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INCORPORATION OF POLYPHENOLS INTO DAIRY MATRICES

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DECLARATION OF ORIGINAL AUTHORSHIP

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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ABSTRACT

Currently, there is a significant interest in incorporating polyphenols into dairy products. However, critical information is required, not only on the impact of polyphenol addition on the technological quality of the resulting dairy products, but also as to how dairy processing operations impact on the incorporated polyphenols. Ultimately such information is required to enable processors to develop successful strategies for polyphenolic supplementation of dairy products. Therefore, the overall aim of this thesis was to determine the optimum processing point at which polyphenols could be added to fluid milk before it is subjected to further processing and ultimately if such additions impact on final product quality. Due to their high levels of consumption acidified dairy products were chosen as an appropriate model product for this research.

Initially, this study determined the effect of addition of a range of phenolic sources, before and after heat treatment on the properties of milk-polyphenol mixtures. The milk-polyphenol mixtures were then used to produce acidified milk gels to determine the impact polyphenol addition on gelation kinetics and the rheological properties of the gels. Secondly, this study investigated the effect of 28 days of refrigerated storage on physicochemical properties of the acidified milk gels. Four sources of phenolic compounds (green tea, white grape, tannic acid, gallic acid) were used in the study and incorporated into pasteurized skim milk. A heat treatment (85ºC for 30 min) was applied to pasteurized-skim milk either before (M_hP) or after polyphenols addition (M_hP_h).

Heat treatment decreased the total phenolic content (TPC) and ferric ion reducing antioxidant power (FRAP) values of the samples. Although the stage of polyphenol addition had no significant effects on TPC and FRAP, the addition of gallic acid before heat treatment resulted in a significant increase in casein micelle size (CMS) due to the lower pH of this

sample prior to heating. Acid gelation decreased the extractable total polyphenols, however there was no significant difference between the FRAP of the acid gel and M_hP_h milk samples.

The stage of gallic acid addition had a significant effect on the rheological properties of the acidified milk gel sample. The addition of gallic acid before heat treatment (M_hP_h) resulted in the longest gelation time and significantly decreased gelation pH, final storage modulus (G′) and fracture stress. This was attributed to higher attachment of denatured whey proteins to casein micelles during heat treatment. The addition of polyphenols, with M_hP_h treatment, had an effect on physicochemical properties of acidified milk gels during 28 days of refrigerated storage, depending on source of polyphenols.

The findings of this thesis demonstrated that polyphenols could sucessfully be incorporated into milk used for production of acidified dairy products. It was found that the nutritional profile of the products could be improved without a significant impact on final product stability and quality. However consideration as to the properties of the phenolic source used, as well as the stage at which it is added to the milk must be given in order to achieve this. In particular the pH of phenolic compounds should be taken into consideration when fortifying dairy products with polyphenols.

