming bacteria including *Bacillus* and or asporogenous

thermodurics including *Micrococcus* can survive. Most of these microorganisms grow slowly in refrigerated milk (Eneroth et al., 1998), however, when the total aerobic plate count reaches $10^7$ cfu/ml (Deeth et al., 2002) spoilage (mainly odor) becomes evident.

The occurrence of post-pasteurization contamination in milk also leads to spoilage. The most common sources are filling-machines and pasteurizers that have not been cleaned and/or sanitized properly (Hayes et al., 2002). Contamination occurs because filling is an open process and exposes the milk to aerosolized organisms. Condensed water on machinery also can enter milk and cause contamination if the machinery has not been thoroughly cleaned or if the water is contaminated. For example, Eneroth et al. (2000) reported that although the municipal water used in several dairy plants was not contaminated with gram-negative bacteria, their presence was nevertheless detected on the interior surface of rubber hoses. In addition, biofilms were created. The study also detected the presence of gram-negative organisms in the residual water at the bottom of the filling machines as well as on the filling nozzles.

### 2.2.2. Important spoilage organisms

Approximately 50% of retail pasteurized milk contains a flora consisting of gram-negative psychrotrophic bacteria including *Pseudomonas, Aeromonas* and *Enterobacteriaceae* (Eneroth et al., 2000). In many cases their presence is due to post-process contamination and represents the primary cause of spoilage in retail milk (Hayes et al., 2002). The contamination level of these organisms is relatively low (< 30 cfu/ml) as to be virtually undetectable in freshly pasteurized milk. However, due to their psychrotrophic nature they can not only grow under refrigeration temperatures but also

synthesize extracellular enzymes that contribute to a shortened shelf life (Eneroth et al., 1998).

*Pseudomonas* is the main spoilage bacterium found in refrigerated, pasteurized milk. *Pseudomonas fluorescens* is usually the most prevalent species, followed by the non-fluorescing *Pseudomonas fragi* (Ternström et al., 1993). The typical aroma of milk spoiled by *Pseudomonas* is often described as fruity. This aroma is caused by the production of esters including ethyl butyrate and ethyl hexanoate (Hayes et al., 2002). Rancid or "cheesy" aromas may also be present due to free fatty acids produced by lipolysis (Hayes et al., 2002). The growth of these organisms also causes milk to have a bitter flavor. This type of spoilage is due to proteases produced during growth, which leads to the breakdown of milk proteins producing bitter peptides (Deeth et al., 2002). Free fatty acids due to lipolysis of milk are also products of *Pseudomonas* growth and contribute to the sour flavor of spoiled milk (Deeth et al., 2002). Low molecular weight organic acids including lactic and acetic acid may also contribute to sourness. Severe bacteriological spoilage is often accompanied by rotten flavors due to sulfur compounds such as sulphides, disulphides and mercaptans produced during proteolysis (Deeth et al., 2002).

## 2.3. Yoghurt

### 2.3.1. Health benefits

Yoghurt is a food product made by the fermentation of milk using starter cultures normally consisting of *Streptococcus thermophilus* and *Lactobacillus delbruekii* spp. *bulgaricus* (McKinley, 2005). The addition of these microorganisms leads to the

development of a product with a lower pH and a firmer texture compared to milk. Overall, the shelf life of yogurt is greater compared to fluid milk primarily due to its acid pH (4.2-4.5).

Extensive research has confirmed the human health benefits of eating yoghurt (McKinley, 2005). The first and most obvious benefit is derived through the nutrients contained in the food product. The primary component of yoghurt is milk, and as such it has a nutritional profile similar to that of milk. Table 2.2 shows the nutrient values of milk and yoghurt. The addition of fruit, which is very popular in commercial yoghurt, will slightly alter its nutrient profile (McKinley, 2005). Yoghurt is a good source of proteins, vitamin B1, vitamin B2, vitamin B12, calcium and phosphorous (McKinley, 2005). Milk proteins are of excellent biological quality because they contain all the amino acids necessary for good health. Proteolysis caused by starter cultures also makes the proteins more digestible (Tamime and Robinson 1985).

**Table 2.2.** Values of the major constituents of milk and yoghurt

| Constituent (units/100g) | Milk | | Yoghurt | | |
|---|---|---|---|---|---|
| | Whole | Skim | Full fat | Low fat | Fruit |
| Calories | 67.5 | 36 | 72 | 64 | 98 |
| Protein (g) | 3.5 | 3.3 | 3.9 | 4.5 | 5 |
| Fat (g) | 4.25 | 0.13 | 3.4 | 1.6 | 1.25 |
| Carbohydrate (g) | 4.75 | 5.1 | 4.9 | 6.5 | 18.6 |
| Calcium (mg) | 119 | 121 | 145 | 150 | 176 |
| Phosphorous (mg) | 94 | 95 | 114 | 118 | 153 |
| Sodium (mg) | 50 | 52 | 47 | 51 | - |
| Potassium (mg) | 152 | 145 | 186 | 192 | 254 |

adapted from Tamime and Robinson, 1985

The vitamins and minerals contained in yoghurt are also beneficial to human health. It has been shown that consumption of dairy products increases the likelihood that children and teenagers will meet the recommended intakes of calcium, folate, phosphorous, vitamin B12, vitamin A and magnesium (McKinley, 2005). Calcium in particular is an important mineral found in dairy products. Although it can be found in other foods, the calcium contained in dairy products is high on a per serving basis and is highly bioavailable, meaning it is available for absorption and use by the human body (McKinley, 2005).

As mentioned, calcium is an essential nutrient found in abundance in dairy products. Unfortunately, many people suffer from lactose intolerance and are unable to consume milk. Lactose is a disaccharide composed of the sugars galactose and glucose. People who suffer from lactose intolerance lack sufficient amounts of the enzyme lactase necessary to break lactose down into its primary sugars, which can then be absorbed by the small intestine and used by the body (McKinley, 2005). If lactose is not broken down, it passes to the large intestine where it is fermented by microorganisms in the colon, leading to flatulence, diarrhea and abdominal pain (McKinley, 2005). The reaction to lactose appears to decrease or disappear entirely, however, when it is consumed in the form of yoghurt. Although the exact nature of this phenomenon is not known, there are several possible explanations. It could be explained by the breakdown of lactose by the starter cultures, causing a lower level of lactose to reach the small intestine so that an adverse reaction is not instigated (Tamime and Robinson, 1985). Lactic acid bacteria produce β-galactosidase during yoghurt production, which may still be active in yoghurt

at the time of consumption. Some of the organisms may also survive the passage through the stomach and continue to metabolize lactose in the small intestine (Tamime and Robinson, 1985). Yoghurt also has a different texture than that of milk; it is a coagulated product. The yoghurt coagulum remains partially intact after digestion and so is diffused more slowly through the walls in the intestine. This may allow the lactase within the yoghurt to break down the lactose (Tamime and Robinson, 1985). Whatever the exact mechanism, the reduction of the adverse reaction to lactose facilitates the calcium intake of those people suffering from lactose intolerance.

Health benefits of eating yoghurt are also attributed to the bacterial starter cultures used in its manufacture and to other lactic acid bacteria, which survive passage through the human stomach and are known as probiotics (Haines, 2004). McKinley (2005) described probiotics as "viable non-pathogenic microorganisms which, when ingested, exert a positive influence on host health or physiology". Probiotics have been proven to exert several benefits to human health especially maintenance of a healthy microflora in the human gastrointestinal tract (GIT) (Stanton et al., 2003). Probiotics including *Bifidobacterium* and *Lactobacillus* species aid in maintaining human health by contributing to digestion, stimulating the immune system and inhibiting pathogen growth (Stanton et al., 2003). Several lactobacilli, for example, produce alpha-galactosidic activity and are therefore able to eliminate the gas-producing carbohydrates from legumes and soy bean milks by breaking down alpha-galactosidic bonds in raffinose and stachyose (Kalantzopoulos, 1997). The antagonistic activity of probiotics toward pathogens in the intestine is due to several factors. These include competition for available nutrients, decrease in pH due to production of lactic and acetic acids,

production of inhibitory metabolites such as hydrogen peroxide and diacetyl, a decrease in redox potential and the production of antimicrobial compounds such as antibiotics and bacteriocins (Kalantzopoulos, 1997). Some of the health benefits of probiotics exhibited in human clinical trials are shown in Table 2.3. In order to be effective in improving human health, these organisms must be present in a viable form and in sufficient numbers. *Bifidobacteria* in fermented milk of $10^8$ cfu/g, for example, have been shown to have major effects, while one clinical trial found that *Lactobacillus casei* levels of $10^{11}$ cfu per day were necessary to increase levels of immunoglobulins in children with acute diarrhea (Kalantzopoulos, 1997). Probiotics must also survive passage through both the stomach and small intestine to reach the large intestine and be capable of colonization. This colonization is optimized if the organisms are versatile and efficient in the substrates they utilize for metabolism, have the ability to adhere to epithelial cells of the gut walls and be able to tolerate gastric acid. It is important for probiotics to be as hardy and efficient as possible, since the bacteria already residing in the gut are highly adapted to their environment and conditions, providing intense competition for any incoming organisms (McKinley, 2005). The consumption of probiotics with foods such as milk, cheese or yoghurt buffers stomach acid, thereby increasing the chance of bacterial survival (Haines, 2004). It is also believed that certain dairy components may have a synergistic effect on probiotics. Lactoferrin, which is naturally present in milk, has been shown in several *in vitro* studies to enhance the growth of bifidobacteria (Haines, 2004).

**Table 2.3.** Probiotic organisms and their documented health benefits in human clinical trials

| Genus | Species | Example strains | Health benefit |
|---|---|---|---|
| *Lactobacillus* | *acidophilus* | La5 | reduced diarrhea |
| | *casei* | Shirota | shortening of diarrhea episodes |
| | | | reduced recurrence of superficial bladder cancer |
| | | | immune modulation |
| | *reuteri* | SD2112 | shortening of diarrhea episodes |
| | *rhamnosus* | GG | shortening of diarrhea episodes |
| | | | immune modulation |
| | | | relief of inflammatory bowel disease (IBD) |
| | | | treatment/prevention of allergy |
| *Bifidobacterium* | *salivarius* | UCC118 | reduced IBD |
| | *longum* | Bb536 | reduced IBD |
| | *lactis* | Bb12 | allergy treatment |

adapted from Stanton et al., 2003

### 2.3.2. Production methods

The milk used for yoghurt production is standardized and fortified to ensure it complies with standards for % fat and solids-not-fat (SNF), as well as protein since the consistency of the resulting yoghurt is affected by the amount of protein present in the milk (Tamime and Robinson, 1985). Legal standards for the SNF of yoghurt are a minimum of 8.2-8.6%. In the USA, yoghurt must contain no less than 8.25% SNF (Gale, 2006). If the SNF content is higher than 25% it can reduce the activity of the starter culture organisms by limiting available moisture. SNF in milk can be increased by adding skim milk powder to liquid milk, which is a popular method in the industry. Whey powder or casein powder may also be used. The fat content of the milk is also standardized by removing fat or adding cream to the milk in conjunction with the use of standardizing centrifuges (Tamime and Robinson, 1985).

The next step is homogenization. This ensures a homogenous emulsion of the mixture. The milk used in yoghurt production is an oil-in-water emulsion and has a tendency to undergo separation when left standing. After homogenization, the mixture is pasteurized (85° C for 30 min; 90-95°C for 5-10 min) to destroy pathogens and most non-sporulating spoilage organisms. The fermentation of the mixture takes place after it has been cooled to the incubation temperature. This varies between producers. A variety of temperatures can be used as long as they are within the tolerance of the starter culture organisms. A temperature of 40-45°C is the maximum, and ensures a short incubation time of approximately 2.5 h if the starter culture concentration is sufficiently high (3%). A longer incubation period of 18 h is needed when the temperature is 30°C. The

fermentation of the yoghurt by the starter culture causes the formation of a gel. This occurs due to the utilization of lactose by the organisms as an energy source, leading to the production of lactic acid. The lactic acid destabilizes the casein micelle and denatures the whey protein complex. Aggregates of casein micelles coalesce as the pH drops to the isoelectric point, which is around 4.6-4.7 (Tamime and Robinson, 1985). The casein particles link together in clusters and chains with pores where the aqueous phase is confined (Lucey and Singh, 1998). Fermentation also leads to the production of compounds that define the flavour and odour of yoghurt. The sequence of fermentation during production differs depending on the type of yoghurt being produced. For set yoghurt, the fermentation takes place in the final retail container. Once the incubation period is over, the yoghurt is cooled to refrigeration temperature to decrease the metabolic activity of the starter cultures and their enzymes (Tamime and Robinson, 1985). For stirred yoghurt, the fermentation is conducted in a bulk tank. After cooling the yoghurt can be mixed with fruit or flavoring and is pumped into retail containers and kept under refrigeration.

### 2.3.3. Criteria for determining quality

The quality of finished yoghurt is assessed based on its chemical composition, physical characteristics, microbiology and sensory characteristics. Chemical analysis involves determination of SNF, % lactic acid or pH and% fat, while physical characteristics are evaluated based on the nature of three different products; set yoghurt, stirred yoghurt and drinking yoghurt (Tamime and Robinson, 1985). These products each have different standards for viscosity and firmness, which can be increased by

denaturation of whey proteins (Lucey and Singh, 1998). Set yoghurt has a firm gel structure while stirred yoghurt is a semi-viscous liquid and has a lower viscosity as the coagulum is broken up during packaging (Tamime and Robinson, 1985). In set-style yoghurt, firmness (Newtons) decreases during storage (Torre et al., 2003). Viscosity is measured using dynamic testing employing a series of oscillatory or applied strain stress tests (Lucey and Singh, 1998). These tests yield a physical profile of the sample, which includes a measure of the energy stored in the material per oscillation cycle, which is called the elastic or storage modulus (G') and the viscous or loss modulus (G"), which represents the energy dissipated as heat per cycle of deformation. The loss tangent (tan $\delta$) is also determined and is the ratio of viscous to elastic properties (Lucey and Singh, 1998). These parameters are affected by the strength and number of the bonds between casein particles in the yoghurt, as well as their structure and spatial distribution (Lucey and Singh, 1998).

Whey separation is another important physical measurement of yoghurt quality. It refers to the formation of liquid on the surface of the yoghurt (Lucey and Singh, 1998). Separation occurs if there is substantial rearrangement of the yoghurt gel or if the gel network is damaged. Whey separation sometimes occurs with syneresis, which is caused by shrinkage of the yoghurt gel and expulsion of liquid from the gel structure. Syneresis occurs due to instability in the gel network and loss of the ability to hold all of the serum phase. It can be prevented by increasing the total solids content of the milk in set-style yoghurt, or by adding stabilizers such as pectin or gelatin to stirred yoghurt (Lucey and Singh, 1998).

Starter culture activity also has an effect on the sensory quality of yoghurt. It is important that *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are present in a 1:1 ratio in order to optimize their activity (Tamime and Robinson, 1985). During incubation, *L. bulgaricus* produces amino and formic acids that stimulate the growth of *S. thermophilus*, which grows rapidly until a pH of 5.5 is reached. This favours the growth of *L. bulgaricus*. If incubation is not halted when a pH of 4.0 to 4.4 is reached, then *L. bulgaricus* will continue to grow and produce a yoghurt that is overly sour (Barraquio et al., 1981). It is also important that the starter culture is free of contamination. An "unclean" smell or gas bubbles in the culture indicates contamination. This could lead to off-flavours in the yoghurt or, if yeast or molds are present, a decrease in the shelf life of the product (Tamime and Robinson, 1985).

Microbiology of yoghurt is important because low populations of *S. thermophilus* and *L. bulgaricus* will lead to poor flavour development while a high level will lead to excessive acidification of the product, syneresis, and imbalance in the flavour components and spoilage due to acid production during storage. Although there are no official regulations or standards for the level of organisms present in yoghurt, the industry standard is a minimum of $10^7$ to $10^8$ cfu/ml depending on the country of production. Yoghurt is also evaluated for contaminating organisms, particularly coliforms and yeast and moulds. Yeast and mould are particularly troublesome as they are capable of withstanding the high acidity of yoghurt (Tamime and Robinson, 1985).

### 2.3.4. Spoilage

Although the increased acidity of yoghurt allows for an extended shelf life, it is still susceptible to spoilage. For example, fungi and yeast are capable of surviving and growing in yoghurt despite its acid nature (Viljoen et al., 2003). In particular, growth of yeasts is aided by the addition of sugar and fruits, which act as fermentable substrates. Normally resistant to metabolites produced by the starter culture during fermentation, yeast and mould are also resistant to preservatives that are sometimes added to yoghurt, and this increases their competitive advantage (Viljoen et al., 2003). In order to assure a high quality product, yeast levels should be < 10 cfu/g, although a level of < 1 cfu/g is ideal. Under refrigeration temperatures, a shelf life of 4 weeks can be attained, assuming all other manufacturing conditions are in place (Fleet, 1990). This shelf life is diminished when yeasts are introduced with raw ingredients such as fruits, nuts and honey (Fleet, 1990). Contamination can also occur from equipment that has been improperly sanitized. In addition, some dairy-associated yeasts have been shown to be resistant to commercial sanitizers and cleaning compounds (Viljoen, 2001). *Debaryomycese hanseii, Candida versatilis* and *Torulaspora delbrueckii* were shown by Laubscher and Viljoen (1999) to resist sanitizers even after 60 min of exposure. Generally, it is not unusual to find yeast levels of $10^3$ cfu/g or greater in commercial yoghurt samples. A survey of retail yoghurt in Canada and the UK found that 20-30% of samples had yeast numbers > $10^3$ cfu/g (Fleet, 1990) and yeast numbers as high as $10^6$-$10^7$ cfu/g have been found in commercial yoghurt products (Viljoen et al., 2003). This is a problem as spoilage becomes evident when yeast populations reach $10^5$ to $10^6$ cfu /g (Viljoen et al., 2003). This spoilage takes

the form of excessive gas formation leading to package swelling, as well as off-flavours and discoloration (Viljoen et al., 2003).

Several yeasts are associated with yoghurt spoilage, however *Kluyvermyces, Candida* and *Saccharomyces* are the most commonly isolated genera (Fleet, 1990).

### 2.3.5. Functional yoghurts

In addition to phytosterol-enriched products, there are several types of nutraceutical yoghurts that are available. For example, Naturally Nutritious Yoghurt™ includes dietary fiber and is 99.5% fat-free (Mountain High Yoghurt, 2003). In 2004 Chr. Hansen A/S developed a yoghurt called Cardio-04™ that purports to lower blood pressure through a mixture of lactic acid bacteria (Chr. Hansen A/S, 2004). Several yoghurts enriched with EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) are also available. These two fatty acids play an important role in brain and nervous system development and aid in the maintenance of cardiovascular function (Ocean Nutrition Canada Limited, 2006). In Canada in 2006, Danone introduced Danino®, a yoghurt containing omega-3 fatty acid and DHA (Danone Canada, 2006). The first drinkable yoghurt containing EPA and DHA was launched in Japan in 2006 (Ocean Nutrition Canada Limited, 2006).

The market for probiotics is rapidly growing, with the sales in Europe set to triple from 2004 to 2010, reaching $137.9 million (Haines, 2004). The U.S. market is somewhat smaller, but is growing very rapidly and is projected to reach $394 million (Haines, 2004). As previously mentioned, the addition of probiotics to yoghurt is becoming widespread. The global probiotic and dairy drink market is estimated at $10

billion (The Dannon Company, 2005). Available products contain a range of *Lactobacillus* and *Bifidobacterium* species including *L. casei, L. acidophilus, L. delbreuckii ssp. bulgaricus, L. brevis, L. fermentum, L. plantarum, B.bifidum, B. longum, B. infantis, B. thermophilum* and *B. animalis* (Kalantzopoulos, 1997). In 1987, Danone introduced Activia® brand yoghurt in France. The product became available in the U.S.A. and Canada in 2006 (The Dannon Company, 2005). It is marketed by the Dannon Company as "the first and only probiotic yoghurt that is clinically proven to help naturally regulate your digestive system in two weeks, when eaten daily as part of a healthy and balanced diet". In 2006, CoolBrands International Inc. introduced Breyers Light Probiotics Plus Yoghurt®. This product contains 70% less sugar than the leading low-fat sweetened yoghurt, contains *Bifidobacterium* cultures and is enriched with vitamins A and D as well as calcium (CoolBrands, 2006).

## 2.4. Antioxidant Activity

### 2.4.1. Action of antioxidants

Like many phytochemicals, phytosterols exhibit antioxidant activity (Wang et al., 2002). These compounds are receiving increased attention because consumers desire products with "all natural" labels. There have also been safety concerns over the use of common synthetic antioxidants such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) and TBHQ (tert-butylhydroquinone) (Hung and Yen, 2001). Antioxidants are necessary in many fat-containing foods to combat the damage to sensory and nutritional quality caused by lipid oxidation. Antioxidants are capable of donating hydrogen radicals and, therefore, reducing primary radicals to nonradicals (Jadhav et al.,

1996). Once the hydrogen atom is donated, a radical with low reactivity is formed so that further reactions with lipids are halted. Antioxidants are classified as either primary or secondary. Primary antioxidants react with peroxide radicals prior to their reaction with unsaturated lipid molecules and convert them to more stable compounds. Secondary antioxidants slow the rate of chain initiation (the autoxidation of a fat due to the contact of an unsaturated lipid with oxygen leading to the formation of free radicals) by methods such as scavenging oxygen, absorbing UV radiation, binding metal ions and deactivating singlet oxygen (Jadhav et al., 1996).

Phytochemicals with antioxidant activity include phenolics, flavonoids, cinnamic acids, proteins, carotenoids and lignans as well as sterols (Jadhav et al., 1996). Tocopherols and ascorbic acid are highly effective as antioxidants and are commonly added to foods (Jadhav et al., 1996). Also, herbs and spices have attracted interest with respect to their content of antioxidant compounds (Rajalakshmi and Narasimnhan, 1996).

### 2.4.2. Antioxidants as antimicrobials

Several researchers have reported the use of antioxidants to inhibit fungal and bacterial growth and in some cases toxin production (Eubanks and Beuchat, 1983).

For example, Fung et al. (1977) showed that both growth and aflatoxin production by *Aspergillus parasiticus* were inhibited by BHA. A study by Eubanks and Beuchat (1983) showed that *Saccharomyces cerevisiae* viability was reduced when TBHQ was used at 100 ppm at –18°C over 25 d compared to controls. The authors also showed that 100 ppm BHA at 4°C had a lethal effect against *S. cerevisiae* over a period of 25 d.

Essential oils obtained from herbs and spices as well as extracts from the leaves and bark of traditional medicinal plants are natural antioxidants that have been investigated for their antimicrobial activity (Rajalakshmi and Narasimnhan, 1996). The impetus for the development of natural antimicrobials is the same as that for natural antioxidants: consumer concern about synthetic ingredients. In addition, some organisms have developed resistance to antibiotics and antimicrobials that are commonly used. Several natural antioxidants have been shown to exhibit antimicrobial activity. The mechanism of action differs between compounds, but activity has been shown to be dependant on the plant from which the extract has been produced and the bacterial strain against which it was tested (Dykes et al., 2003). Flavonoids act as primary antioxidants as well as chelators and superoxide anion scavengers. Their antioxidant activity is enhanced by the presence of a 3-hydroxyl group in their structure as well as a 2-3 double bond in their C ring (Rajalakshmi and Narasimnhan, 1996). Spices and herbs contain many antioxidant compounds and some have yet to be identified and isolated (Rajalakshmi and Narasimnhan, 1996). Phenolic acids are a group of compounds found in plants whose antioxidant activity is correlated with the number of hydroxyl groups in the molecule (Rajalakshmi and Narasimnhan, 1996). Carotenoids are a group of antioxidants found in plants that prevent the formation of hydroperoxides in the presence of singlet oxygen by quenching the singlet oxygen. This ability is related to the number of double bonds in the molecule (Rajalakshmi and Narasimnhan, 1996).

Flavonoids reportedly have antibacterial activity against microorganisms including *Proteus vulgaris*, *Staphylococcus aureus* and *Staphylococcus epidermis* (formerly *Staphylococcus albus*) (Tereschuk et al., 1997). A study by Tereschuk et al. (1997)

investigated the effect of flavonoids extracted from *Tagetes minuta* tea leaves against several microorganisms in comparison to chloramphenicol. They found that *Escherichia coli* growth was significantly inhibited by the flavonoid extract even at low concentrations in comparison to the antibiotic chloramphenicol. Extracts of the tea leaves were also effective in inhibiting *Bacillus subtilis* and *Pseudomonas aeruginosa*. However, *S. cerevisiae, Lactobacillus rhamnosus, L. plantarum* and *Zymomonas mobilis* were not inhibited by the extracts.

Although the antimicrobial activity of phytosterols has not been extensively researched, several studies investigating the antimicrobial activity of herbs have led to the isolation and subsequent evaluation of ß-sitosterol. In a study by Beltrame et al. (2002) ß-sitosterol was extracted from the tropical plant *Cissus sicyoides* in Brazil. The compound inhibited the growth of *B. subtilis* at a concentration of 50 µg/ml. Against *Staphylococcus aureus, E. coli,* and *Pseudomonas aeruginosa*, however, no inhibition was observed at a concentration of 100 µg/ml. While investigating the antimicrobial activity of the Tanzanian plant *Uvaria scheffleri*, Moshi et al. (2004) extracted a mixture of stigmasterol and ß-sitosterol from the leaves. This 1:1 mixture of the phytosterols exhibited antifungal activity against *C. albicans*, but failed to inhibit the growth of *Aspergillus niger, A. fumigatus, E. coli, S. aureus, P. aeruginosa* and a *Penicilium* species.

### 2.4.3. Antioxidant activity of phytosterols

Lipid oxidation is an important factor influencing the quality of oils. It is one of the major detrimental reactions that occur in oils used for frying and in fried foods. Losses in microbial stability as well as functional, nutritive and sensory values are often

the result of lipid oxidation. Therefore, in the food industry, antioxidants are often added to oils, fats and foods containing fats to combat the development of oxidation (Jaswir et al., 2000a). As previously mentioned, consumers prefer to purchase foods that contain natural additives rather than commercial synthetic compounds. For this reason, there has been a growing interest in natural sources of antioxidants that can be used in foods.

Several studies have investigated the antioxidant activity of phytosterols. It is believed that their activity is due to formation of an allylic free radical, which is isomerized to other stable free radicals (Yoshida and Niki, 2003). In plants, it is believed that phytosterols also act as antioxidants by packing and stabilizing membranes (Yoshida and Niki, 2003). Yoshida and Niki (2003) investigated the effect of β-sitosterol, stigmasterol and campesterol as well as a mixture of the three compounds on the oxidation of oils during heating. The researchers reported that the compounds inhibited oxidation of methyl linoleate, with the mixture having the greatest activity followed by campesterol, β-sitosterol and stigmasterol. However, when evaluated in liposomal membranes prepared from phosphatidylcholine they did not exhibit antioxidant activity. In addition, stigmasterol appeared to have a pro-oxidant effect.

Avenestrol, a phytosterol found in rice bran oil, has also been reported to act as an antioxidant during frying due to a ethyldiene group on its side chain (Wang et al., 2002). Wang et al. (2002) investigated the antioxidant effect of phytosterols/phytostanols and their ferulic acid esters in corn fiber oil and soybean sterols. All of the samples exhibited antioxidant activity, with rice bran oil showing the highest value, followed by sitostanol-ferulate. The results of this study are summarized in Table 2.4.

**Table 2.4.** Antioxidant activity (OSI)[1] of several natural and synthetic compounds in FAME[2] and commercial soybean oil

| | FAME[2] (h) | | Soybean oil (h) | |
|---|---|---|---|---|
| | 6 µmol/5g oil | 12 µmol/5g oil | 6 µmol/5g oil | 12 µmol/5g oil |
| Sitostanol-ferulate | 5.48 | 6.28 | 11.30 | 11.70 |
| Sitostanol | 4.80 | 4.90 | 11.53 | 11.05 |
| Soy sterols | 5.08 | 4.80 | 12.43 | 11.28 |
| Corn fiber oil | 5.23 | 5.45 | 10.75 | 10.30 |
| Rice bran oil | 9.18 | 11.95 | 13.78 | 14.68 |
| TBHQ | 21.98 | - | 35.30 | - |
| δ-Tocopherol | 12.40 | 17.78 | 12.25 | 12.55 |
| Blank | 4.69 | | 11.33 | |

[1]OSI = oxidative stability index

[2]FAME = distilled soybean oil fatty acid methyl esters

adapted from Wang et al., 2002

### 2.4.3. Evaluating antioxidant activity

Antioxidant activity of natural compounds is usually determined by evaluating the stability of food lipids after the addition of the compound. The sample is oxidized under standard conditions and the amount of oxidation is measured by one of a variety of chemical, instrumental or sensory methods. In sensory analysis, effectiveness of an antioxidant is evaluated by determining the lengthening of the induction period, which is the time it takes for initiation of oxidation coinciding with the development of off-flavour in the lipids (Rajalakshmi, and Narasimnhan, 1996). Chemical methods for determining oxidative stability include estimation of the peroxide or anisidine values, the Kreis test and the thiobarbituric acid test (Rajalakshmi, and Narasimnhan, 1996). These chemical tests utilize reagents, which react with the products of oxidation, such as peroxides, hydroperoxide, free fatty acids and aldehydes. Each test expresses results in different values, based on the manner in which oxidation is being measured (Rajalakshmi, and Narasimnhan, 1996).

# 3. MATERIALS AND METHODS

## 3.1. Effect of CPP On Growth of Milk Microflora

### 3.1.1. Preparation of milk samples

Retail skim (0.5% m.f.) and whole (3.2% m.f.) pasteurized milk were stored at 4°C and used within one day of experimentation. CPP powder (Forbes Medi-Tech Inc., Vancouver, BC) consisting of a mixture of (% w/w): ß-sitosterol (68.5), campesterol (8), ß-sitostanol (18.5), campestanol (1.5%) was added to a series of beakers containing skim milk in order to obtain final concentrations of 0.26%, 0.72% and 1.8%, (w/v) CPP. Aqueous stock solutions of kappa carrageenan (CM 750; TIC Gums, Belcamp, MD.) and maltodextrin (Cerestar /Cargill, Wayzata, MN) were boiled 5 minutes and added to the milk to obtain final concentrations of 0.016% (w/v) and 0.186% (w/v), respectively. Carrageenan and maltodextrin were added to help stabilize the milk-CPP mixture. Carrageenans are used in food as gelling agents, stabilizers, water binding agents and thickeners (ISI Inc., 2005). K-carrageenan forms strong gels and combines with milk proteins forming a salt bridge (Hazen, 2006). Maltodextrins are water soluble polysaccharides that are used to regulate viscosity and act as binders or diluents (Pformualte, 2004). Following addition of both stabilizers and CPP to the milk samples (12 ml) were transferred aseptically to a series of sterile test tubes, vortex-mixed and stored at 4-7°C. Two control treatments were also included and consisted of milk without stabilizer and CPP and milk with stabilizers only.

Samples were held at 4°C for 21-22 d and analyzed at regular intervals for autochthonous standard plate count (SPC) and psychrotrophs. In addition, growth of

pseudomonads was evaluated using challenge studies employing *Pseudomonas fluorescens* and a cocktail consisting of 2 strains of *Pseudomonas syringae* and *Pseudomonas aeruginosa.* Each treatment at each sampling period was evaluated in duplicate. The preceding protocol was repeated with whole milk to determine whether fat content played a role in the efficacy of phytosterols as antimicrobials.

### 3.1.2. SPC

Samples (1.0 ml) were serially diluted in sterile 0.1% peptone and plated on Standard Methods Agar (BBL, Becton Dickinson and Co., Sparks, MD) using both the spread plate and pour plate methods. Plates were incubated at 35°C for 48 h.

### 3.1.3. *Pseudomonas* growth

*P. syringae* (ATCC 11528; ATCC 19304), *P. aeruginosa* (ATCC7700) and *P. fluorescens* (Presque Isle) were obtained from the Department of Microbiology, University of Manitoba, Winnipeg, MB. Cultures were maintained at 4-6°C on trypticase soy agar (TSA) slants (BBL). Inocula were prepared by transferring loopfuls of culture from slants to tryptic soy broth (TSB, BBL) and grown overnight at 22°C. Following centrifugation (x 490g; 15 min) the pellets were suspended in 0.85% sterile saline and adjusted to their original volume and again centrifuged (x 490g; 10 min). The final pellets were each suspended in sterile 0.85% saline solution, vortex-mixed and standardized to an $OD_{600\ nm} = 0.700$ corresponding to approximately 8 $\log_{10}$ cfu/ml. CFU were determined by direct plating using *Pseudomonas* base agar (Oxoid Ltd., Hampshire,

England) containing centrimide-fucidin-cephalosporin (CFC) supplement (Oxoid Ltd.). Plates were incubated at 25°C for 48 h.

Standardized *P. syringae*, and *P. aeruginosa* were each diluted to about $10^3$ cfu/ml in sterile 0.85% saline, combined in equal amounts, vortex-mixed and 0.1ml inoculated into 12ml milk samples. *Pseudomonas* growth was evaluated using *Pseudomonas* base agar with CFC supplement. Plates were incubated at 25°C for 48 h. Colonies were confirmed as *Pseudomonas* using the criteria described in Table 3.1.

Separately, standardized *P. fluorescens* culture was diluted to $10^3$ cfu/ml using sterile 0.85% saline and 0.1ml was inoculated into 12ml milk samples and vortex-mixed. Growth of *P. fluorescens* was evaluated as described above for the other strains of *Pseudomonas*.

### 3.1.4. Psychrotrophic plate count

Milk samples (1.0 ml) were serially diluted in sterile 0.85% saline and plated on Standard Methods Agar using both the spread plate and pour plate methods. During the first week of storage (4-7°C) samples were recorded as cfu/10 ml. This was accomplished by pouring 5 plates each with 2 ml of the undiluted sample. Plates were incubated at 7°C for 7-10 d following which the cfu among the five plates was totaled. After the first week samples were evaluated as cfu/ml.

### 3.1.5. Water activity

Water activity of milk samples was determined using a Novasina AW Sprint water activity machine (Axair AG Ltd., Pfäffikon, Switzerland).

### 3.1.6. pH

The pH of milk samples was measured using an Accumet pH meter (Fisher Scientific, Nepean, Ontario). Standard buffer solutions (Fisher Scientific; Nepean ON) at pH 4.0 and 7.0 were used to calibrate the meter.

**Table 3.1.** Criteria used for identifying *Pseudomonas* species

| Species | Oxidase | Gram | Catalase | Fluorescence under UV | Growth at 42°C | Growth at 4°C | Gelatin liquefaction | Sugar fermentation on TSI[1,2] |
|---|---|---|---|---|---|---|---|---|
| *fluorescens* | + | - | + | + | - | + | + | none |
| *aeruginosa* | + | - | + | + | + | - | + | none |
| *syringae* | + | - | + | + | - | - | - | none |

[1]TSI = triple sugar iron agar (BBL)

[2]Growth on TSI conducted at 37°C

### 3.2. Microbial Growth in Milk with CPP Following Homogenization

In order to determine whether homogenization would better disperse CPP in milk, thereby increasing its effectiveness, the following protocol was used. Carrageenan, previously dissolved in water heated to 65°C, maltodextrin and CPP were slowly added to milk heated to 65°C and stirred for 10 min. The final concentrations of stabilizers were 0.186 and 0.018% (w/v) respectively, and CPP was added at 0.26% (w/v). The milk samples were subsequently homogenized using an APV 1000 homogenizer (APV Systems, Albertsbord, Denmark) at 3000 psi, first stage, and 2500 psi, second stage. Samples were collected in sterile flasks transferred to sterile test tubes and pasteurized in a water bath at 80°C for 5 min. Milk samples were stored at 7°C and evaluated for SPC and psychrotrophs. In addition, a challenge study using *P. fluorescens* was performed as outlined previously. This procedure was repeated using 0.72% (w/v) final concentration CPP. Studies employing 1.8% CPP were not performed since it clogged the homogenizer.

### 3.3. Effect of Dispersible Phytosterols on Milk Microflora

Retail pasteurized 1% m.f. milk was stored at 4°C for no more than one day prior to experimentation. Three treatments were carried out, including a control containing only milk, milk containing a dispersible mixture designated ADP (dispersible phytosterol mixture A) consisting of 36% (w/v) phytosterols, and milk containing a dispersible mixture designated BDP (dispersible phytosterol mixture B) consisting of 57% (w/v) phytosterols (Forbes Medi-Tech Inc.). The dispersible phytosterol mixtures consisted of spray-dried preparations of CPP, sodium stearoyl lactylate (SSL), maltodextrin and

carrageenan (ADP and BDP differed from each other only in their CPP and maltodextrin concentration). The formulation of SSL in a mixture of phytosterols to increase dispersibilty in water was patented by Forbes Medi-Tech Inc. (USA Patent Application Dr. Jerzy Zawisotski #60/790,749 April 2006). Initially, 10ml milk was aseptically added to a series of sterile test tubes. Each dispersible phytosterol mixture was dispensed into the milk to give a final concentration of 0.72% (w/v) phytosterols and thereafter vortex-mixed. All treatment samples were stored at 4°C for 21-22 d and monitored periodically for SPC and psychrotrophs as described previously.

*P. fluorescens* was grown and standardized as previously indicated. Standardized *P. fluorescens* was diluted to $10^3$ cfu/ml using sterile 0.85% saline and 0.1 ml was inoculated into the milk samples and vortex-mixed. Growth of *P. fluorescens* was evaluated for approximately 10 d using *Pseudomonas* base agar containing centrimide-fucidin-cephalosporin (CFC) supplement. Plates were incubated at 25°C for 48 h. Colonies were confirmed as *Pseudomonas* using the criteria described in Table 3.1. All treatments were carried out in triplicate.

### 3.4 Effect of Sodium Stearoyl Lactylate on SPC Growth in Milk.

Sodium stearoyl lactylate (SSL), a surface active compound used to aid dispersal of CPP in milk, was examined for potential antimicrobial properties. In this respect, 10 ml portions of freshly pasteurized milk containing 0.03% (w/v) SSL (ADM Arkady, Olathe, Kansas) was added to a series of sterile test tubes and incubated at 4-7° C for 14 d. A similar protocol was followed using BDP for comparison (0.03% SSL plus CPP;

Forbes Medi-Tech Inc.). The SPC of milk stored at 4-7°C was determined periodically over 2 weeks.

### 3.5. Effect of Cavamax® on Growth of *P. fluorescens* in Tryptic Soy Broth

In order to confirm the effect of CPP on microbial growth additional studies were performed using *P. fluorescens* in TSB (BBL). In this respect TSB was heated to 40°C and Cavamax® (1.21%, w/v) a dispersible form of phytosterols containing CPP was slowly added with mixing (10 min) such that a final concentration of 0.26% (w/v) CPP was obtained. This concentration was utilized as higher concentrations were not dispersible. Cavamax® contains the compound cyclodextrin which is used to increase the aqueous solubility of compounds and acts as a protectant and natural carrier for functional ingredients (CTD Inc., 2006). Following cooling to 25°C the broths were inoculated (0.1 ml; 4 $\log_{10}$ cfu/ml into 100 ml of broth) with a standardized culture of *P. fluorescens* and incubated at 22°C on a shaker (100 rpm; 24 h). Growth of pseudomonads was evaluated as previously described.

### 3.6. Effect of CPP on Yoghurt Microflora

#### 3.6.1. Cultures

The yoghurt starter cultures consisted of *Lactobacillus bulgaricus* (strain TR160) and *Streptococcus thermophilus* (strain TC120) and were obtained from Danisco (Madison, USA). *L. bulgaricus* cultures were maintained on De Man, Rogosa Sharpe agar (MRS agar; Oxoid Ltd.) and grown (45°C for 18 h) in sterile reconstituted skim milk

powder (11%, w/v). *S. thermophilus* was maintained on M17 agar (Oxoid Ltd.) and grown at 33°C in reconstituted skim milk powder (11%, w/v) for 16 h.

### 3.6.2. Yoghurt production

The yoghurt was prepared according to the commercial formulation used by Forbes Medi-Tech Inc. using pasteurized milk (2% m.f.), skim milk powder and the two commercial starter cultures with and without the addition of 0.39% (w/v) CPP (final concentration). Initially, the milk was heated to 50°C in sterile beakers and commercial skim milk powder was slowly added to achieve a final concentration of 8% (w/v). CPP powder was added concurrently. The samples were vortex-mixed for 10 min and pasteurized in a 65°C water bath for 30 min. Following cooling to 30°C in a thermostatically controlled water bath, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were each added at 1% (v/v) ($3.5 \times 10^8$ and $6.5 \times 10^8$ cfu /ml, respectively) After mixing, 100 ml samples were transferred to sanitized, lidded plastic containers (175 g capacity) and incubated at 33°C for 12 h. The samples were subsequently maintained at 4°C. All trials were performed in triplicate.

Growths of *L. bulgaricus* and *S. thermophilus* were monitored during the 12 h yoghurt fermentation and during storage at 4°C using a modified Elliker's agar (Matalon and Sandine, 1986; see Table 3.2). Samples (1.0 ml) were serially diluted in sterile 0.1% peptone and incubated at 37°C in jars under anaerobic conditions (BBL GasPak Plus™) for 48 h. On Elliker's medium *L. bulgaricus* appear as large (2-3 mm in diameter) white colonies while *S. thermophilus* colonies are much smaller and red. Typical colonies were

confirmed as *S. thermophilus* or *L. bulgaricus* using the criteria listed in Table 3.3. The pH of yoghurt was measured as described previously.

**Table 3.2.** Formula for modified Elliker's agar

| Component | Concentration( g/L) |
|---|---|
| Tryptone | 20.0 |
| Yeast extract | 5.0 |
| Gelatin | 2.5 |
| Dextrose | 5.0 |
| Lactose | 5.0 |
| Sucrose | 5.0 |
| Sodium chloride | 4.0 |
| Sodium acetate | 1.5 |
| Ascorbic acid | 0.5 |
| Agar | 15.0 |
| Tween 80 | 0.10% |
| TTC[1] | 50 µg / ml |

[1] TTC = 2,3,5-triphenyltetrazolium chloride

**Table 3.3.** Criteria for confirming identities of yoghurt starter culture organisms

| | *L. bulgaricus* | *S. thermophilus* |
|---|---|---|
| Growth in 2% NaCl[1,2] | + | - |
| Growth in 6.5% NaCl[1,2] | - | - |
| Growth at 45°C[2] | + | - |
| Growth at 10°C[2] | - | - |
| Catalase | - | - |
| Oxidase | - | - |
| Gram stain | + | + |
| Sugar fermentation[1,3] | glucose | glucose and lactose and/or sucrose |

[1] Conducted at 37°C

[2] Conducted in TSB (Trypticase soy broth)

[3] Conducted on TSI

### 3.6.3. Effect of CPP on growth of yeast

*Saccharomyces cerevisiae* (ATCC 9763) was obtained from the Department of Microbiology at the University of Manitoba and was maintained on potato dextrose agar slants (PDA; BDH, Darmstadt, Germany). Growth was performed by transferring loopfuls of culture to yeast extract glucose peptone (2% yeast extract, 1% peptone, 2% dextrose) broth (Wong et al., 2002). Following incubation at 30°C for 16 h, the culture was centrifuged (x 490g) for 15 min and the pellet suspended in 0.85% NaCl and similarly centrifuged. The final pellet was suspended in sterile 0.85% saline and standardized to approximately 4 $\log_{10}$ cfu/ml using sterile 0.85% saline. Yoghurt (800 ml) was prepared as described previously, however, 0.1 ml of the standardized yeast culture was added concurrently with the starter bacteria. Following mixing, 100 ml portions of the inoculated yoghurt mix was dispensed among 8 lidded sanitized containers. Growth of yeast was monitored during the 12 h fermentation period as well as during refrigerated storage using PDA acidified with 0.1% tartaric acid (22°C, 5 d). Samples were plated in duplicate. Yoghurt with yeast but without CPP served as a control.

## 3.7. Effect of CPP on Mould Growth

*Aspergillus ochraceus* (NRRL 3174) was obtained from the National Regional Research Laboratories (Agricultural Research Service, USDA, Peoria, Ill.) and was maintained on Czapek agar at 4°C. Conidia were prepared by culturing *A. ochraceous* on Czapek agar (5 d at 20-22°C) and were harvested by flooding the culture plates with sterile 0.85% saline; mycelial fragments were removed by filtration using a sterile

Pasteur pipette packed with glass wool. Yoghurt containing 0.39% (w/v) CPP was prepared as described previously, however, 0.1 ml of the conidial suspension (approximately $10^4$ cfu/ml) was added concurrently with the starter bacteria. Triplicate samples were visually monitored every 3 d over a period of 32 d for mold growth. Yoghurt with mold but without the CPP served as a control.

### 3.8. Effect of Phytosterol Mixtures on Yoghurt Microflora

Pasteurized 2% m.f. milk was purchased at a retail outlet and stored at 4°C not more than one day prior to experimentation. Yoghurt containing 0.39% CPP (w/v) was prepared as described previously. In addition, four phytosterol mixtures (designated wood sterols, FCP, FMP and MP4) were used, each at a final concentration of 0.39%. The mixtures differed in their concentration of the phytosterols sitosterol, sitostanol, campesterol, campestanol, and brassicasterol. The phytosterol content of each is shown in Table 3.4. *S. cerevisiae* was also inoculated into samples as previously described. A control sample containing *S. cerevisiae* but no phytosterols was also conducted. Triplicate samples of each treatment were evaluated for yeast, *S. thermophilus* and *L. bulgaricus* counts.

**Table 3.4.** Composition (%) of phytosterol mixtures applied to yoghurt

| | Sitostanol | Sitosterol | Campesterol | Campestanol | Stigmasterol | Brassicasterol |
|---|---|---|---|---|---|---|
| Wood sterols | 10 | 79.2 | 7.3 | 1.4 | 0 | 0 |
| FMP | 0 | 35.22 | 21.11 | 0 | 19.15 | 3.87 |
| FCP | 79.56 | 0 | 0 | 9.69 | 0 | 0 |
| MP4 | 56.34 | 0 | 0 | 34.54 | 0 | 0 |

### 3.9. Effect of CPP on Yoghurt Rheology

#### 3.9.1. Sample preparation

Samples (20 ml) of milk containing skim milk powder, phytosterol and starter culture (as previously outlined) were dispensed into the sample well of the AR 2000 Advanced Rheometer (TA Instruments, New Castle, USA) containing a concentric conical cylinders fixture. The sample was allowed to undergo fermentation for 12 h at 33°C followed by holding at 4°C for 5 h. Employing *in situ* yoghurt production prevented disturbance of its structure during loading or preparation and aided in stabilizing the temperature of the sample. Samples were assessed in triplicate.

#### 3.9.2. Evaluation of viscoelastic properties

Evaluation of the viscoelastic properties of yoghurt consisted of two frequency sweeps to determine the G' and G". A frequency sweep from 0.10 to 10.0 Hz was carried out at 1.0 Pa followed by a 5 min waiting period and a second frequency sweep (using the same parameters). For preliminary experimentation, log stress sweeps were conducted at 0.1, 1.0 and 10.0 Hz from 0.10 to 10.0 Pa. A stress of 1.0 Pa was within the linear viscoelastic region for the yoghurt samples (see results below).

#### 3.9.3. Evaluation of flow properties

Following the evaluation of the linear viscoelastic properties of the yoghurt samples, their flow properties were determined on the same sample. A test was run to

determine shear stress vs. shear rate. This consisted of two continuous ramp tests in which shear rate was increased from 1.452 to 145.2 $s^{-1}$ over a 1.0 min period. After a 10 min holding period, the test was conducted again to determine whether the yoghurt gel structure recovered after it was broken down by the first continuous ramp test. All tests were carried out at 4°C and interpreted using the TA Data Analysis program.

### 3.10. Antioxidant Activity of Free Phytosterols and Phytosterol Esters

All antioxidant activity tests were performed by Forbes Medi-Tech Inc., Vancouver, BC as outlined below.

#### 3.10.1. Sample preparation

Free phytosterols consisted of CPP and the coniferous phytosterols sitosterol, campesterol, sitostanol and campestanol. Phytosterol esters used were coniferous phytosterols esterified with triacylglycerides extracted from sunflower oil. Antioxidant activities of phytosterols were evaluated in a MCT (medium chain triglyceride) oil blend of mainly medium chain triacylglycerides that consisted of 65% tropical oils, 12% olive oil, 7% canola oil, 7% flaxseed oil and 6% coconut oil. Performance was also evaluated in canola oil. Four treatments were applied to each oil: a control oil without phytosterols, a treatment containing 5.44% sterol esters, oil with 2.77% sterol esters and 1.7% free sterols, and a treatment with 3.4% free sterols. These treatments were applied to MCT oil and canola oil and blended. Each treatment was carried out in four trials.