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LIST OF ABBREVIATIONS

a*: Redness

AA: Antioxidant Activity

AAE: Ascorbic Acid Equivalent

ABTS: 2,2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid

AC: Antioxidant Capacity

ANOVA: Analysis of Variance

b*: Yellowness

BSA: Bovine Serum Albumin

c*: Chroma

C: Catechin

Ca: Calcium

CCP: Colloidal Calcium Phosphate

CI: Chlorine

CMS: Casein Micelle Size

CUPRAC: Copper Reducing Antioxidant Activity

DLS: Dynamic Light Scattering

DPPH: 1,1-diphenyl-2-picrylhydrazyl

EC: Epicatechin

ECG: Epicatechin Gallate

EDTA: Ethylene Diamine Tetraacetic Acid

EGC: Epigallocatechin

EGCG: Epigallocatechin Gallate

ET: Electron Transfer

FCR: Folin Ciocalteu reagent

FRAP: ferric reducing antioxidant power

Fe^{3+}-TPTZ: Ferric Tripyridyltriazine

G′: Elastic or Storage Modulus

G″: Viscous or Loss Modulus

GAE: Gallic Acid Equivalent

GDL: Glucono-deltalactone

h*: Hue Angle

HAT: Hydrogen Atom Transfer

HPLC: High Performance Liquid Chromatography

HCI: Hydrochloric Acid

Ig: Immunoglobulins

K: Potassium

L*: Lightness

LC-MS: Liquid chromatography–Mass Spectrometry

Mg: Magnesium

MP: Pasteurized Skim Milk-Polyphenol Mixtures

M_hP: Pasteurized skim milk heated before polyphenols addition

M_hP_h: Pasteurized skim milk heated after polyphenols addition

Na: Sodium

NaCI: Sodium Chloride

NaOH: Sodium Hydroxide

ORAC: Oxygen Radical Absorbance Capacity

P: polyphenols solutions without heat treatment

P_h: heated polyphenol solutions

PTFE: Polytetrafluoroethylene

PVDF: Polyvinylidene Difluoride

SE: Standard Error

SPSS: Statistical Package for the Social Sciences

TEAC: Trolox Equivalence Antioxidant Capacity

TPTZ: 2,4,6-tripyridyl-s-triazine

TPC: Total Phenolic Content

TRAP: Total Radical Trapping Antioxidant Parameter

UHT: Ultra High Temperature

UV: Ultraviolet

WPNI: Whey Protein Nitrogen Index

α-LA: Alpha-lactalbumin

β-LG: Beta-lactoglobulin

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Consumer awareness of the importance of healthy eating and lifestyle is increasing. In the UK 63% of adults reported that they try to consume healthy foods to maintain and improve their health (Mintel, 2017). In older people (over-65s) this figure increases to 78% due to age related health problems (Mintel, 2017). The increasing interest in health promoting foods has led to greater attention being focused on the development of functional foods, including those which incorporate bioactive components (Bhat and Hina, 2011). Polyphenols provide good sources of bioactive compounds which have been associated with prevention of various diseases such as degenerative diseases, cancer and cardiovascular disease. They are mainly found in vegetables and fruits (Gharras, 2009). However, only 46% of the UK population consume the recommended 5 portions of fruit and vegetables a day (Mintel, 2014). Therefore, polyphenol extracts from various fruit and vegetables can be used to produce functional foods to improve public health.

Milk is a complex mixture of protein, fat, lactose, minerals and vitamins. It is widely consumed around the world. Milk proteins are often used as ingredients in food formulations due to their physicochemical and functional properties (Bimbo et al., 2017). They are an important source of bioactive peptides and their beneficial health effects such as metabolic health, bone health and weight control have been well documented in the literature (Singh et al., 2014). Milk and milk products are potentially ideal carriers to convey bioactive compounds such as polyphenols (Tavares et al., 2014), allowing dairy products with improved antioxidant capacity to be produced. Polyphenols may affect the physical, functional and structural properties of dairy products as they have strong affinity to interact with proteins through covalent and non-covalent bonds (Spencer et al., 1988; Najgebauer-

Lejko et al., 2014). These interactions are affected by several factors: pH, temperature, type and concentration of both protein and polyphenol and molecular size of the polyphenol (Bandyopadhyay et al., 2012).

Several studies have tried to evaluate the effect of polyphenol-milk protein interactions on the antioxidant capacity of polyphenols. However, the results of such studies have shown conflicting results due to either the methods used for the measurement of antioxidant capacity or phenolic types utilised (Korir et al., 2014; Kyle et al., 2007). Therefore, in this thesis as well as using phenolic rich extracts which contain a range of phenolic compounds (green tea and white grape), single phenolic compounds of both high (tannic acid) and low (gallic acid) molecular weight were employed. This would enable an assessment of the critical role that the physical and chemical properties of the phenolic source may play in their successful incorporation into dairy matrices.

To date, various dairy products have been fortified with polyphenols (Serafini et al., 2009; Karaaslan et al., 2011; Rashidinejad et al., 2013) in order to improve the nutritional value of the final product. However, there is still limited information about the effect of dairy product processing on milk-polyphenol mixtures and final product properties. Yoghurt and other fermented milk products are one of the most popular dairy product categories worldwide due to their high nutritional value and health benefits (Oliveira et al., 2015). Acidified milk gel, produced by adding glucono-deltalactone (GDL), is an ideal model to develop understanding of how dairy processing operations impact on successful polyphenol addition during low pH dairy product production. Therefore, acidified milk gel was chosen as a suitable matrix to enrich with polyphenols in this study. Currently there are only a limited number of studies that have fortified acidified milk gels with polyphenols (Vega and Grover, 2011; Harbourne et al., 2011).

Thermal processing is one of the most commonly used treatments applied to dairy products to ensure their safety and to manipulate their physical properties. Thermalization of milk (85 °C for 30 min) is an important step in fermented milk product manufacturing. As well as ensuring microbiological safety the heat treatment significantly affects the water holding capacity and textural quality of the final product. The use of polyphenols as a food ingredient in dairy matrices will necessitate understanding of their effects on final product quality under typical processing conditions such as heat treatment and acid gelation. The outcome of this thesis will provide critical information for the food industry in terms of optimum processing techniques and strategies to improve the nutritional values, textural properties and physicochemical properties of dairy products during their shelf life.

1.2 RESEARCH OBJECTIVES

The main hypothesis of this study is that the incorporation of various phenolic compounds into milk before and after heat treatment would impact on the physical, functional and nutritional properties of acidified milk product.

Therefore, the major objectives of this research are:

1. Understand the effect of polyphenol addition, of various types, before and after heat treatment on the the total phenolic content, antioxidant capacity, casein micelle size & whey protein denaturation of milk and acidified gel samples enriched with polyphenols.