### 3.10.2. Effect of phytosterols on the shelf life of MCT oil

The shelf life stability of MCT oil was evaluated using three methods as outlined below. The oil was stored at room temperature (22°C) for 12 months and samples were evaluated monthly.

#### 3.10.2.1 Peroxide value

The peroxide value (POV) of MCT oil was evaluated over the 12 month storage period to determine initial lipid peroxidation products (LOPs). Peroxide value is the most common measure of antioxidant activity (Rajalakshi and Narashimhan, 1996). Samples (5 g) were initially reacted with 30 ml of an acetic acid chloroform solution followed by 0.5 ml potassium iodide, which causes peroxide molecules to release iodine (Rajalakshi and Narashimhan, 1996). Samples were subsequently titrated with 0.1N sodium thiosulfate using a 1% starch solution (0.5 ml) as indicator. Peroxide values were determined based on the level of sodium thiosulfate titrated and its normality (Horowitz, 1980).

#### 3.10.2.2. *p*-Anisidine value (PAV)

The PAV is a measurement of the aldehyde content of an oil, particularly the 2,4-dienals and 2-alkenals. These are secondary products of lipid oxidation and so can be used as an indication of the degree of oxidation that has occurred in an oil. In soybean oil, for example, almost 50% of volatiles from lipid oxidation are aldehydes (Tompkins and Perkins, 1999). PAV was determined as described in AOACS Official Method Cd 18-90

(AOACS, 1992). Samples (0.5 g) were dissolved in 25 ml of n-hexane and the absorbance (Ab) measured at 350 nm with n-hexane serving as the blank; 5 ml of the same solution was reacted with *p*-anisidine (1 ml) for 10 min and the increase in absorbance (As) was measured at 350 nm, 5 ml n-hexane and 1 ml *p*-anisidine was used as the blank. The level of secondary LOPs was determined by the following equation: $PAV = 25 \times (1.2As - Ab) \times (m^{-1})$, where $m$ is the mass of the sample.

### 3.10.2.3 Percent loss of essential fatty acids

The percent loss of C18:2, n-6 (linoleic) and C18:2, n-3 (linolenic) fatty acids were evaluated after 6 months using GLC (Hewlett-Packard gas chromatography Model 6890; Palo Alto, CA.) equipped with a 25 m x 0.53 mm capillary column and a flame-ionization detector. The initial temperature of the column was 140°C, set to increase at 4°C / min to 250°C. The injector and detector temperature were set at 250°C. The air flow rates for the carrier gas which consisted of nitrogen, hydrogen and air were 65, 44 and 440 ml /min, respectively (Jaswir et al., 2000b). Analysis of the peaks was used in calculating the fatty acid levels.

### 3.10.3. Effect of phytosterols on the thermal stability of oils

Thermal stability of oils was evaluated using both MCT and canola oils. The oils were heated to 100 and 190°C for 6 h. POV, PAV and polymerization degradation products (i.e. alkaline contaminant materials; ACM) were evaluated. ACM include soaps and other surface-active components that form in oils during frying and have the ability to lower the retention of the oil on food products. They are formed when fatty acids and

metal ions react during oil degradation and can be used as an indication of thermal abuse in cooking oil (Jaswir et al., 2000a). ACM were measured by the same GLC method used to determine loss of essential fatty acids. POV and PAV were evaluated after heating for 2 and 6 h while the degradation products were measured after 7-12 h of heating.

### 3.11. Data Analysis

The data gathered from this study was analyzed using ANOVA and Tukey's test (SPSS 14.0; SPSS Inc., Chicago, IL).

# 4. RESULTS

## 4.1. Effect of CPP on Milk Microflora, pH and $a_w$

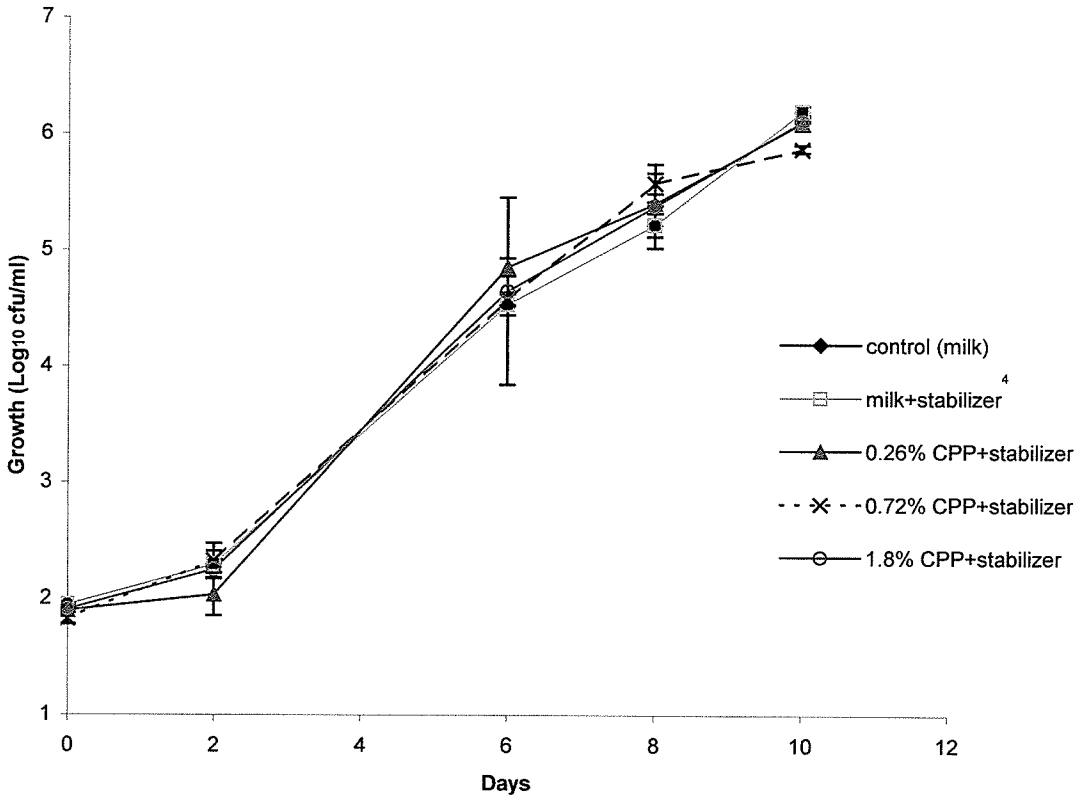
As shown in Figure 4.1, the CPP (0.26 to 1.8%; w/v) exhibited no significant ($p>0.05$) effect on SPC growth in whole milk stored at 4 -7°C ($\leq 0.25$ $\log_{10}$ difference compared to the control for each time period). Compared to the control, skim milk containing 1.8% (w/v) CPP also exhibited a minimal effect ($\leq 0.25$ $\log_{10}$ difference compared to the control for each time period) on SPC growth (Figure 4.2), although the effect of treatment in this trial was determined to be statistically significant ($p<0.05$).

The growth of *P. fluorescens* in skim (Figure 4.3) and whole (Figure 4.4) milk appeared unaffected by the addition of the CPP. For example, in whole milk at 7°C, an initial inoculum of $10^1$ cfu/ml *P. fluorescens* increased by approximately 7 $\log_{10}$ cfu/ml within 8 d regardless of the CPP concentration. Also, the addition of CPP had no effect on the growth of a mixed culture of *Pseudomonas* species in skim milk (Figure 4.5). In whole milk, significant differences (based on Tukey's test) in population were only observed with 1.8% (w/v) CPP, however, differences between the treatment and the control were $\leq 0.5$ $\log_{10}$ cfu/ml (Figure 4.6).

As shown in Figure 4.7, the addition of CPP had no effect on the growth of psychrotrophs in 1% m.f. milk during storage at 4-7°C for 24 days.

The effect of CPP on the initial pH and $a_w$ of skim and whole milk are shown in Table 4.1. In all cases the initial pH of the milk products did not differ by more than 0.04 units. Also, compared to the control treatment CPP appeared to have no effect on the initial $a_w$.

**Figure 4.1.** Effect of CPP[1] on the SPC[2] of pasteurized whole milk stored at 4-7°C. Values[3] are means of duplicate samples each performed in duplicate (n = 4). Error bars represent standard deviation of means.



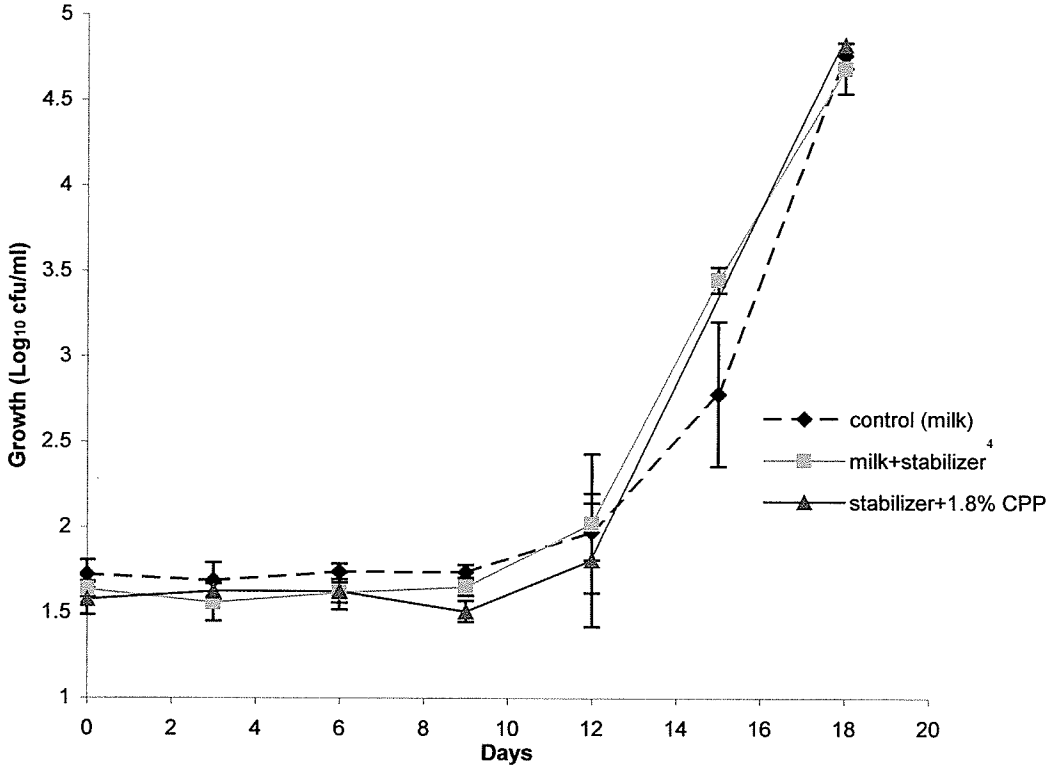

[1] Commercial phytosterol preparation

[2] Standard plate count

[3] Appendix Table 1

[4] Stabilizer: 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

**Figure 4.2.** Effect of 1.8% CPP[1] on the SPC[2] of pasteurized skim milk stored at 4-7°C. Values[3] are means of duplicate samples each performed in duplicate (n = 4). Error bars represent standard deviation of means.



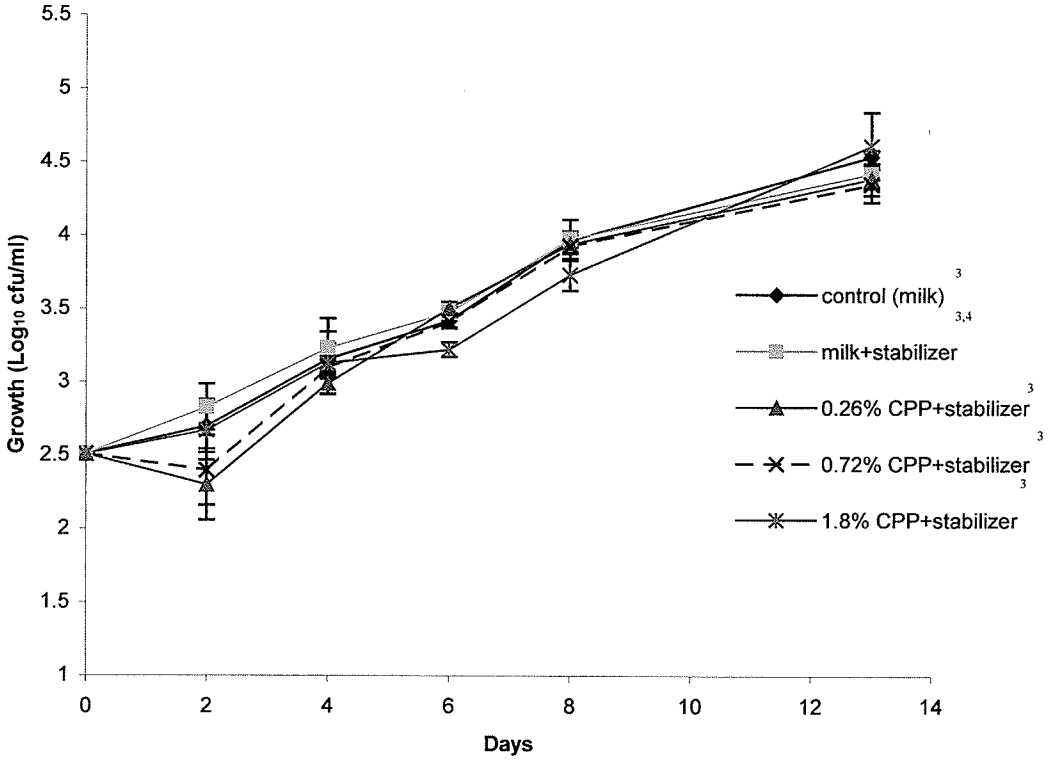

[1] Commercial phytosterol preparation

[2] Standard plate count

[3] Appendix Table 2

[4] Stabilizer: 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

**Figure 4.3.** Effect of CPP[1] on *P. fluorescens* growth in pasteurized skim milk stored at 4-7°C. Values[2] are means of duplicate samples each performed in duplicate (n=4). Error bars represent standard deviation of means.



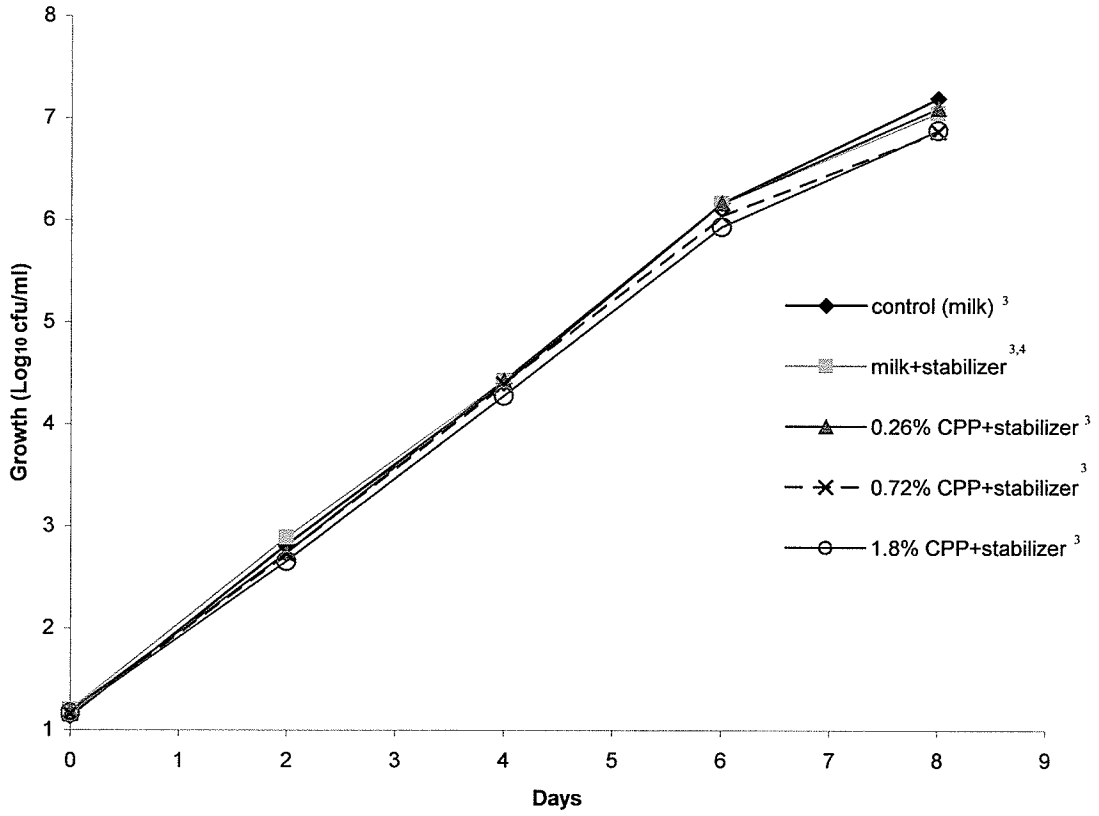

[1] Commercial phytosterol preparation

[2] Appendix Table 3

[3] Includes *P. fluorescens* inoculum

[4] Stabilizer: 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

**Figure 4.4.** Effect of CPP[1] on *P. fluorescens* growth in pasteurized whole milk stored at 4-7°C. Values[2] are means of duplicate samples each performed in duplicate (n=4). Error bars not represented: standard deviations of means are below 0.07.



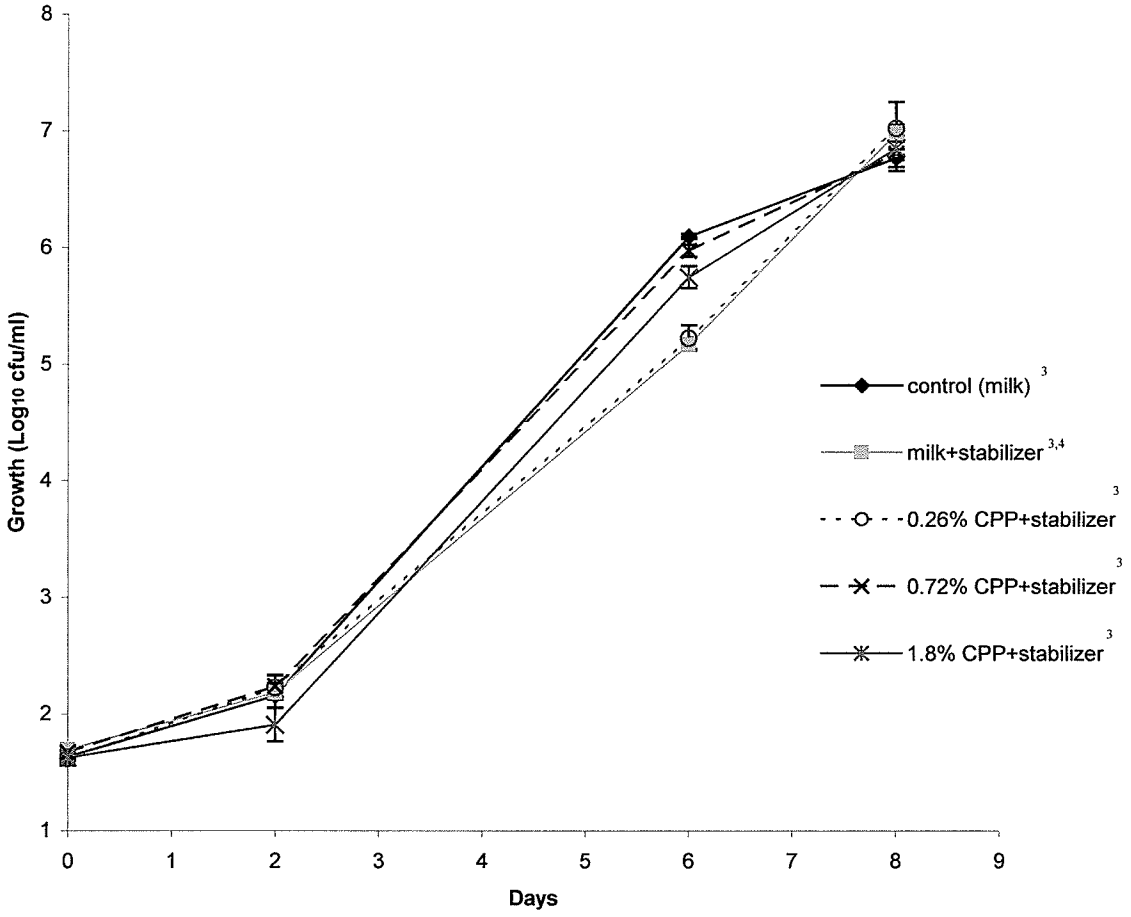

[1] Commercial phytosterol preparation

[2] Appendix Table 1

[3] Includes *P. fluorescens* inoculum

[4] Stabilizer: 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

**Figure 4.5.** Effect of CPP[1] on growth of a cocktail of 2 strains of *P. syringae* and *P. aeruginosa* in pasteurized skim milk stored at 4-7°C. Values[2] are means of duplicate samples each performed in duplicate (n = 4). Error bars represent standard deviation of means.



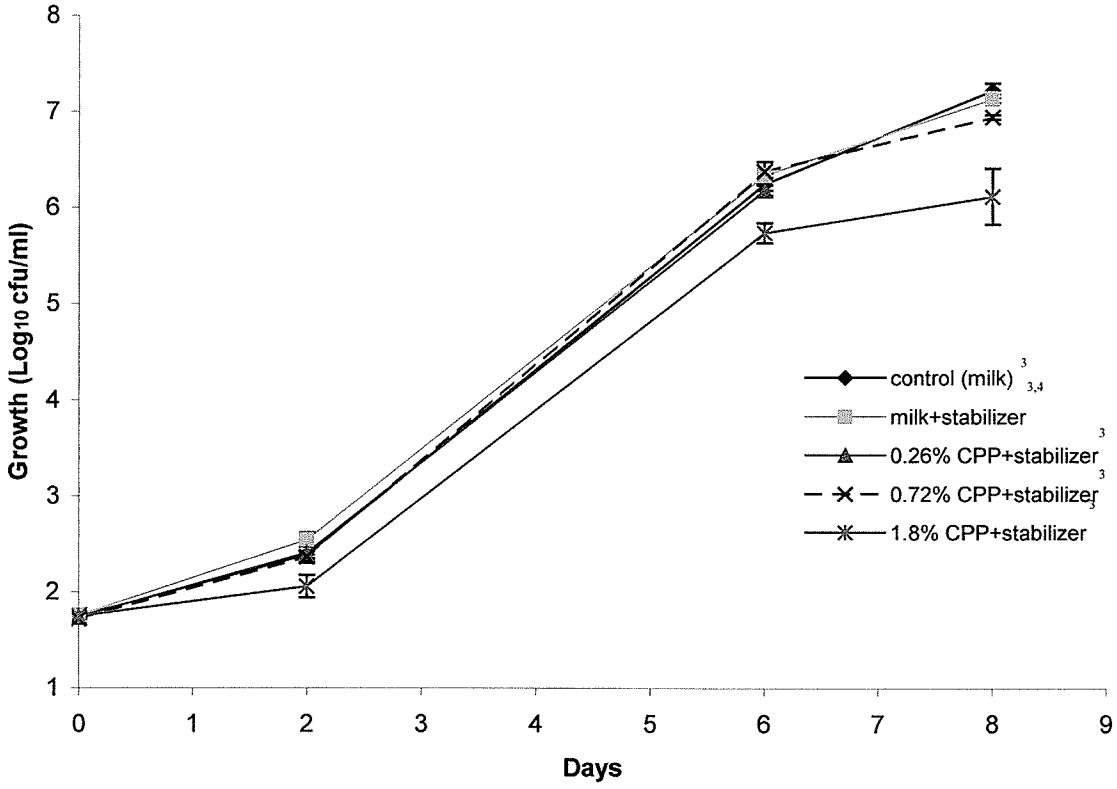

[1] Commercial phytosterol preparation

[2] Appendix Table 3

[3] Includes *Pseudomonas* inoculum

[4] Stabilizer: 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

**Figure 4.6.** Effect of CPP[1] on growth of a cocktail of 2 strains of *P. syringae* and *P. aeruginosa* in pasteurized whole milk stored at 4-7°C. Values[2] are means of duplicate samples each performed in duplicate (n = 4). Error bars represent standard deviation of means.



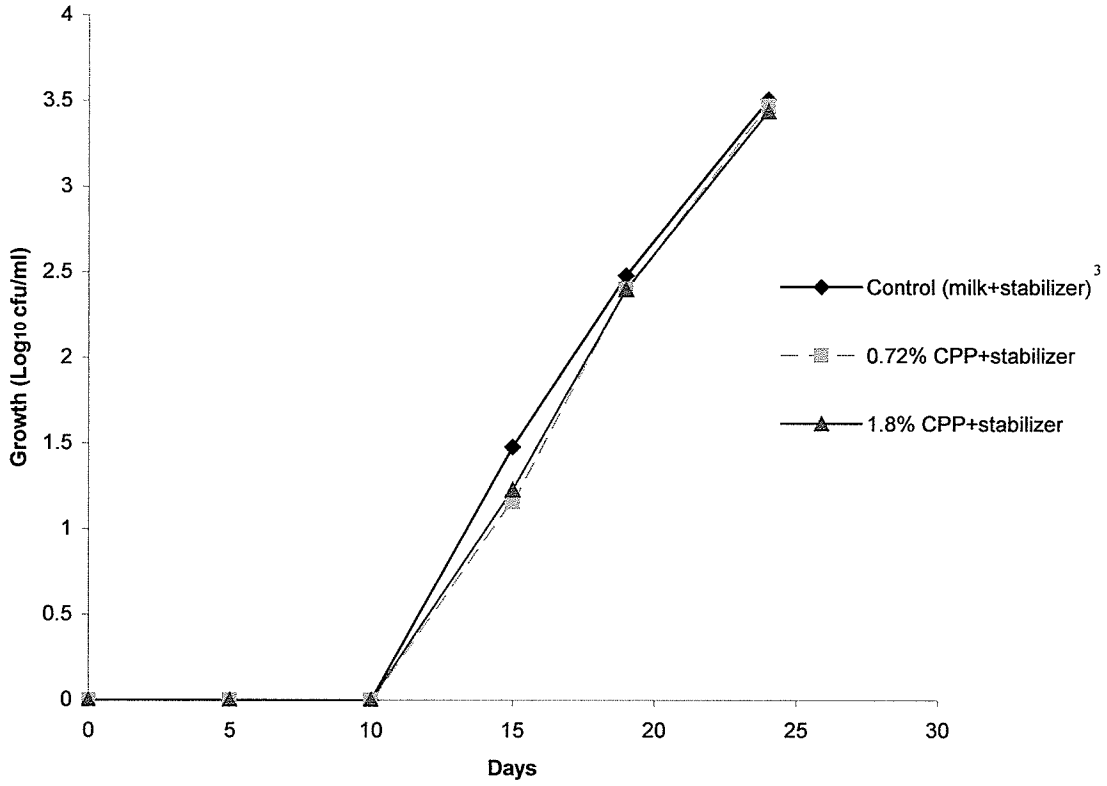

[1] Commercial phytosterol preparation

[2] Appendix Table 1

[3] Includes *Pseudomonas* inoculum

[4] Stabilizer: 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

**Figure 4.7.** Effect of CPP[1] on the psychrotrophic plate count in 1% m.f. milk stored at 4-7°C. Values[2] are means of triplicate samples each performed in duplicate (n=6).




[1] Commercial phytosterol preparation

[2] Appendix Table 4

[3] Stabilizer: 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

**Table 4.1.** Effect of CPP[1] on the initial pH and $a_w$ of pasteurized skim and whole milk

| | Skim milk | | | | Whole milk | | | |
|---|---|---|---|---|---|---|---|---|
| | | CPP (% w/ v) [3] | | | | CPP (% w/ v) [3] | | |
| | Control [2,4] | 0.26[4] | 0.72[4] | 1.8[4] | Control[2,4] | 0.26[4] | 0.72[4] | 1.8[4] |
| pH | 6.81 | 6.80 | 6.80 | 6.80 | 6.77 | 6.77 | 6.76 | 6.76 |
| $a_w$ | 0.978 | 0.979 | 0.978 | 0.978 | 0.974 | 0.974 | 0.975 | 0.974 |

[1] Commercial phytosterol preparation

[2] Milk without CPP

[3] Milk with 0.016% (w/v) carrageenan, 0.185% (w/v) maltodextrin and CPP

[4] Values are means of triplicate samples

### 4.2. Effect of Dispersible Phytosterols on Milk Microflora

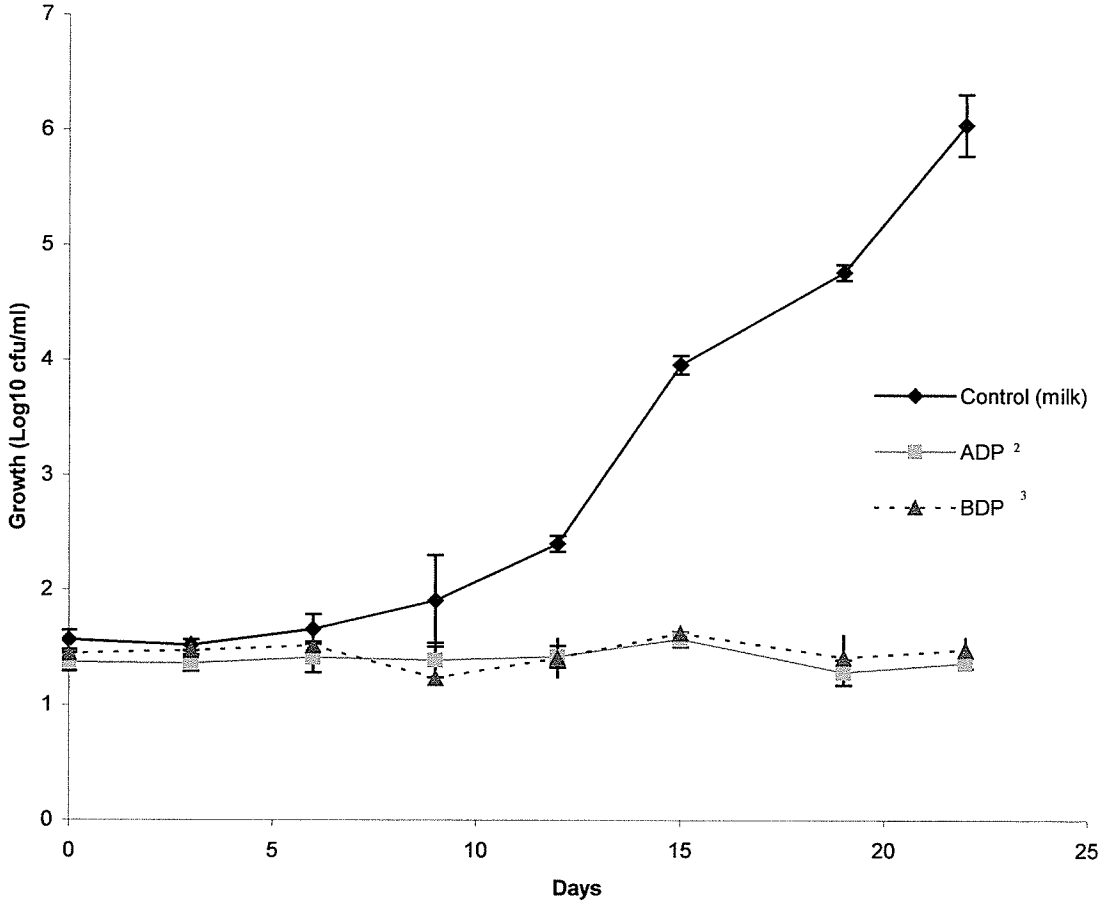
Both the ADP and BDP dispersible phytosterol mixtures appeared to have an inhibitory effect on the SPC of milk stored at 4°C (Figure 4.8). By day 12, the SPC of the control samples increased while the SPC in the phytosterol samples remained relatively constant. This trend continued for 21 d after which the SPC in the control was approximately 5 $\log_{10}$ higher than that of the phytosterol samples. Although there was a significant difference between the control and phytosterol treatments ($p<0.05$), no significant difference ($p> 0.05$) in SPC between the two phytosterol treatments was observed.

As shown in Figure 4.9, the psychrotrophic plate count in milk decreased to less than 1 cfu/10 ml by 3 d in the presence of the ADP and BDP phytosterol mixtures, however, the *P. fluorescens* population remained unaffected (Figure 4.10). In this regard, differences $\leq 0.25$ $\log_{10}$ cfu/ml were observed in the number of pseudomonads between the control and CPP samples even after 12 d of storage at 4°C. It would, therefore, appear that the activity of CPP towards *P. fluorescens* was not enhanced by an increase in the dispersibility of the phytosterol.

### 4.3. Effect of CPP on Milk Microflora Following Dispersal by Homogenization

The growth of *Pseudomonas* or the SPC following homogenization treatment of CPP in milk differed only by $\leq 0.25$ $\log_{10}$ (Table 4.2) when compared to their respective controls at all time periods, although CPP dispersibility appeared to be improved (CPP did not appear to float to the surface of the samples as readily).

**Figure 4.8.** Effect of dispersible phytosterol mixture A (ADP) and dispersible phytosterol mixture B (BDP) on the standard plate count of 1% m.f. milk stored at 4-7°C. Values[1] are means of triplicate samples each performed in duplicate (n = 6). Error bars represent standard deviation of means.

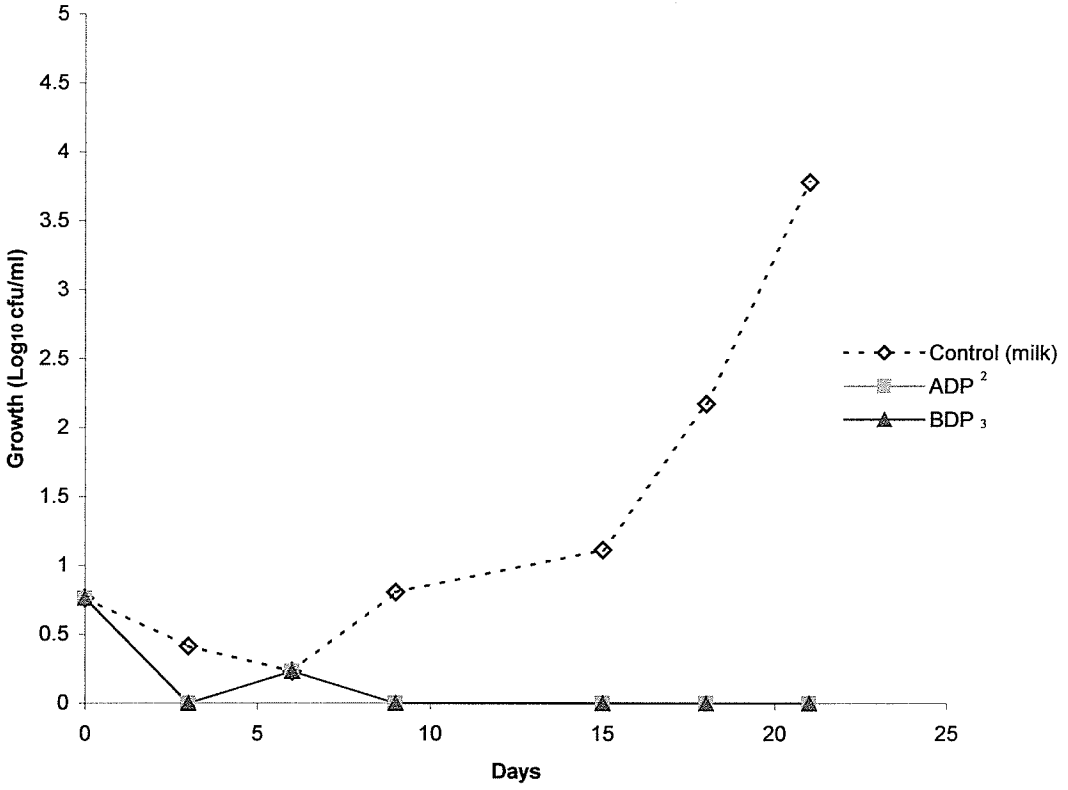



[1] Appendix Table 5

[2] Mixture consists of: carrageenan, maltodextrin, 36% (w/v) CPP and 1.54% (w/v) sodium stearoyl lactylate, added such that final phytosterol concentration in milk was 0.72% (w/v)

[3] Mixture consists of: carrageenan, maltodextrin, 57% (w/v) CPP and 1.54% (w/v) sodium stearoyl lactylate, added such that final phytosterol concentration in milk was 0.72% (w/v)

**Figure 4.9.** Effect of dispersible phytosterol mixture A (ADP) and dispersible phytosterol mixture B (BDP) on the psychrotrophic plate count in 1% m.f. milk stored at 4-7°C. Values[1] are means of triplicate samples each performed in duplicate (n = 6).

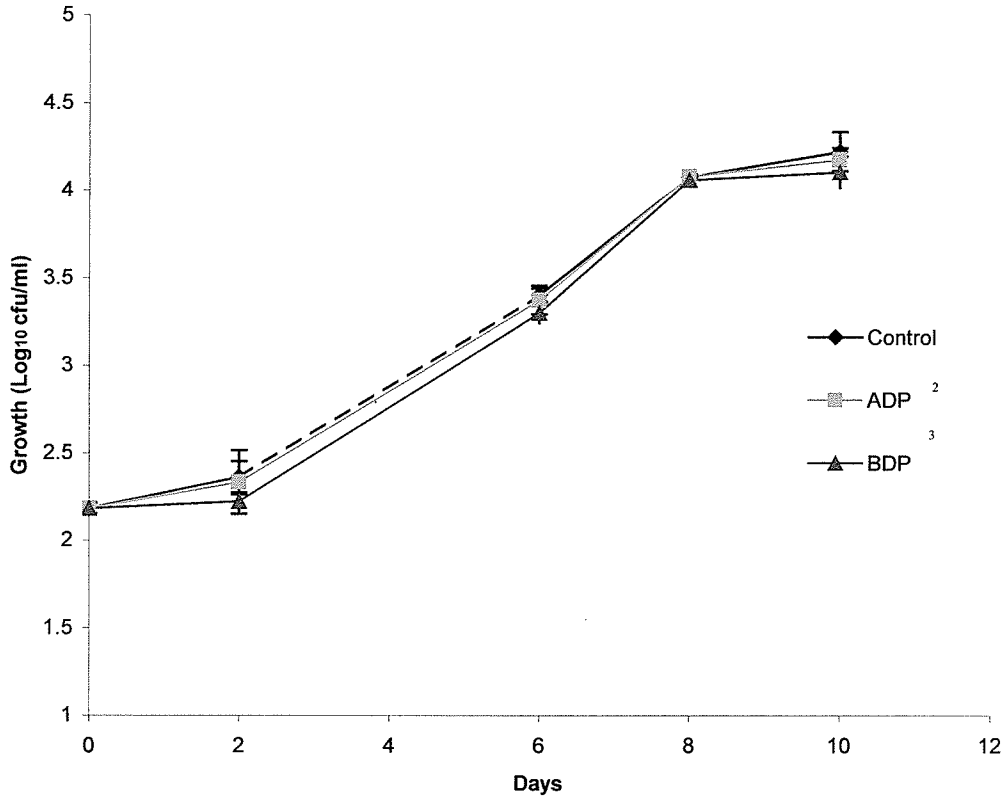



[1] Appendix Table 5

[2] Mixture consists of: carrageenan, maltodextrin, 36% (w/v) CPP and 1.54% (w/v) sodium stearoyl lactylate, added such that final phytosterol concentration in milk was 0.72% (w/v)

[3] Mixture consists of: carrageenan, maltodextrin, 57% (w/v) CPP and 1.54% (w/v) sodium stearoyl lactylate, added such that final phytosterol concentration in milk was 0.72% (w/v)

**Figure 4.10.** Effect of dispersible phytosterol mixture A (ADP) and dispersible phytosterol mixture B (BDP) on the growth of *P. fluorescens* in 1% m.f. milk stored at 4°C. Values[1] are means of triplicate samples each performed in duplicate (n = 6). Error bars represent standard deviation of means.




[1] Appendix Table 6

[2] Mixture consists of: carrageenan, maltodextrin, 36% (w/v) CPP and 1.54% (w/v) sodium stearoyl lactylate, added such that final phytosterol concentration in milk was 0.72% (w/v)

[3] Mixture consists of: carrageenan, maltodextrin, 57% (w/v) CPP and 1.54% (w/v) sodium stearoyl lactylate, added such that final phytosterol concentration in milk was 0.72% (w/v)

**Table 4.2.** Effect of CPP[1] on microflora following its dispersal by homogenization in 1% m.f. milk stored at 4-7°C

| | | $Log_{10}$ cfu/ml [2] | |
|---|---|---|---|
| Time (d) | CPP (% w/v) | SPC | *P. fluorescens* |
| 0 | 0 [3] | 0.86 | 0.98 |
| | 0.26 [3] | 0.76 | 0.80 |
| | 0.72 [3] | 0.94 | 0.72 |
| 2 | 0 | 0.94 | 2.79 |
| | 0.26 | 0.94 | 2.69 |
| | 0.72 | 0.86 | 2.73 |
| 5 | 0 | 0.86 | 4.37 |
| | 0.26 | 0.86 | 4.15 |
| | 0.72 | 0.94 | 4.28 |
| 8 | 0 | 1.15 | 6.29 |
| | 0.26 | 0.99 | 6.32 |
| | 0.72 | 0.83 | 6.31 |
| 10 | 0 | 0.96 | 7.55 |
| | 0.26 | 0.74 | 7.53 |
| | 0.72 | 0.90 | 7.62 |
| 13 | 0 | 1.18 | N.D. [4] |
| | 0.26 | 1.00 | N.D. |
| | 0.72 | 1.00 | N.D. |
| 15 | 0 | 1.16 | N.D. |
| | 0.26 | 1.11 | N.D. |
| | 0.72 | 1.08 | N.D. |

[1] Commercial phytosterol preparation

[2] Values are means of duplicate samples each performed in duplicate

[3] Includes 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

[4] Not determined

Also, CPP dispersed in milk after homogenization had no significant effect ($p > 0.05$) on the growth of psychrotrophs (Figure 4.11). Because the milk had been pasteurized after the addition of CPP, levels of psychrotrophic organisms remained below 1 cfu/10ml for at least 21 d. By 30 d, samples with 0.26% and 0.72% (w /v) CPP contained lower psychrotrophic counts compared to the control samples until 35 d of storage. By 40 d, however, populations in milk containing the phytosterols were at least 0.5 $\log_{10}$ higher.

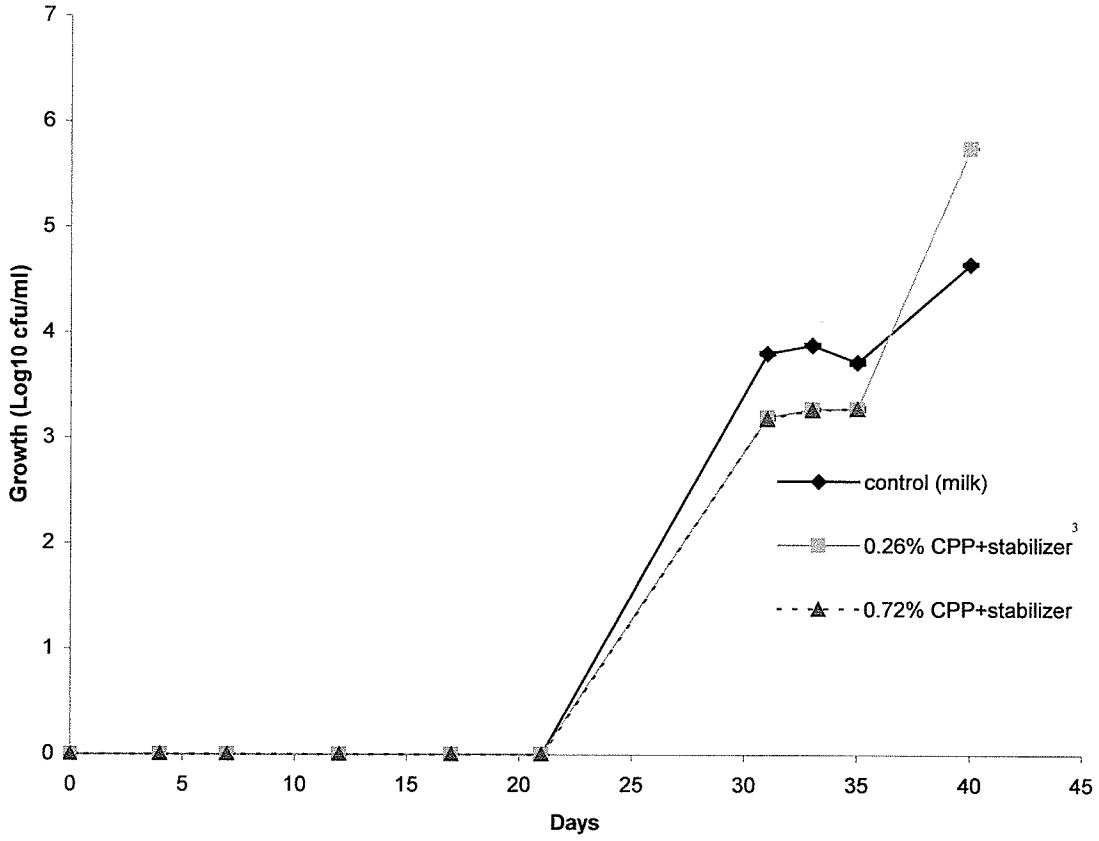
### 4.4. Effect of CPP on the Growth of *P. fluorescens* in TSB

The addition of CPP containing Cavamax® to increase dispersion (1.21% w/v, corresponding to 0.26% CPP) appeared to have no effect on the growth of *P. fluorescens* in TSB at 21-22°C when compared to the control ($\leq 0.25$ $\log_{10}$ cfu/ml difference) through the entire time course (Figure 4.12). Higher concentrations of Cavamax® were not used due to dispersal problems.

### 4.5. Effect of Sodium Stearoyl Lactylate (SSL) on SPC Growth in Milk

As shown in Figure 4.13 the SPC time course profile of milk with and without the addition of SSL remained similar. By 14 d the SPC in both treatments exceeded 2 $\log_{10}$ cfu/ml. In comparison, the SPC of milk containing the CPP mixture remained relatively constant ($\leq 1$ $\log_{10}$ cfu/ml) throughout the entire storage period.

**Figure 4.11.** Effect of CPP[1] on psychrotrophs following dispersal by homogenization in 1% m.f. milk stored at 4-7°C. Values[2] are means of duplicate samples each performed in duplicate (n = 4). Standard deviations of means are below 0.025

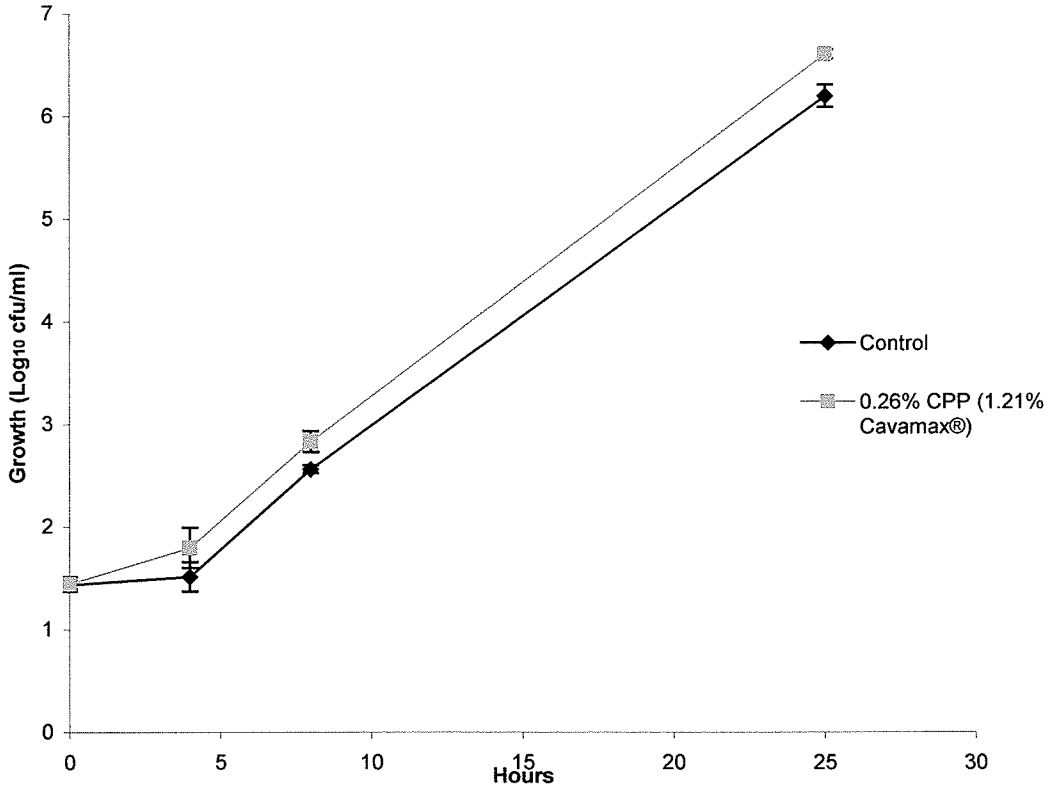



[1] Commercial phytosterol preparation

[2] Appendix Table 7

[3] Stabilizer: 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

**Figure 4.12.** Effect of Cavamax®[1] on *P. fluorescens* growth in TSB[2] at 22°C. Values[3] are means of duplicate samples each performed in duplicate (n = 4). Error bars represent standard deviation of means.