2. Evaluate the impact of stage of addition of polyphenols, of various types, to milk (i.e. before or after it is heat-treated) on the gelation kinetics and rheological properties of acidified milk gels.

3. Determine the impact of refrigerated storage (28 days) on the total phenolic content, antioxidant capacity, pH, syneresis, texture, and colour of acidified gel samples enriched with polyphenols.

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CHAPTER 2

LITERATURE REVIEW

2.1 POLYPHENOLS

In the plant kingdom, one of the most various and abundant plant metabolites are polyphenols. Polyphenols are produced by plants as secondary metabolites which provide the plants resistance against pathogens and predators by increasing food astringency (Bravo, 1998). The importance of polyphenols in human diet and their possible health benefits have led to an increase in the interest of these compounds in recent years (Gupta et al., 2013).

2.1.1 Classification

The basic chemical structure of polyphenols is an aromatic ring linked to hydroxyl groups. However, they show great structural diversity ranging from simple molecules (monomers) to polymers (Tiwari et al., 2013). Polyphenols can be classified into different groups according their origin, structure and physicochemical functions. Therefore, they can be subdivided into several groups based on the number of phenolic rings and structural elements which bind these rings (Gharras, 2009). The main polyphenol classes are: phenolic acids, flavonoids, tannins, stilbenes and lignans (**Figure 2.1**). Gallic acid is a phenolic acid, which belongs to hydroxybenzoic acid group (**Table 2.1**). Tannins have a much more complex structure than phenolic acids and there are mainly two groups: condensed tannins and hydrolysable tannins. Tannins are large water soluble compounds and have a molecular weight ranging from 500 to 3,000 daltons (Chung et al., 1998; Han et al., 2007). Hydrolysable tannins are composed of gallic acid and glucose molecules and condensed tannins are polymers of flavanols. Hydrolysable tannins can be divided into two groups based on their structures: ellagitannins and gallotannins (Han et al., 2007). Flavonoids are the largest and most ubiqutious groups of all plant phenolics. There are currently more than 8000 known phenolic structures and among them 4000 flavonoids have been identified

(Tsao, 2010). Many flavonoids may either be attached to sugar units (glycosylated or esterified) or free from sugars (acylated), which affects their absorption and bioavailability in the body (Tsao, 2010; Gupta et al., 2013).

2.1.2 Sources of Polyphenols

Polyphenols are commonly found in both edible and non-edible plants. The main sources of phenolic compounds in the human diet are vegetables, fruits, herbs, cereals, legumes, tea, coffee and wine (Dai and Mumper, 2010; Manach et al., 2004). Flavanols (catechin and proanthocyanins), anthocyanidins and their oxidation products are the most abundant flavonoids in the diet (Han et al., 2007). The sources of dietary polyphenols and their phenolic content are presented in **Table 2.1**.

In this thesis, two sources rich in polyphenols: green tea and white grape and two phenolic compounds: tannic acid and gallic acid were used. They differ in their molecular weight, pH, and the class of polyphenols that they contain. In addition, the interactions of phenolic rich extracts (green tea and white grape) with milk proteins would differ from the single phenolic compounds (tannic and gallic acids) under processing conditions. Therefore, the model system was set up with one concentration (1 mg ml^{-1}) based on the previous work (Han et al, 2011b) to compare the impact of selected phenolic rich extracts on the physical, functional and nutritional values of acidified milk gels with the impact of single phenolic compounds. These dried extracts/pure phenolics have been previously used at the same concentration as reported in this thesis in the fortification of dairy products (da Silva et al, 2015; Harbourne et al., 2011). It was reported that higher concentrations, for example 2 mg ml^{-1}, may cause adverse effects on the textural and sensory properties of final product (da Silva et al, 2015; Giroux et al., 2013).