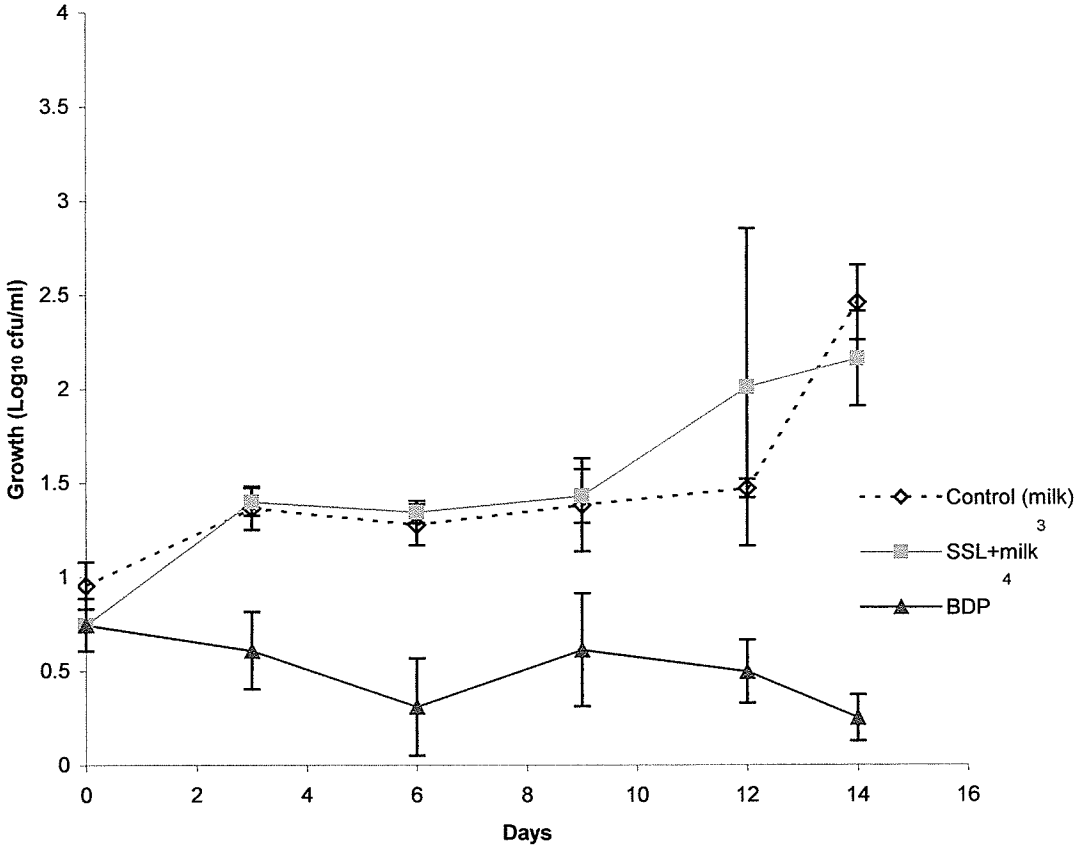



[1] Dispersible form of commercial phytosterol preparation containing cyclodextrin

[2] Trypticase soy broth

[3] Appendix Table 8

**Figure 4.13.** Effect of Sodium stearoyl lactylate (SSL) and BDP[1] on the SPC of 1% m.f. milk stored at 4-7°C. Values[2] are means of triplicate samples each conducted in triplicate (n = 6). Error bars represent standard deviation of means.




[1] Dispersible phytosterol mixture B

[2] Appendix Table 9

[3] SSL was added to milk at a concentration of 0.03% (w/v)

[4] Mixture consists of: carrageenan, maltodextrin, 57% (w/v) CPP and 1.54% (w/v) sodium stearoyl lactylate, added such that final phytosterol concentration in milk was 0.72% (w/v)

### 4.6. Effect of CPP on Yoghurt Microflora and pH

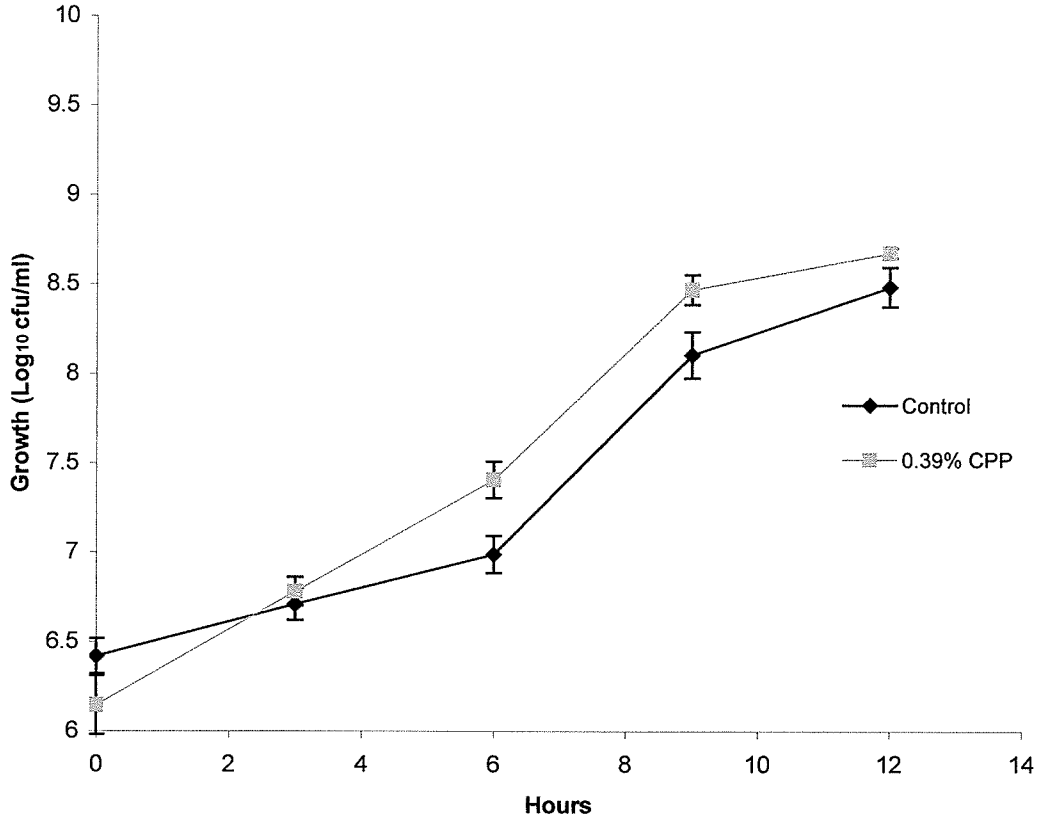
Overall, growth of either *L. bulgaricus* or *S. thermophilus* during yoghurt production at 33°C over 12 h with or without the addition of 0.39% (w/v) CPP did not differ by more than 0.5 $\log_{10}$ cfu/ml (Figure 4.14 and 4.15) at each sampling period. Survivor levels for both starter culture organisms during storage at 4°C over 40 d with and without CPP were not significantly different ($p>0.05$). Differences between the controls and treatments were $\leq 0.25$ $\log_{10}$ cfu/ml (Table 4.3).

Changes in pH during yoghurt production (Figure 4.16) and storage (Table 4.3) remained unaffected by the addition of 0.39% (w/v) CPP. The ultimate pH of the yoghurt preparations following 12 h of fermentation was approximately 4.63, which decreased slightly to 4.50 following 40 d of storage at 4°C.

Growth of *S. cerevisiae* in yoghurt during fermentation was unaffected by the presence of 0.39% (w/v) CPP (Figure 4.17). By the end of fermentation yeast populations in yoghurt with and without CPP remained relatively unchanged at approxiamtely 2 $\log_{10}$ cfu/ml. In contrast, *S. cerevisiae* exhibited growth over the entire storage period at 4°C regardless of treatment (Figure 4.18). Compared to the control, yeast levels in preparations with the phytosterol were numerically lower throughout the storage period increasing only at 20 d.

CPP did not appear to exhibit any effect on the growth of mould in yoghurt stored at 4°C. Mould growth in yoghurt was observed within 26 d of storage (Table 4.4) with and without CPP.

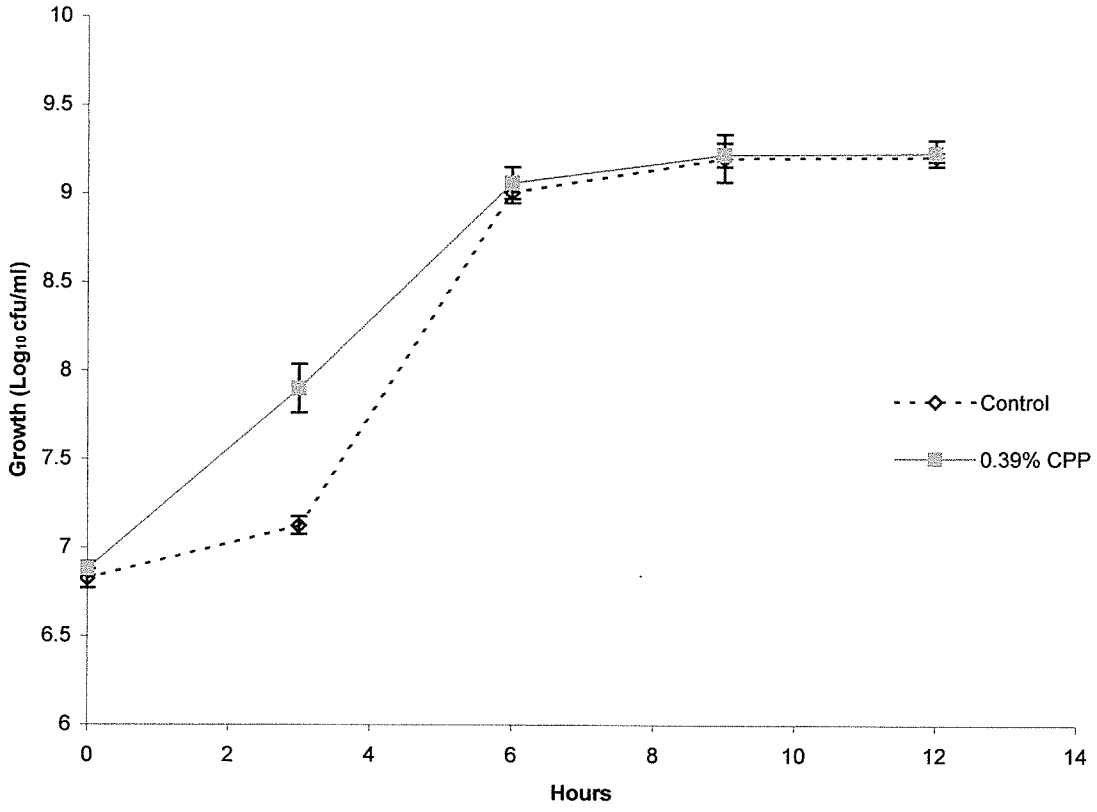
**Figure 4.14.** Effect of CPP[1] on *L. bulgaricus* growth during yoghurt production at 33°C. Values[2] are means of triplicate samples each performed in duplicate (n = 6). Error bars represent standard deviation of means.




[1] Commercial phytosterol preparation

[2] Appendix Table 10

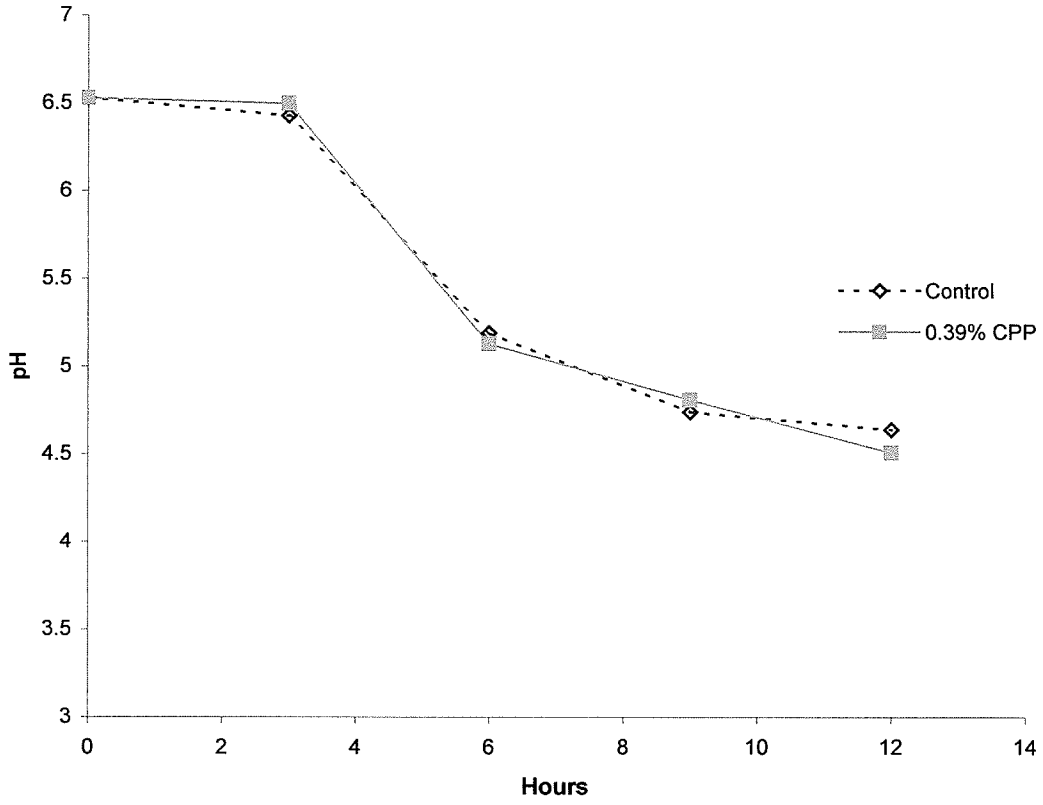
**Figure 4.15.** Effect of CPP[1] on *S. thermophilus* growth during yoghurt production at 33°C. Values[2] are means of triplicate samples each performed in duplicate (n = 6). Error bars represent standard deviation of means.




[1] Commercial phytosterol preparation

[2] Appendix Table 10

**Figure 4.16.** Effect of CPP[1] on pH during yoghurt production at 33°C. Values[2] are means of triplicate samples performed in duplicate (n = 6).




[1] Commercial phytosterol preparation

[2] Appendix Table 10

**Table 4.3.** Effect of CPP[1] on the pH and growth of starter culture organisms in yoghurt stored at 4°C

| | | $Log_{10}$ cfu/ml [2] | | |
|---|---|---|---|---|
| Time (d) | CPP (% w/v) | *S. thermophilus* [3] | *L. bulgaricus* [3] | pH [4] |
| 0 | 0 | 9.45±0.12 | 5.75±0.35 | 4.63 |
| | 0.39 | 9.42± 0.07 | 5.40±0.19 | 4.65 |
| 4 | 0 | 9.32±0.02 | 5.95±0.06 | 4.51 |
| | 0.39 | 9.28±0.06 | 5.74±0.29 | 4.50 |
| 8 | 0 | 9.29±0.02 | 5.79±0.08 | 4.56 |
| | 0.39 | 9.26±0.06 | 5.79±0.05 | 4.54 |
| 12 | 0 | 9.33±0.38 | 5.88±0.09 | 4.47 |
| | 0.39 | 9.32±0.05 | 5.85±0.10 | 4.45 |
| 16 | 0 | 9.44±0.04 | 5.95±0.04 | 4.61 |
| | 0.39 | 9.32±0.03 | 5.90±0.04 | 4.52 |
| 26 | 0 | 9.32±0.03 | 5.95±0.06 | 4.50 |
| | 0.39 | 9.30±0.06 | 5.89±0.04 | 4.50 |
| 31 | 0 | 9.27±0.02 | 5.73±0.06 | 4.54 |
| | 0.39 | 9.23±0.04 | 5.74±0.05 | 4.50 |
| 40 | 0 | 9.34±0.07 | 5.80±0.03 | 4.52 |
| | 0.39 | 9.27±0.08 | 5.79±0.04 | 4.50 |

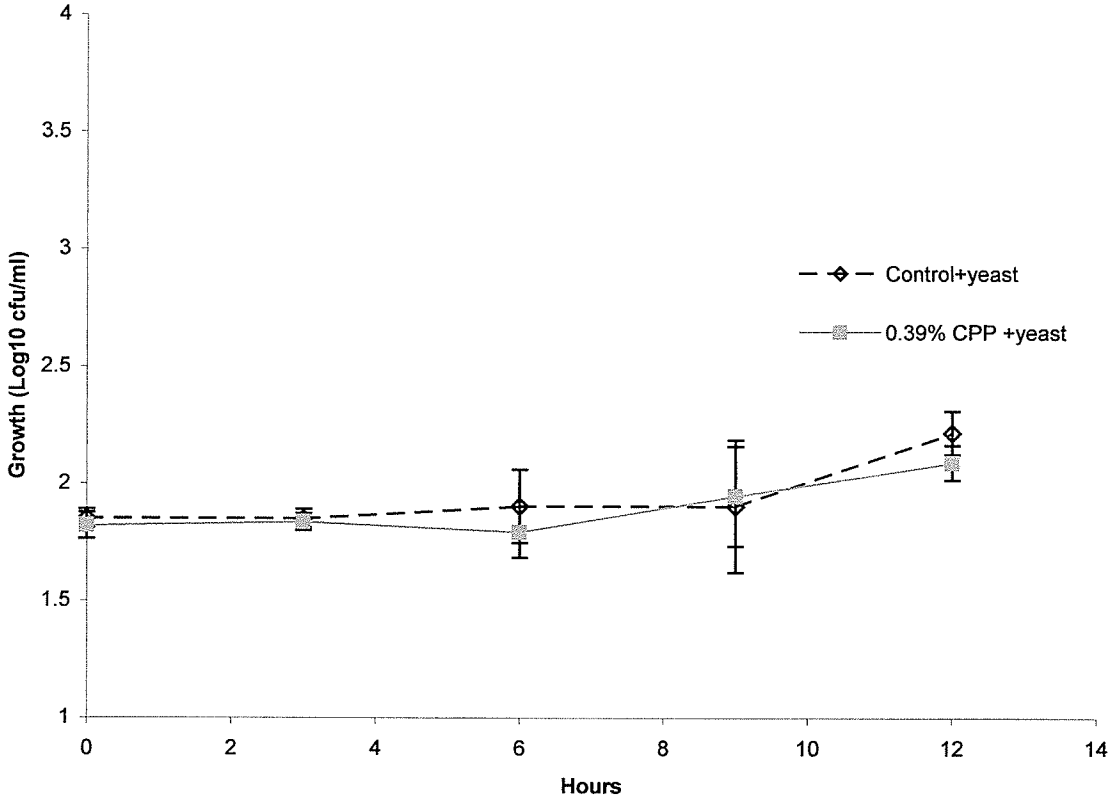
[1] Commercial phytosterol preparation

[2] Values are means of triplicate samples each performed in duplicate

[3] Mean values ± S.D.

[4] Values are means of triplicate samples

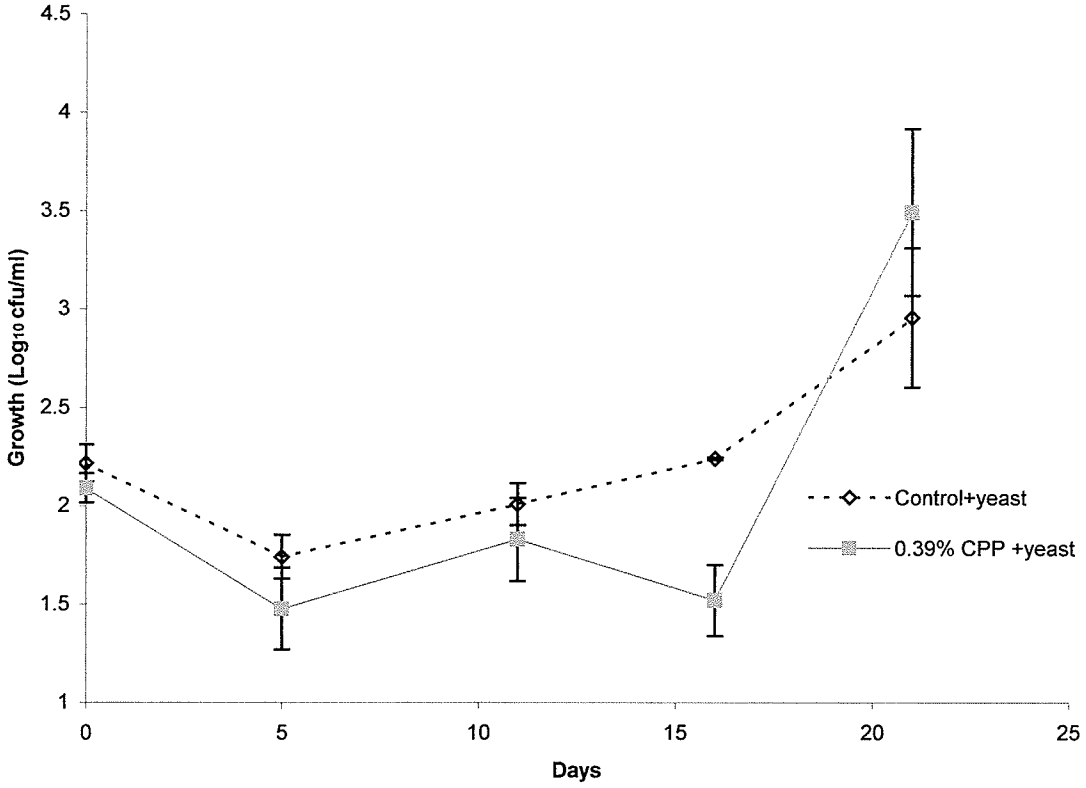
**Figure 4.17.** Effect of CPP[1] on *S. cerevisiae* growth during yoghurt production at 33°C. Values[2] are means of triplicate samples each performed in duplicate (n = 6). Error bars represent standard deviation of means.




[1] Commercial phytosterol preparation

[2] Appendix Table 10

**Figure 4.18.** Effect of CPP[1] on *S. cerevisiae* growth in yoghurt stored at 4°C. Values[2] are means of triplicate samples each performed in duplicate (n =6). Error bars represent standard deviation of means.




[1] Commercial phytosterol preparation

[2] Appendix Table 11

**Table 4.4.** Effect of CPP[1] on the visible growth of mould in yoghurt stored at 4°C

| | Mould growth[2] | |
|---|---|---|
| Time (d) | Yoghurt | Yoghurt with CPP (0.39% w/ v) |
| 0 | 0 | 0 |
| 10 | 0 | 0 |
| 17 | 0 | 0 |
| 21 | 0 | 0 |
| 26 | 1 | 1 |
| 30 | 1 | 1 |
| 35 | 2 | 2 |

[1] Commercial phytosterol preparation

[2] Number of samples out of three exhibiting visible mold growth

### 4.7. Effect of Phytosterol Mixtures on Yoghurt Microflora

Differences in the growth of *S. thermophilus* during the manufacture of control and yoghurt containing phytosterol mixtures at similar sampling periods were not significantly different ($p<0.05$) and were $< 0.5$ $\log_{10}$ / ml (Table 4.5). Similar trends were observed for *L. bulgaricus* (Table 4.6) and *S. cerevisiae* (Table 4.7).

### 4.8. Effect of CPP on Yoghurt Rheology

#### 4.8.1. Stress sweeps

Stress sweeps performed on yoghurt with the rheometer are shown in Figure 4.19. An oscillation stress value, in which all three frequencies achieved a constant G'/oscillation stress ratio, was chosen, where evaluation of the properties of the yoghurt dependent on its native structure would be acquired. This controlled variable was used to conduct frequency sweeps on yoghurt samples in order to determine viscoelastic properties. A value of 1.0 Pa was chosen.

#### 4.8.2. Viscoelastic properties

Results of tests performed on yoghurt with and without 0.39% CPP are shown in Figure 4.20. The G' and G" for both treatments appear similar. As expected, G' is higher than G", indicating that the yoghurt behaves more like a solid than a liquid. The tan δ of the yoghurts also remained unaffected by the presence of CPP. The tan δ for both the control and the 0.39% CPP-containing sample was 0.23.

**Table 4.5.** Effect of phytosterol mixtures [1] on *S. thermophilus* growth during yoghurt production at 33°C

| | $Log_{10}$ cfu/ml | | | | |
|---|---|---|---|---|---|
| Hour | Control [2] | FCP [3, 4] | FMP [3, 5] | Wood sterols [3, 6] | MP4 [3, 4] |
| 0 | 6.80±0.09 | 6.81±0.08 | 6.84±0.04 | 6.89±0.11 | 6.73±0.11 |
| 3 | 7.37±0.09 | 7.62±0.04 | 7.57±0.03 | 7.85±0 | 7.73±0.12 |
| 6 | 8.81±0.19 | 8.91±0.09 | 8.92±0.04 | 9.09±0.07 | 9.08±0.02 |
| 9 | 9.17±0.13 | 8.89±0.07 | 9.11±0.07 | 9.19±0.05 | 9.25±0.05 |
| 12 | 9.07±0.05 | 9.04±0.03 | 8.94±0.10 | 9.29±0.05 | 9.31±0.05 |

[1] All mixtures added to final concentration of 0.39% (w/v)

[2] Milk only

[3] Values are means of triplicate samples each performed in duplicate ± S.D.

[4] FCP and MP4 contain different ratios of sitostanol and campestanol

[5] Consists of sitosterol, stigmasterol and brassicasterol

[6] Consists of sitosterol, sitostanol, campestanol and campesterol

**Table 4.6.** Effect of phytosterol mixtures [1] on *L. bulgaricus* growth during yoghurt production at 33°C

| | $Log_{10}$ cfu/ml | | | | |
|---|---|---|---|---|---|
| Hour | Control [2] | FCP [3, 4] | FMP [3, 5] | Wood sterols [3, 6] | MP4 [3, 4] |
| 0 | 6.47±0.09 | 6.39±0.04 | 6.44±0.15 | 6.23±0.14 | 6.16±0.15 |
| 3 | 6.67±0.03 | 6.67±0.08 | 6.70±0.04 | 6.53±0.15 | 6.72±0.11 |
| 6 | 6.78±0.19 | 6.99±0.18 | 7.17±0.09 | 8.17±0.19 | 8.22±0.11 |
| 9 | 8.19±0.21 | 8.05±0.08 | 8.24±0.11 | 8.30±0.07 | 8.40±0.04 |
| 12 | 8.45±0.20 | 8.38±0.08 | 8.48±0.10 | 8.69±0.07 | 8.68±0.04 |

[1] All mixtures added to final concentration of 0.39% (w/v)

[2] Milk only

[3] Values are means of triplicate samples each performed in duplicate ± S.D.

[4] FCP and MP4 contain different ratios of sitostanol and campestanol

[5] Consists of sitosterol, stigmasterol and brassicasterol

[6] Consists of sitosterol, sitostanol, campestanol and campesterol

**Table 4.7.** Effect of phytosterol mixtures[1] on *S. cerevisiae* growth during yoghurt production at 33°C

| | $Log_{10}$ cfu/ml | | | | |
|---|---|---|---|---|---|
| Hour | Control [2] | FCP [3, 4] | FMP [3, 5] | Wood sterols [3, 6] | MP4 [3, 4] |
| 0 | 1.94±0.03 | 1.88±0.02 | 1.91±0.04 | 1.91±0.03 | 1.89±0.05 |
| 3 | 1.95±0.09 | 1.86±0.04 | 1.89±0.05 | 1.85±0.06 | 1.86±0.04 |
| 6 | 1.94±0.10 | 2.01±0.09 | 2.05±0.28 | 1.90±0.12 | 1.94±0.14 |
| 9 | 2.08±0.12 | 2.16±0.26 | 1.78±0.11 | 2.17±0.14 | 2.13±0.20 |
| 12 | 2.20±0.13 | 2.22±0.09 | 2.15±0.10 | 2.33±0.18 | 2.20±0.12 |

[1] All mixtures added to final concentration of 0.39% (w/v)

[2] Milk only

[3] Values are means of triplicate samples each performed in duplicate ± S.D.

[4] FCP and MP4 contain different ratios of sitostanol and campestanol

[5] Consists of sitosterol, stigmasterol and brassicasterol

[6] Consists of sitosterol, sitostanol, campestanol and campesterol

**Figure 4.19.** Stress sweeps performed with a rheometer on yoghurt at 4°C at various frequencies

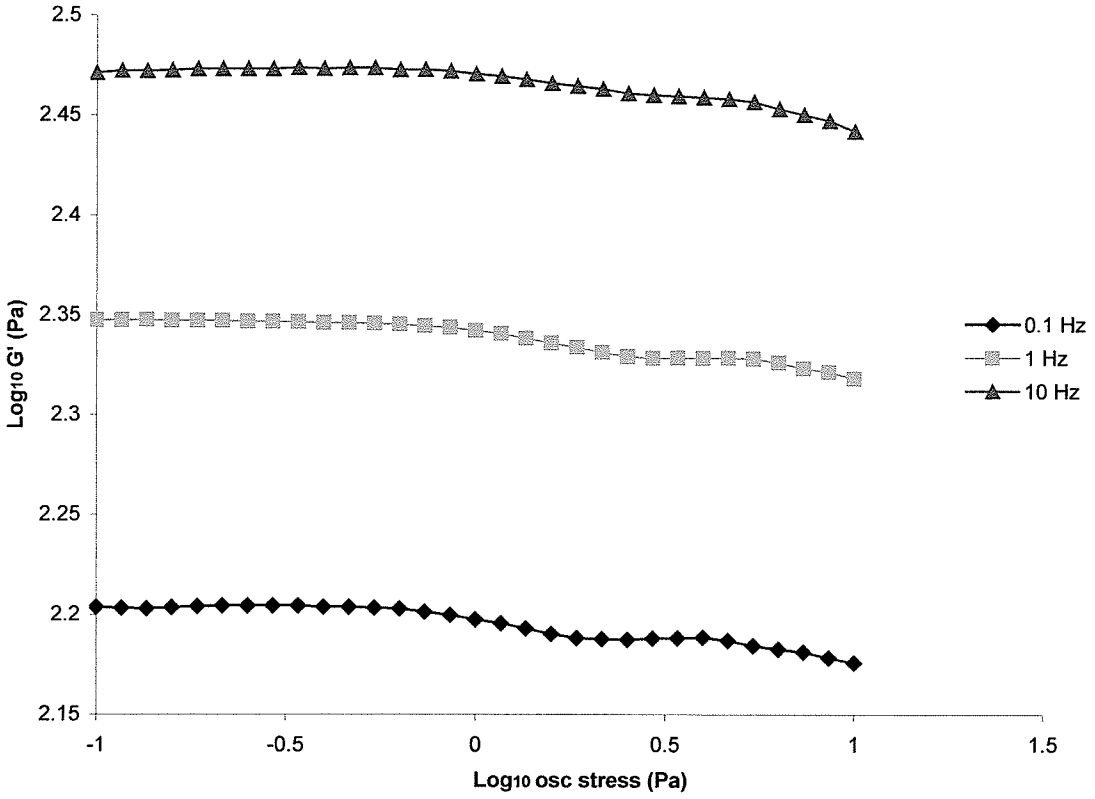
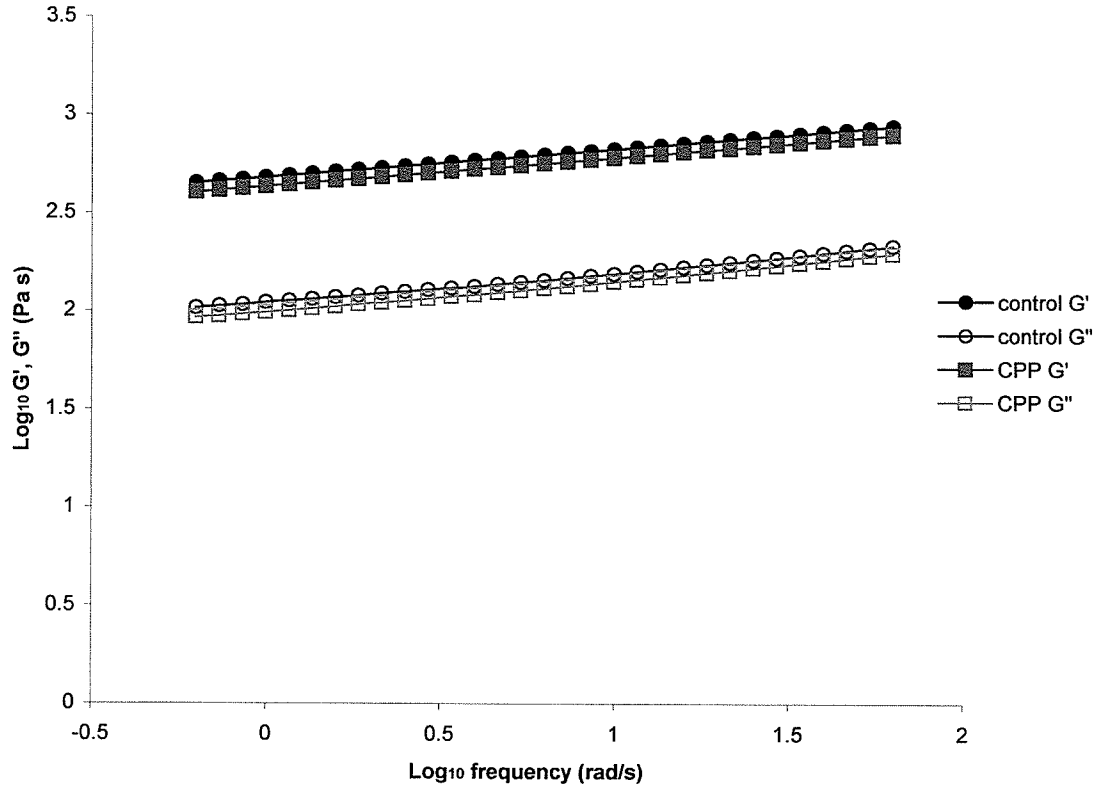


**Figure 4.20.** Effect of 0.39% CPP[1] on the viscoelastic properties of yoghurt. Values are means of triplicate samples each performed in duplicate (n = 6)




[1] Commercial phytosterol preparation

### 4.8.3. Flow properties

The addition of 0.39% CPP had no effect on the flow properties of the yoghurt samples (Figure 4.21). Both the control and the CPP-containing treatments produced a yoghurt having an initial stiff structure. As the shear rate increased, the structure of the samples quickly broke down and became more free-flowing. As shown in Figure 4.21, yoghurt viscosity decreased rapidly even at low initial shear rates, and quickly entered the shear-thinning range where viscosity decreases according to a power law relationship (Rao, 1999). Thereafter, increases in shear rate no longer resulted in a rapid decrease in viscosity. A second strain sweep was performed on the sample after the yoghurt was left standing for 10 min. Although the gel structure recovered somewhat (based on a higher initial viscosity compared to that at the end of the first trial) it was quickly broken down again.

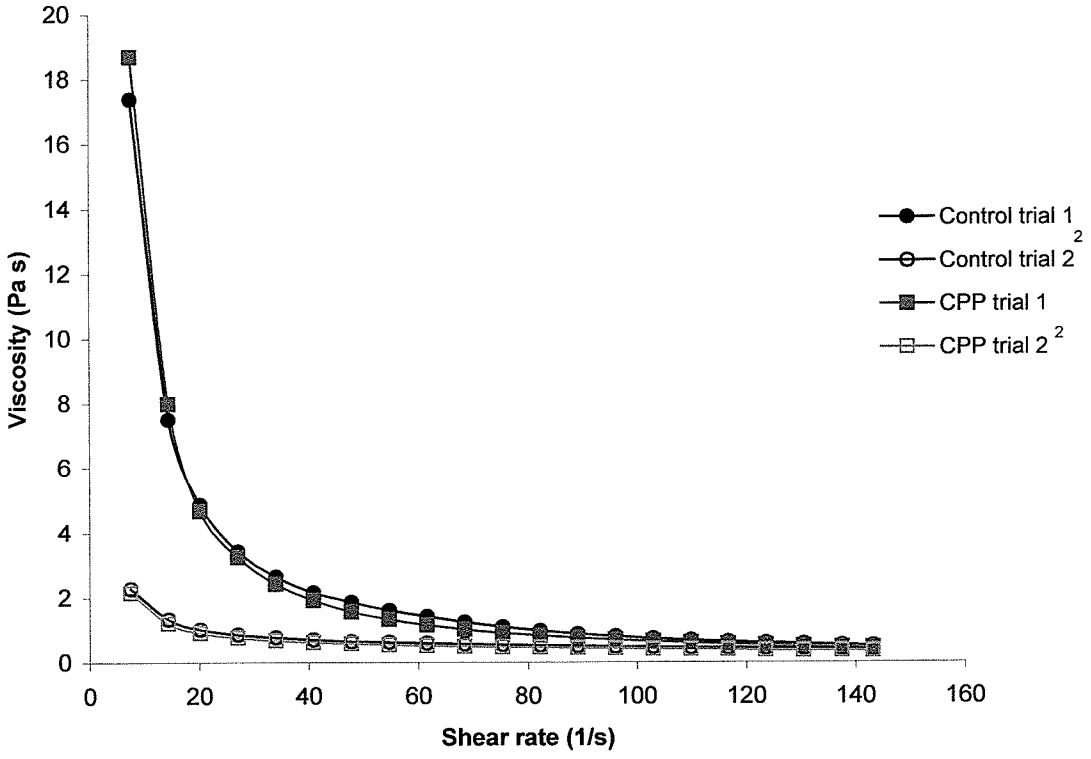
## 4.9. Antioxidant Activity of Free Phytosterols and Phytosterol Esters

Results are those presented by Dr. Jerzy Zawistoski at the 6th ISF (International Society for Fat Research) World Congress "Modern Aspects of Fats and Oils - A Fascinating Source of Knowledge" Prague, 25-28 September 2005.

### 4.9.1. POV and PAV

As shown in Figure 4.22 and 4.23 free phytosterols significantly ($p<0.05$) reduced the oxidation of MCT oil over a 12 month period while phytosterol esters did not. The mixture of phytosterol esters and free phytosterols significantly ($p<0.05$) reduced oxidation. The lack of a reduction in oxidation of oil containing only phytosterol esters indicated that this activity was caused by the free phytosterols.

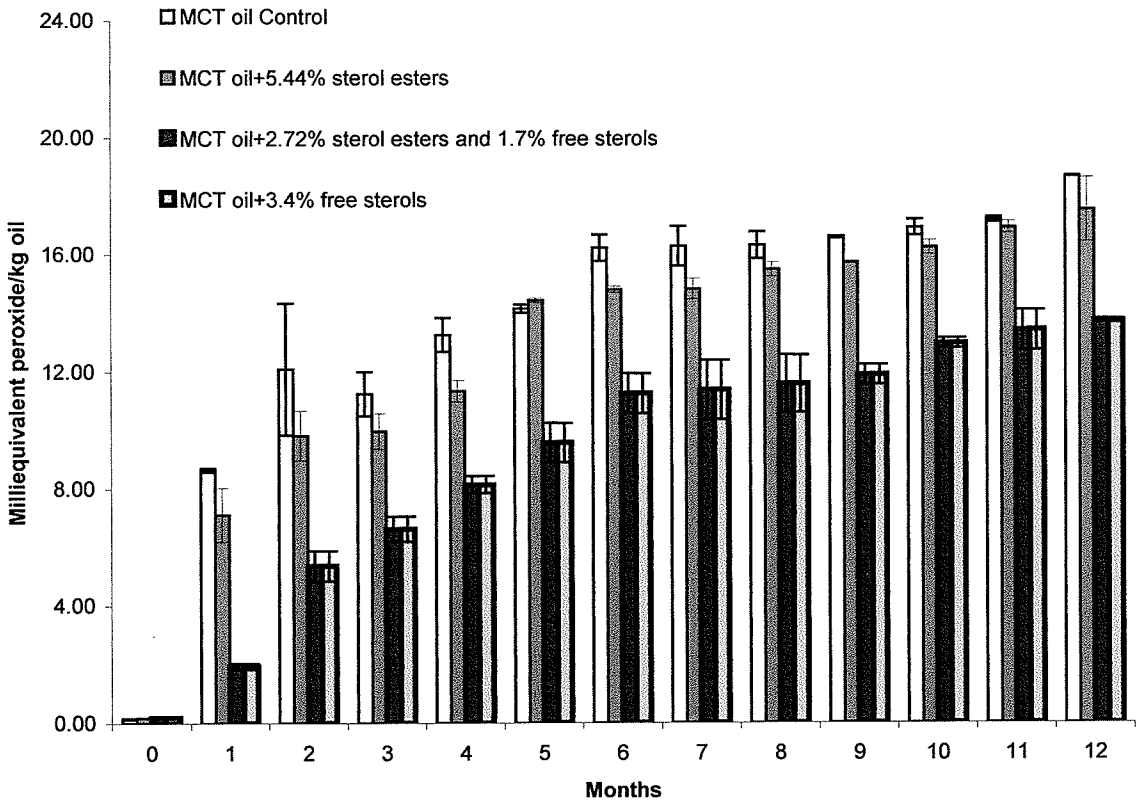
**Figure 4.21.** Effect of 0.39% CPP[1] on the flow properties of yoghurt at 4°C. Values are means of triplicate samples (n = 3).




[1] Commercial phytosterol preparation

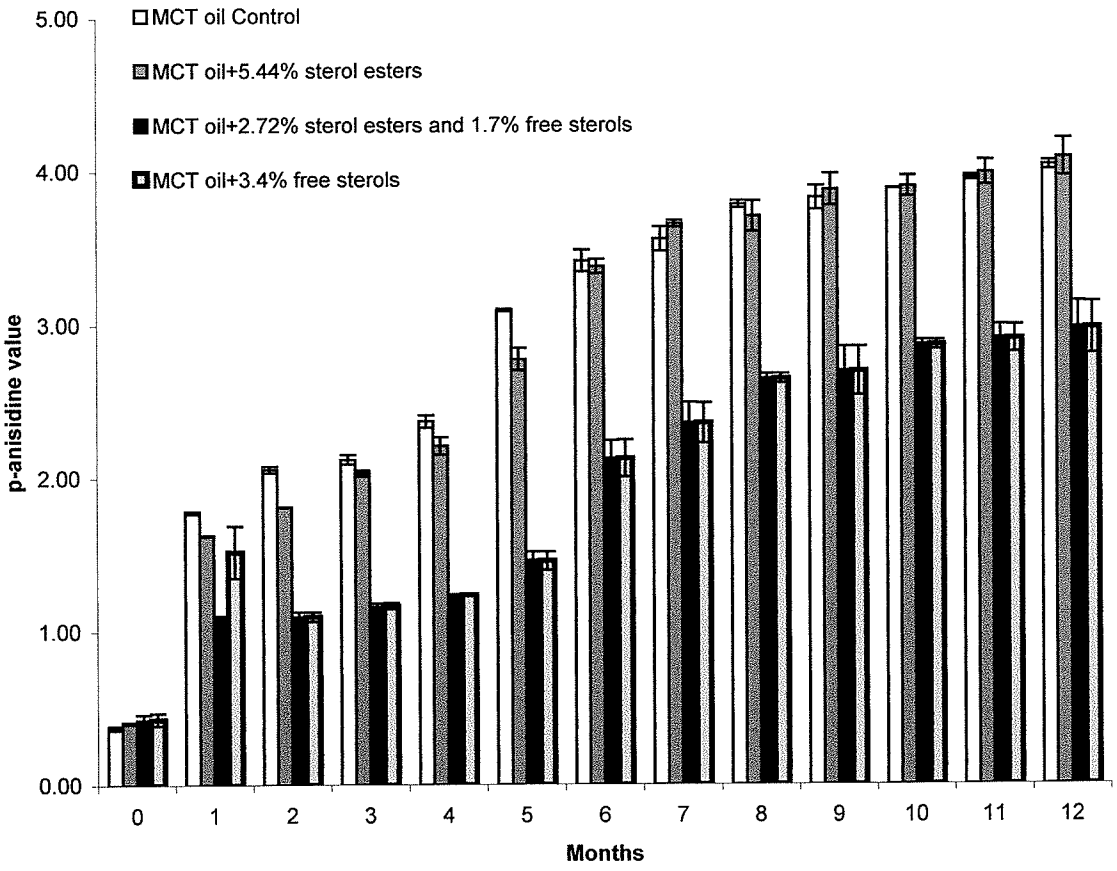
[2] Conducted 10 min after Trial 1 to assess recovery of gel structure

**Figure 4.22.** Effect of phytosterols on the formation of primary lipid oxidation products in MCT oil [1]




[1] Error bars represent standard deviations of means (n=4)

**Figure 4.23.** Effect of phytosterols on the formation of secondary lipid oxidation products in MCT oil [1]




[1] Error bars represent standard deviations of means (n=4)

### 4.9.2. Percent loss of essential fatty acids

Compared to the control the free phytosterols significantly reduced the loss of linolenic acid in oil after 6 months of storage by at least 25% (Figure 4.24). This is significant as linolenic acid is an omega-3 PUFA (polyunsaturated fatty acid) and is one of the two fatty acids that are essential to human nutrition (the other being linoleic acid) because vertebrates lack enzymes known as $\Delta^{12}$ and $\Delta^{15}$ desaturases. These enzymes incorporate double bonds at those positions in the carbon chain. This means that vertebrates are not able to form double bonds beyond the $\Delta^{9}$ carbon in the chain and cannot synthesize fatty acids with double bonds beyond that point from simple precursors. Omega-3 PUFAs have also been shown to be hypocholesterolemic and exert antiatherogenic properties (Groff and Gropper, 2000). In contrast, the loss of linoleic acid in oil during storage was not affected by the addition of free phytosterols.

### 4.9.3. Effect of phytosterols on thermal stability of oils

As shown in Figure 4.25 the addition of sterol esters and or sterol esters with free sterols to MCT oil resulted in an increase in primary oxidation products following heating at 100°C. Overall, however, it appeared that the sterol esters were largely responsible for the increase. The secondary lipid oxidation products were not affected by the addition of phytosterol esters to MCT oil (Figure 4.26), however, there was a slight decrease in secondary LOPs with the addition of free phytosterols. Also, during the heating of canola oil to 100°C (Figure 4.27) the addition of free sterols to MCT oil appeared to decrease the production of secondary LOPs. Compared to the controls, the inclusion of sterol esters in both MCT and canola oils when heated to 190°C also resulted

in higher primary LOP levels (Figures 4.28 and 4.29). Extended heating of canola oil for up to 12 h at 190°C resulted in a gradual increase in ACM products in the control treatment (Figure 4.30). The addition of free sterols and to a lesser extent the mixture consisting of sterol esters and free sterols reduced the levels of these contaminants in the canola oil.

**Figure 4.24.** Effect of free phytosterols on loss of essential fatty acids in MCT oil after 6 months storage

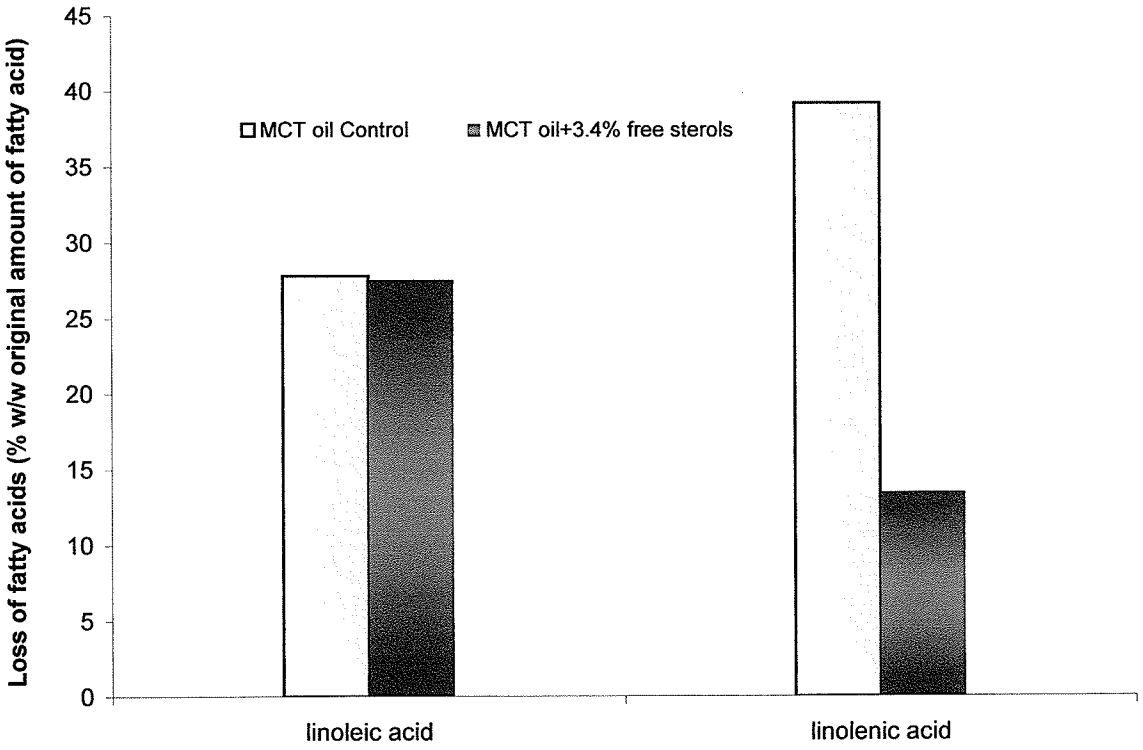
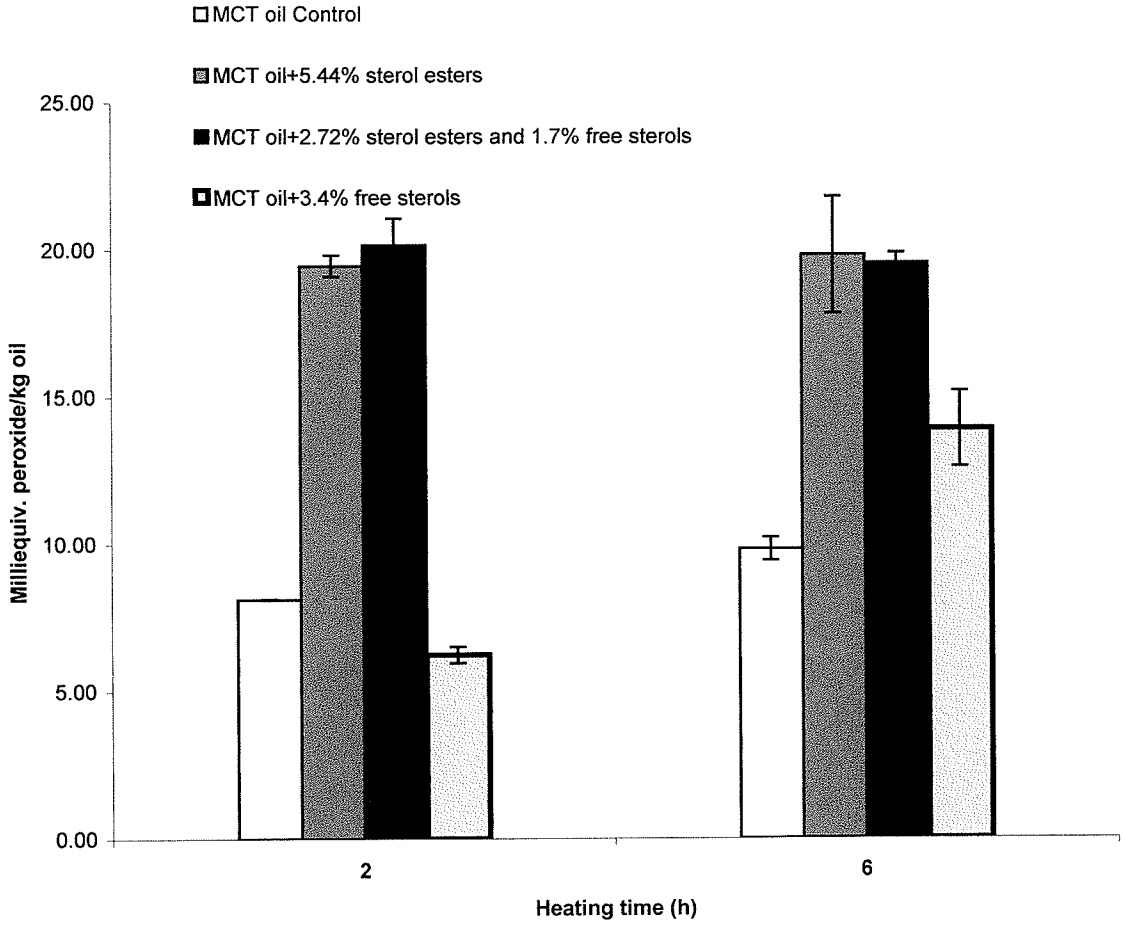
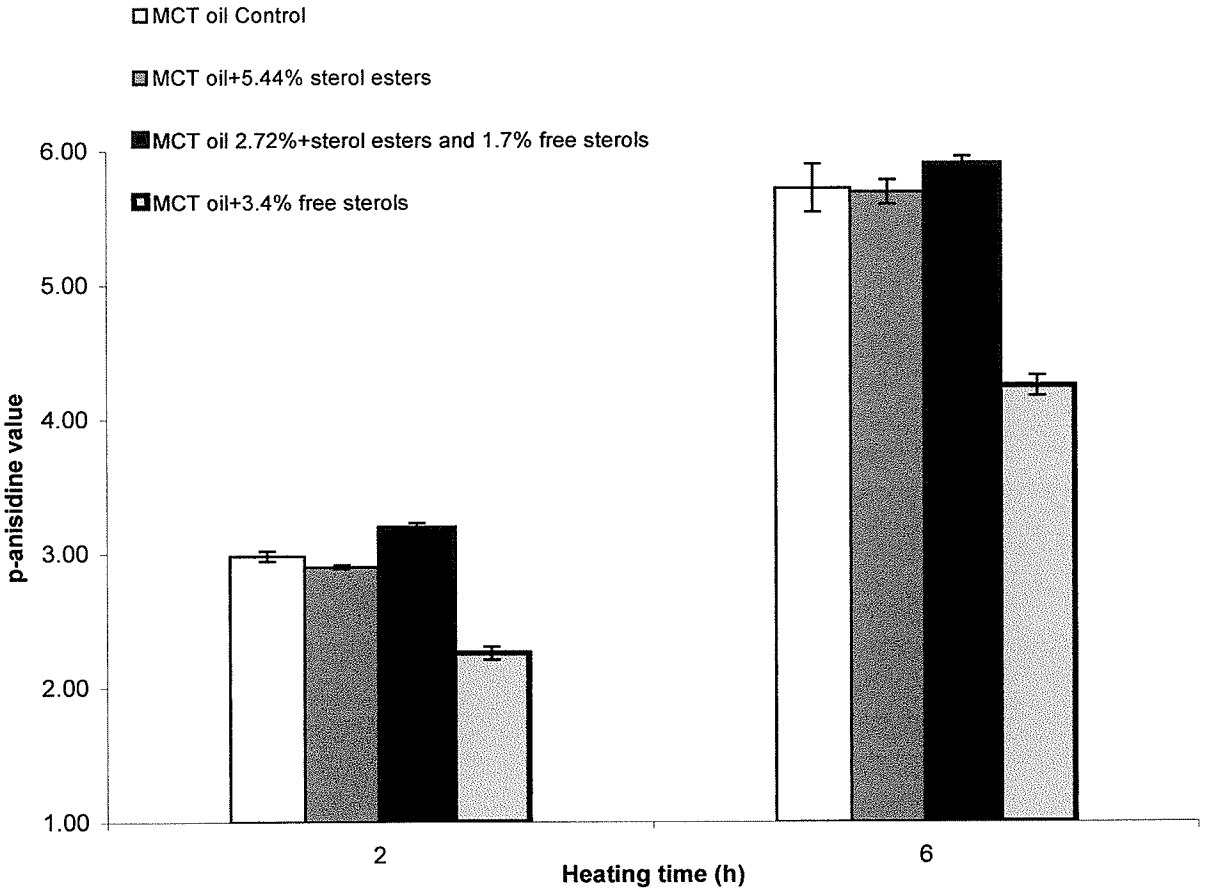


**Figure 4.25.** Effect of phytosterols on primary lipid oxidation products in MCT oil due to thermal degradation at 100°C [1]



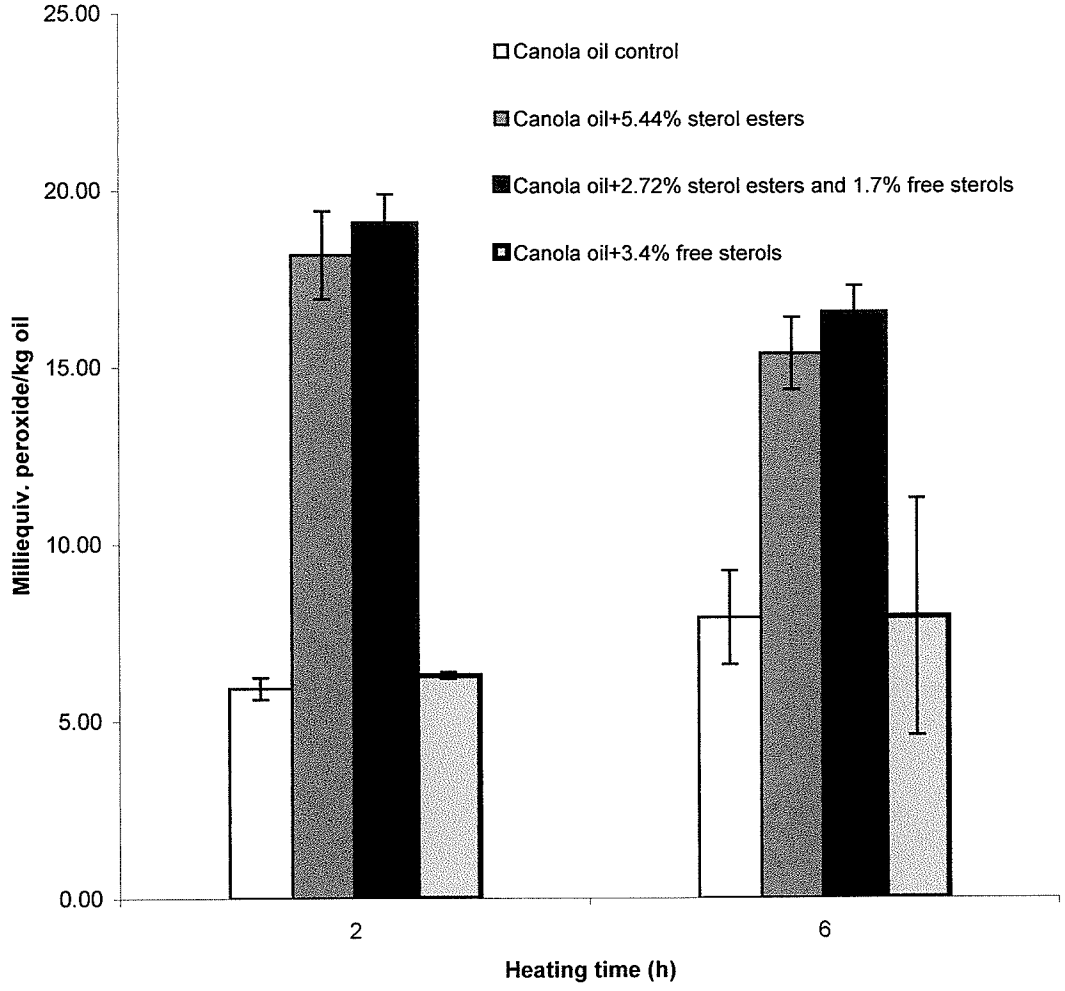

[1] Error bars represent standard deviations of means (n=4).

**Figure 4.26.** Effect of phytosterols on secondary lipid oxidation products in MCT oil due to thermal degradation at 100°C [1]



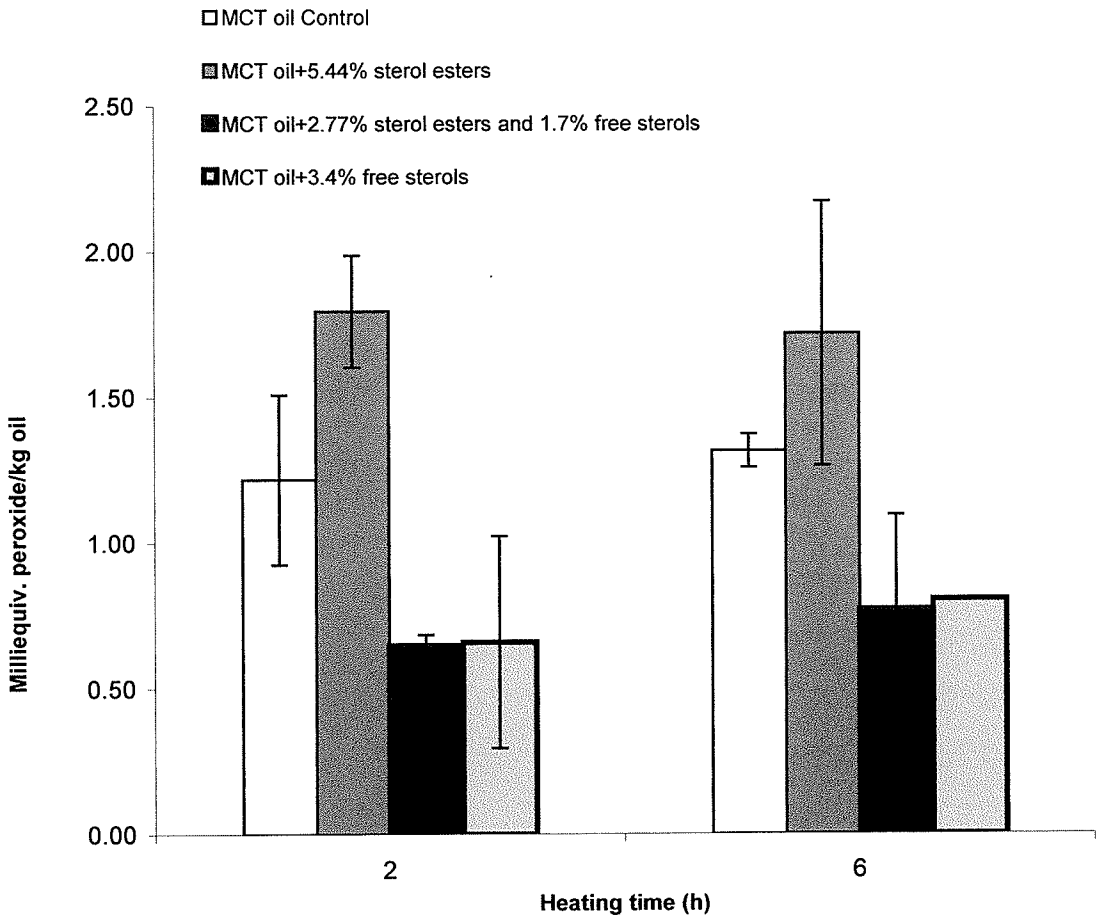

[1] Error bars represent standard deviations of means (n=4).

**Figure 4.27.** Effect of phytosterols on the thermal oxidation of canola oil at 100°C [1]



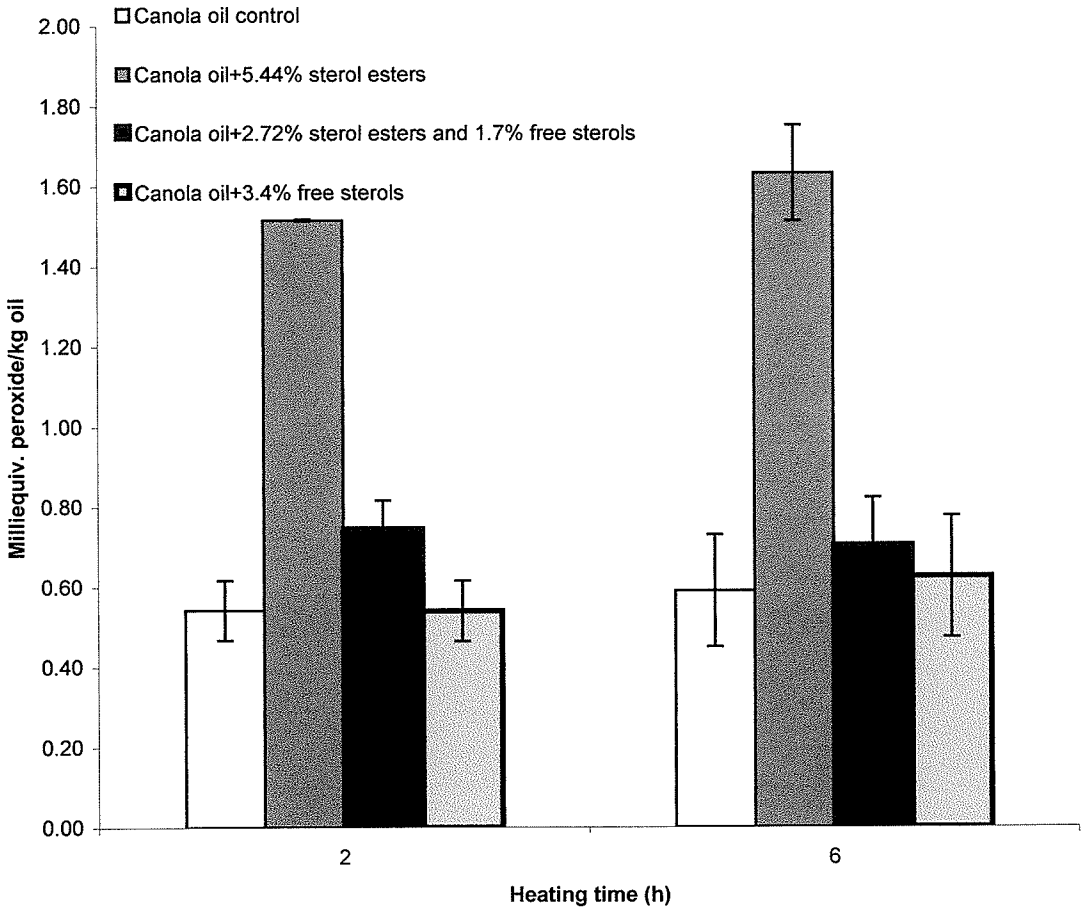

[1] Error bars represent standard deviations of means (n=4).

**Figure 4.28.** Effect of phytosterols on the thermal degradation of MCT oil at 190°C [1]



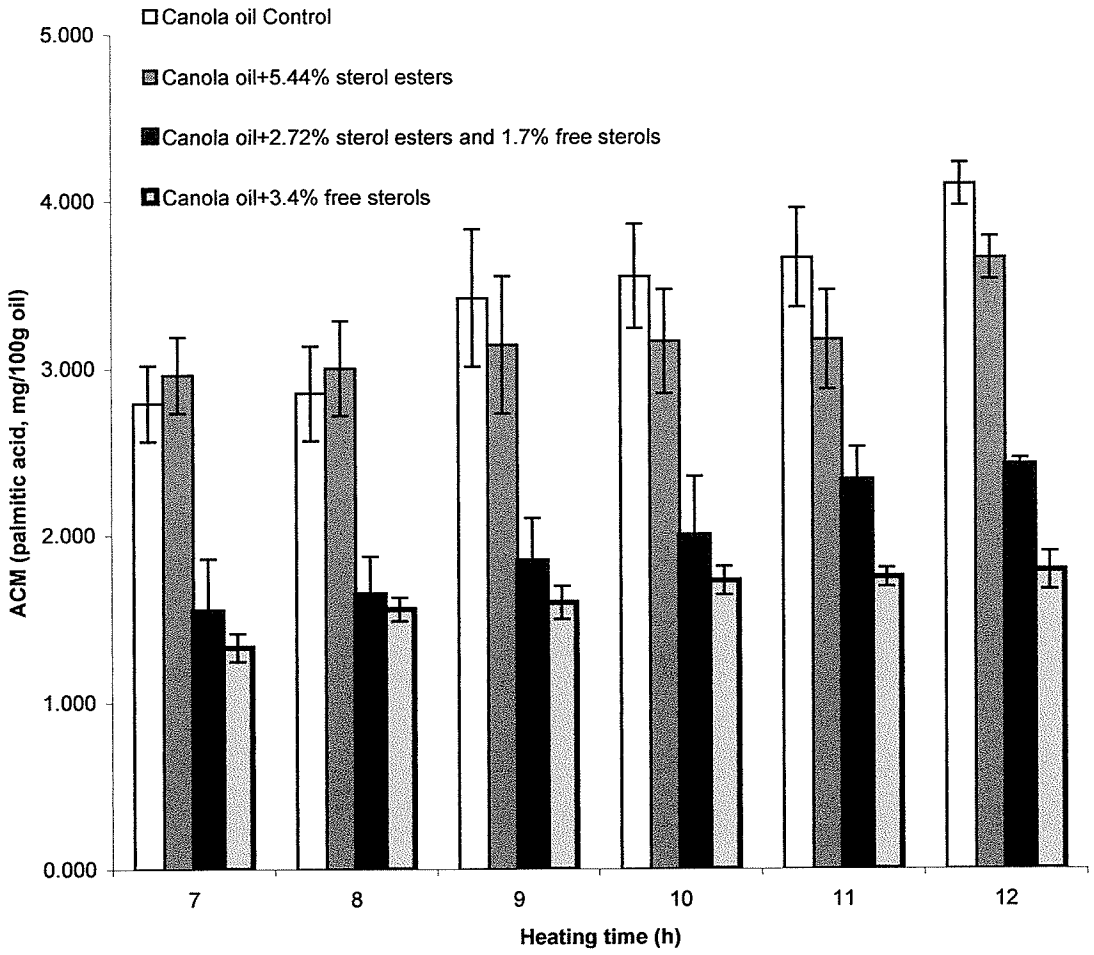

[1] Error bars represent standard deviations of means (n=4).

**Figure 4.29.** Effect of phytosterols on the thermal oxidation of canola oil at 190°C [1]




[1] Error bars represent standard deviations of means (n=4).

**Figure 4.30.** Effect of phytosterols on the levels of ACM [1] in canola oil after heating to 190°C [2]




[1] Alkaline contaminant materials

[2] Error bars represent standard deviations of means (n=4).

# 5. DISCUSSION

In this investigation, CPP concentrations up to 1.8% (w/v) exhibited minimal antimicrobial activity towards the growth of psychrotrophs and the SPC in milk. In addition the growth of various *Pseudomonas* species, normally recovered from pasteurized milk as post process contaminants, were not affected by the presence of the phytosterol. Homogenization of CPP, in order to increase its dispersal in milk, (Vuillemard, 1991) did not improve its efficacy as an antimicrobial.

The use of dispersible blends of phytosterols (ADP and BDP) containing maltodextrin, carrageenan and sodium stearoyl lactylate did, however, exhibit a long-term (up to 23 d) inhibitory effect on the SPC and growth of psychrotrophs in milk maintained at 4-7°C. However, growth of *P. fluorescens* was not affected. Overall, the antimicrobial activity of the dispersibile phytosterol mixtures may be due to the increased solubility in milk. For example, during treatment regular CPP separated, much of it floating to the top of the milk after a few hours of incubation. Since SSL is an anionic lipophilic emulsifier (Ultra Chemical Inc, 2000) its ability to disperse CPP would be advantageous, as the efficacy of hydrophobic antimicrobials is dependant on their ability to travel across the aqueous phase of a solution in order to interact with the membrane of the target organism (Trombetta et al., 2005). In addition, the percent saturation and total concentration in solution would affect antimicrobial efficacy (Taylor et al., 2004). As lipophilic compounds, phytosterols may partition out of the aqueous phase of a solution crossing the lipid membranes of bacteria (Trombetta et al., 2004).

It was also possible that SSL exerted a direct antimicrobial effect based on its lactic and stearic acid content. Both compounds are known to exhibit antimicrobial effects (Jay et al., 2005). For example, SSL has been shown to inhibit germination and growth of *Bacillus cereus* spores at a concentration of 0.1% (Feijoo et al., 1997). In this study, however, the use of SSL without phytosterols did not appear to have any effect on the SPC in milk and therefore should not be considered a significant antimicrobial. Also, neither carrageenan nor maltodextrin exhibited antimicrobial effects on the SPC in milk (data not presented). Ostensibly the antimicrobial effect of the dispersible CPP mixture was due to synergy among its components. Although the antimicrobial mode of action of the dispersible CPP mixture was not investigated, it is possible that (similar to other antioxidant compounds) it functions by impairing bacterial membrane function. Several phenolic antioxidants such as BHA have been demonstrated to cause lysis of bacterial protoplasts by affecting cell permeability and leading to leakage of cell nucleotides (Degre and Sylvestre, 1983). Monoterpenes, which also act as antioxidants, have been reported to increase membrane permeability and fluidity leading to alteration of ion transport and inhibition of cell respiration (Trombetta et al., 2005). Generally, the cell membrane is the choice site of action because many antioxidants including BHA have low water solubility, (as is also the case with phytosterols) which prevents them from diffusing into the cell cytoplasm (Degre and Sylvestre, 1983). Generally, antimicrobial effectiveness is dependant on the lipid composition of the target organism as well as its net surface charge and the permeability of the microbial outer envelope (Trombetta et al., 2005).

It is interesting to note that while the dispersible phytosterol mixtures inhibited psychrotroph growth, they did not have any effect on the growth of *P. fluorescens*. Since the SPC was also inhibited it would be expected that some pseudomonads, which are normally a significant part of the psychrotroph population in refrigerated milk (Ternström et al., 1993) would also be inhibited. Although gram-negative microorganisms may be considered more resistant to hydrophobic compounds due to their lipopolysaccharide outer membrane (Trombetta et al., 2004) the SPC of refrigerated milk is composed mainly of gram-negative microorganisms (Eneroth et al., 2000). Therefore permeability of the organism *per se* may not be important. Alternatively, some species of *Pseudomonas* have the ability to metabolize phytosterols. For example, a study by Mahato et al. (1981) reported that *Pseudomonas* used sitosterol as a sole carbon source by cleaving its side chain. Other microorganisms including *Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Microbacterium, Mycobacterium, Nocardia, Protoaminobacter, Serratia*, and *Streptomyces* secrete enzymes capable of cleaving the side chain of cholesterol (structurally similar to phytosterols) and contribute to the complete decomposition of the compound (Owen et al., 1983). The utilization of phytosterols by *Pseudomonas* would not likely occur in milk, which has more readily available nutrients and, in fact, if it did the SPC population should also have increased due to phytosterol degradation.

It is more likely that the lack of an inhibitory response to the phytosterol mixture was due to the relatively high initial *P. fluorescens* population, following its inoculation. The *P. fluorescens* numbers were at least 1 $\log_{10}$ higher than the naturally present SPC

and psychrotroph populations, and presumably would be expected to require higher levels of the phytosterol mixture in order to be effective.

CPP without dispersants failed to inhibit the growth of *L. bulgaricus* and *S. thermophilus* and did not affect their ability to produce acid as monitored by pH change. In one sense this was advantageous as these organisms are essential in the development of acid and flavour compounds which affect the taste and texture of yoghurt. Inability to inhibit the growth of starter culture organisms may be due to their ability to break down phytosterols. For example, some lactobacilli are capable of assimilating cholesterol (Klaver and Van der Meer, 1993). Alternatively, solubility issues pertaining to phytosterol may also be important. Ultimately, population numbers of starter cultures in yoghurt production can increase from $10^6$ to $10^9$ cfu/ml. It is possible that the levels of phytosterol employed were simply inadequate to elicit a microbial response. The same argument could be made for inhibition of *S. cerevisiae* and *Aspergillus*. Although the addition of CPP did not detrimentally affect the quality of yoghurt, neither did it increase the shelf life by inhibiting the growth of typical spoilage microorganisms.

The rheological profiles of yoghurt also were not affected adversely by the addition of CPP. In this respect, the viscosity and the storage (or elastic) and loss (or viscous) moduli remained similar in yoghurts with and without CPP. Since viscosity and firmness are important attributes of yoghurt quality, it was desirable to minimize changes in these properties. Also, if growth of the starter culture organisms had been inhibited by the presence of phytosterols, acid production would have been inhibited, ultimately affecting coagulum formation and viscosity (Lucey and Singh, 1998).

The antioxidant activity of the free phytosterols was confirmed. Esterified phytosterols, however, failed to protect against oxidation of oils at elevated temperatures although they did reduce the amount of oxidation in oil stored at room temperature for 12 months. Free phytosterols also reduced the loss of linolenic acid during 6 months of storage. The difference in antioxidant activity between free and esterified phytosterols may be due to their structural difference. In esterified phytosterols, the 3β-OH group on the A ring that remains unoccupied in free phytosterols is attached to a fatty acid (Moreau et al., 2002). The unavailability of the OH group to interact with radicals as well as the increased size of the molecule may affect the ability of esterified phytosterols to act as antioxidants.

# 6. CONCLUSION

The addition of CPP did not appear to have any antimicrobial effect. Dispersible phytosterol mixtures (ADP and BDP), however, did appear to increase the shelf life of milk. In this respect, both the development of the SPC and psychrotrophic growth were inhibited, however, *Pseudomonas fluorescens* was not affected. Since psychrotrophic bacteria are the main determinants of shelf life in pasteurized milk it is possible that the inclusion of the phytosterol mixture could not only offer consumers a health benefit but also extend the shelf life of the product.

CPP did not appear to inhibit the growth of spoilage yeast and mold in yoghurt and as such would not be expected to contribute to an increase in its shelf life. However, phytosterols did not inhibit the growth of starter culture organisms or detrimentally affect the rheological properties of yoghurt and as such did not have a negative effect on quality.

The antioxidant activity of CPP (free phytosterols) was confirmed. Phytosterols esterified with triacylglycerides from sunflower oil reduced oil oxidation during storage at room temperature. However, the esterified phytosterols had either no effect or increased the oxidation of oils during heating. Free phytosterols also enhanced the quality of the oil by reducing the loss of linolenic acid. The antioxidant activity of free phytosterols could be advantageous in maintaining the quality of high fat foods by delaying the onset of rancidity due to lipid oxidation and by reducing the loss of essential fatty acids. Based on the results of this study a link between the antimicrobial activity of CPP (which contained free sterols) and antioxidant activity could not be established.

Based on these results, it is possible that phytosterols could play a multifunctional role as food additives. They provide health benefits to consumers as well as extending the shelf life of some food products by delaying microbial spoilage and oxidative rancidity. These benefits are achieved without any detrimental effects to the quality of the food product.

Further studies on the effects of phytosterols on the shelf life of dairy foods should utilize more soluble forms including phytosterol esters. The antimicrobial effect of phytosterol mixtures should be extended to include bacterial pathogens such as *Listeria* and *Salmonella* and on gram-positive and gram-negative bacteria. Starter culture activity should also be evaluated in order to assess the potential for inclusion of the dispersible phytosterol mixtures in fermented dairy products. Also, the antimicrobial effect of the dispersible phytosterol mixtures should be more closely investigated as a means of determining what the active components are.

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# APPENDICES

**Appendix 1.** Effect of CPP[1] on the SPC, growth of *P. fluorescens* and growth ($log_{10}$ cfu/ml) of a *Pseudomonas* cocktail ($log_{10}$ cfu/ml) in whole milk stored at 4-7°C

| | | | | CPP (% w/v) [5] | | |
|---|---|---|---|---|---|---|
| Day | | Control [2,3] | Stabilizer [2,4] | 0.26 [2] | 0.72 [2] | 1.8 [2] |
| 0 | SPC | 1.95±0.02 | 1.95±0.01 | 1.90±0.02 | 1.83±0.05 | 1.91±0.00 |
| | *P. fluorescens* | 1.14±0.05 | 1.20±0.05 | 1.19±0.04 | 1.16±0.04 | 1.17±0.05 |
| | *Pseudomonas* cocktail | 1.75±0.04 | 1.76±0.08 | 1.74±0.01 | 1.72±0.05 | 1.75±0.01 |
| 2 | SPC | 2.30±0.11 | 2.30±0.11 | 2.04±0.18 | 2.33±0.14 | 2.25±0.08 |
| | *P. fluorescens* | 2.81±0.02 | 2.89±0.02 | 2.74±0.06 | 2.73±0.06 | 2.66±0.03 |
| | *Pseudomonas* cocktail | 2.39±0.07 | 2.54±0.08 | 2.41±0.06 | 2.36±0.06 | 2.06±0.11 |
| 4 | SPC | N.D. [6] | N.D. | N.D. | N.D. | N.D. |
| | *P. fluorescens* | 4.40±0.05 | 4.43±0.05 | 4.42±0.02 | 4.39±0.03 | 4.28±0.04 |
| | *Pseudomonas* cocktail | N.D. | N.D. | N.D. | N.D. | N.D. |
| 6 | SPC | 4.53±0.30 | 4.53±0.09 | 4.86±0.08 | 4.58±0.06 | 4.65±0.80 |
| | *P. fluorescens* | 6.17±0.03 | 6.17±0.04 | 6.17±0.06 | 6.04±0.03 | 5.94±0.05 |
| | *Pseudomonas* cocktail | 6.25±0.02 | 6.34±0.07 | 6.18±0.05 | 6.38±0.10 | 5.74±0.10 |
| 8 | SPC | 5.22±0.06 | 5.22±0.10 | 5.40±0.02 | 5.58±0.09 | 5.38±0.36 |
| | *P. fluorescens* | 7.20±0.03 | 7.06±0.05 | 7.10±0.06 | 6.88±0.03 | 6.89±0.01 |
| | *Pseudomonas* cocktail | 7.23±0.04 | 7.14±0.07 | N.D. | 6.95±0.02 | 6.12±0.20 |
| 10 | SPC | 6.20±0.03 | 6.20±0.05 | 6.10±0.07 | 5.88±0.03 | 6.11±0.01 |
| | *P. fluorescens* | N.D. | N.D. | N.D. | N.D. | N.D. |
| | *Pseudomonas* cocktail | N.D. | N.D. | N.D. | N.D. | N.D. |

[1] Commercial phytosterol preparation

[2] Values are means of duplicate samples each performed in duplicate

[3] Milk without CPP

[4] Milk with 0.016% w/v carrageenan and 0.186% w/v maltodextrin

[5] Milk with 0.016% w/v carrageenan, 0.186% w/v maltodextrin and CPP

[6] Not determined

**Appendix 2.** Effect of CPP[1] on the SPC of pasteurized skim milk stored at 4-7°C

| | $Log_{10}$ cfu/ml | | |
|---|---|---|---|
| Day | Control [2,3] | Stabilizer [2,4] | CPP [2,5] |
| 0 | 1.72±0.08 | 1.64±0.05 | 1.58±0.09 |
| 3 | 1.69±0.10 | 1.56±0.10 | 1.63±0.05 |
| 6 | 1.74±0.05 | 1.62±0.06 | 1.63±0.11 |
| 9 | 1.74±0.04 | 1.65±0.05 | 1.51±0.06 |
| 12 | 1.98±0.16 | 2.02±0.04 | 1.81±0.39 |
| 15 | 2.78±0.42 | 3.45±0.08 | 3.40±0.09 |
| 18 | 4.77±0.07 | 4.69±0.15 | 4.83±0.09 |

[1] Commercial phytosterol preparation

[2] Values are means of duplicate samples each performed in duplicate

[3] Milk without CPP

[4] Milk with 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

[5] Milk with 0.016% w/v carrageenan, 0.186% (w/v) maltodextrin and 1.8% (w/v) CPP

**Appendix 3.** Effect of CPP[1] on the growth ($log_{10}$ cfu/ml) of *P. fluorescens* and a *Pseudomonas* cocktail in pasteurized skim milk stored at 4-7°C

| | | | | CPP (% w/v) [5] | | |
|---|---|---|---|---|---|---|
| Day | | Control [2,3] | Stabilizer [2,4] | 0.26 [2] | 0.72 [2] | 1.8 [2] |
| 0 | *P. fluorescens* | 2.51±0.0 | 2.51±0.0 | 2.51±0.0 | 2.51±0.0 | 2.51±0.0 |
| | *Pseudomonas* cocktail | 1.63±0.07 | 1.70±0.03 | 1.62±0.05 | 1.67±0.06 | 1.63±0.07 |
| 2 | *P. fluorescens* | 2.70±0.18 | 2.83±0.16 | 2.30±0.24 | 2.40±0.24 | 2.67±0.20 |
| | *Pseudomonas* cocktail | 2.16±0.11 | 2.19±0.06 | 2.23±0.10 | 2.24±0.09 | 1.91±0.15 |
| 4 | *P. fluorescens* | 3.16±0.11 | 3.23±0.20 | 2.99±0.07 | 3.10±0.08 | 3.13±0.21 |
| | *Pseudomonas* cocktail | N.D. [6] | N.D. | N.D. | N.D. | N.D. |
| 6 | *P. fluorescens* | 3.42±0.05 | 3.48±0.04 | 3.50±0.04 | 3.41±0.04 | 3.22±0.05 |
| | *Pseudomonas* cocktail | 6.10±0.02 | 5.17±0.03 | 5.23±0.11 | 5.98±0.05 | 5.75±0.09 |
| 8 | *P. fluorescens* | 3.97±0.14 | 3.97±0.06 | 3.94±0.07 | 3.93±0.04 | 3.73±0.11 |
| | *Pseudomonas* cocktail | 6.77±0.07 | 6.98±0.07 | 7.02±0.22 | 6.81±0.15 | 6.85±0.07 |
| 13 | *P. fluorescens* | 4.54±0.04 | 4.42±0.15 | 4.39±0.07 | 4.35±0.12 | 4.61±0.23 |
| | *Pseudomonas* cocktail | N.D. | N.D. | N.D. | N.D. | N.D. |

[1] Commercial phytosterol preparation

[2] Values are mans of duplicate samples each performed in duplicate

[3] Milk without CPP

[4] Milk with 0.016% (w/v) carrageenan and 0.186% (w/v) maltodextrin

[5] Milk with 0.016% (w/v) carrageenan, 0.186% (w/v) maltodextrin and CPP

[6] Not determined

**Appendix 4.** Effect of CPP[1] on the psychrotrophic plate count ($log_{10}$ cfu/ml) of 1% m.f. pasteurized milk stored at 4-7°C

| | CPP (% w/v) | | |
|---|---|---|---|
| Day | Control [2,3] | 0.72 [2,4] | 1.8 [2,4] |
| 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 |
| 15 | 1.48 | 1.16 | 1.23 |
| 19 | 2.48 | 2.4 | 2.4 |
| 24 | 3.51 | 3.47 | 3.44 |

[1] Commercial phytosterol preparation

[2] Values are mans of duplicate samples each performed in duplicate

[3] Milk without CPP

[4] Milk with 0.016% (w/v) carrageenan, 0.186% (w/v) maltodextrin and CPP

**Appendix 5.** Effect of dispersible phytosterol mixtures ADP and BDP on the SPC and psychrotrophic plate count ($log_{10}$ cfu/ml) of 1% m.f. pasteurized milk stored at 4-7°C

| | | | Phytosterol mixture (% w/v) | |
|---|---|---|---|---|
| Day | | Control [1, 2] | ADP [1, 3] | BDP [1, 4] |
| 0 | SPC | 1.57±0.08 | 1.37±0.07 | 1.45±0.08 |
| | Psychrotrophs | 0.76±0 | 0.76±0 | 0.76±0 |
| 3 | SPC | 1.52±0.05 | 1.36±0.11 | 1.47±0.08 |
| | Psychrotrophs | 0.41±0 | 0.00±0 | 0.00±0 |
| 6 | SPC | 1.66±0.13 | 1.41±0.08 | 1.52±0.13 |
| | Psychrotrophs | 0.23± | 0.23± | 0.23± |
| 9 | SPC | 1.91±0.39 | 1.39±0.05 | 1.23±0.15 |
| | Psychrotrophs | 0.81± | 0.00± | 0.00± |
| 12 | SPC | 2.40±0.07 | 1.42±0.17 | 1.41±0.10 |
| | Psychrotrophs | N.D. [5] | N.D. | N.D. |
| 15 | SPC | 3.96±0.08 | 1.57±0.05 | 1.63±0.06 |
| | Psychrotrophs | 1.11± | 0.00± | 0.00± |
| 18 | SPC | N.D. | N.D. | N.D. |
| | Psychrotrophs | 2.18± | 0.00± | 0.00± |
| 19 | SPC | 4.76±0.07 | 1.29±0.19 | 1.41±0.11 |
| | Psychrotrophs | N.D. | N.D. | N.D. |
| 21 | SPC | N.D. | N.D. | N.D. |
| | Psychrotrophs | 3.78± | 0.00± | 0.00± |
| 22 | SPC | 6.04±0.27 | 1.37±0.10 | 1.48±0.05 |
| | Psychrotrophs | N.D. | N.D. | N.D. |

[1] Values are means of triplicate samples each performed in duplicate

[2] Milk without phytosterols

[3] Milk and dispersible phytosterol mixture A containing 36% phytosterols added to a final concentration of 0.72% phytosterols

[4] Milk and dispersible phytosterol mixture B containing 57% phytosterols added to a final concentration of 0.72% phytosterols

[5] Not determined

**Appendix 6.** Effect of dispersible phytosterol mixtures ADP and BDP on the growth of *P. fluorescens* ($log_{10}$ cfu/ml) in 1% m.f. pasteurized milk stored at 4-7°C

| | | Phytosterol mixture (% w/v) | |
|---|---|---|---|
| Day | Control [1, 2] | ADP [1, 3] | BDP [1, 4] |
| 0 | 2.18±0.03 | 2.18±0.03 | 2.18±0.03 |
| 2 | 2.36±0.09 | 2.33±0.18 | 2.22±0.04 |
| 6 | 3.40±0.04 | 3.37±0.08 | 3.30±0.06 |
| 10 | 4.08±0.01 | 4.08±0.01 | 4.06±0.02 |
| 12 | 4.22±0.11 | 4.18±0.07 | 4.11±0.09 |

[1] Values are means of triplicate samples each performed in duplicate

[2] Milk without phytosterols

[3] Milk and dispersible phytosterol mixture A containing 36% phytosterols added to a final concentration of 0.72% phytosterols

[4] Milk and dispersible phytosterol mixture B containing 57% phytosterols added to a final concentration of 0.72% phytosterols

**Appendix 7.** Effect of CPP[1] on the psychrotrophs ($log_{10}$ cfu/ml) following dispersal by homogenization in of 1% m.f. homogenized skim milk stored at 4-7°C

| | | CPP (% w/v) [4] | |
|---|---|---|---|
| Day | Control [2,3] | 0.26 [2] | 0.72 [2] |
| 0 | 0±0 | 0±0 | 0±0 |
| 4 | 0±0 | 0±0 | 0±0 |
| 7 | 0±0 | 0±0 | 0±0 |
| 12 | 0±0 | 0±0 | 0±0 |
| 17 | 0±0 | 0±0 | 0±0 |
| 21 | 0±0 | 0±0 | 0±0 |
| 31 | 3.81±0.01 | 3.20±0.02 | 3.19±0.00 |
| 33 | 3.89±0.02 | 3.28±0.01 | 3.27±0.00 |
| 35 | 3.72±0.02 | 3.28±0.02 | 3.29±0.01 |
| 40 | 4.65±0.01 | 5.75±0.00 | N.D. [5] |

[1] Commercial phytosterol preparation

[2] Values are means of duplicate samples performed in duplicate

[3] Milk without CPP

[4] Milk with 0.016% (w/v) carrageenan, 0.186% (w/v) maltodextrin and CPP

[5] Not determined

**Appendix 8.** Effect of Cavamax[1] on *P. fluorescens* growth at 22°C

| | $Log_{10}$ cfu/ml | |
|---|---|---|
| Hour | Control [2, 3] | Cavamax [2, 4] |
| 0 | 1.44±0.04 | 1.44±0.07 |
| 4 | 1.51±0.14 | 1.80±0.20 |
| 8 | 2.56±0.04 | 2.83±0.10 |
| 24 | 6.20±0.11 | 6.61±0.05 |

[1] Dispersible form of CPP

[2] Values are means of duplicate samples each performed in duplicate

[3] TSB only

[4] TSB and 1.21% (w/v) Cavamax, which is equivalent to 0.26% CPP

**Appendix 9.** Effect of Sodium stearoyl lactylate (SSL) and BDP[1] on the SPC ($log_{10}$ cfu/ml) of 1% m.f. milk stored at 4-7°C.

| Day | Control[2,3] | SSL[4] | BDP[5] |
|---|---|---|---|
| 0 | 0.96±0.12 | 0.75±0.14 | 0.75±0.14 |
| 3 | 1.37±0.11 | 1.40±0.07 | 0.61±0.20 |
| 6 | 1.28±0.11 | 1.35±0.06 | 0.31±0.26 |
| 9 | 1.38±0.25 | 1.43±0.14 | 0.61±0.30 |
| 12 | 1.47±0.05 | 2.01±0.84 | 0.50±0.17 |
| 14 | 2.46±0.20 | 2.16±0.25 | 0.25±0.12 |

[1] Dispersible phytosterol mixture B

[2] Milk alone

[3] Values are means of triplicate samples each performed in duplicate

[4] Milk containing 0.03% sodium stearoyl lactylate

[5] Milk and a phytosterol mixture containing 57% phytosterols added to a final concentration of 0.72% phytosterols

**Appendix 10.** Effect of CPP[1] on pH and the growth of *S. thermophilus*, *L. bulgaricus* and *S. cerevisiae* during yoghurt production at 33°C

| Hour | CPP (% w/v) | $Log_{10}$ cfu/ml | | | pH [2] |
|---|---|---|---|---|---|
| | | *S. thermophilus* [2] | *L. bulgaricus* [2] | *S. cerevisiae* [2] | |
| 0 | 0 | 6.82±0.05 | 6.42±0.09 | 1.85±0.04 | 6.53 |
| | 0.39 | 6.88±0.04 | 6.15±0.16 | 1.82±0.06 | 6.53 |
| 3 | 0 | 7.12±0.05 | 6.71±0.09 | 1.85±0.04 | 6.43 |
| | 0.39 | 7.90±0.14 | 6.78±0.08 | 1.84±0.04 | 6.5 |
| 6 | 0 | 9.01±0.06 | 6.99±0.10 | 1.90±0.15 | 5.19 |
| | 0.39 | 9.06±0.09 | 7.41±0.10 | 1.79±0.11 | 5.13 |
| 9 | 0 | 9.20±0.13 | 8.11±0.13 | 1.90±0.28 | 4.74 |
| | 0.39 | 9.22±0.07 | 8.47±0.08 | 1.95±0.21 | 4.81 |
| 12 | 0 | 9.21±0.03 | 8.48±0.11 | 2.22±0.09 | 4.64 |
| | 0.39 | 9.23±0.07 | 8.67±0.03 | 2.09±0.07 | 4.51 |

[1] Commercial phytosterol preparation

[2] Values are means of triplicate samples each performed in duplicate

**Appendix 11.** Effect of CPP[1] on *S. cerevisiae* growth in yoghurt stored at 4°C

| Day | $Log_{10}$ cfu/ml | |
|---|---|---|
| | Control [2,3] | CPP [2,4] |
| 0 | 2.22±0.09 | 2.09±0.07 |
| 5 | 1.74±0.11 | 1.48±0.21 |
| 11 | 2.01±0.11 | 1.83±0.21 |
| 16 | 2.24±0.01 | 1.52±0.18 |
| 21 | 2.96±0.35 | 3.49±0.42 |

[1] Commercial phytosterol preparation

[2] Values are means of triplicate samples each performed in duplicate

[3] Yoghurt only

[4] Yoghurt with 0.39% w/v CPP