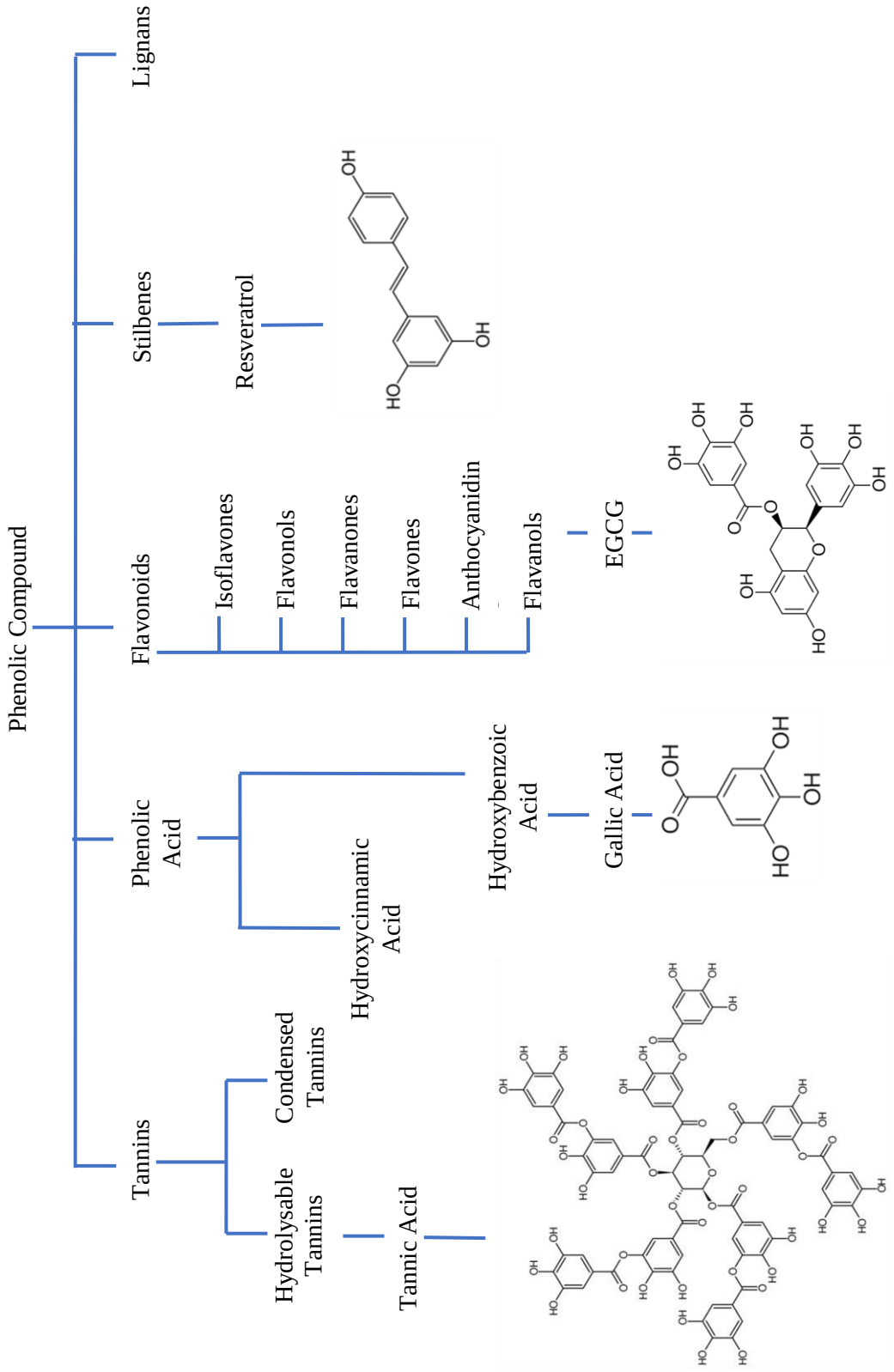

Figure 2.1 Classification of polyphenols and their chemical structure (Han et al., 2007).

Table 2.1 Polyphenol content in foods.

	Source[A]	Polyphenol content By wt or vol mg kg^{-1} Fresh wt (or mg L^{-1})
Flavonols	Yellow onion	350-1200
Quercetin	Curly kale	300-600
Kaempferol	Leek	30-225
Myricetin	Cherry tomato	5-200
	Broccoli	40-100
	Blueberry	30-160
	Black currant	30-70
	Apricot	25-50
	Apple	20-40
	Beans, green or white	10-50
	Black grape	15-40
	Tomato	2-15
	Black tea infusion	30-45
	Green tea infusion	20-35
	Red wine	2-30
Flavones	Parsley	240-1850
Apigenin	Celery	20-140
Luteolin	Capsicum pepper	5-10
Flavanones	Orange juice	215-685
Hesperetin	Grapefruit juice	100-650
Naringenin	Lemon juice	50-300
Isoflavones	Soy flour	800-1800
Daidzein	Soybeans, boiled	200-900
Genistein	Miso	250-900
Glycitein	Tofu	80-700
	Tempeh	430-530
	Soy milk	30-175
Monomeric flavanols	Chocolate	460-610
Catechin	Beans	350-550
Epicatechin	Apricot	100-250
	Cherry	50-220
	Grape	30-175
	Peach	50-140
	Blackberry	130
	Apple	20-120
	Green tea	100-800
	Black tea	60-500
	Red wine	80-300
	Cider	40
Anthocyanidins	Aubergine	7500
Cyanidin	Blackberry	1000-4000
Pelargonidin	Black currant	1300-4000
Peonidin	Blueberry	250-5000
Delphinidin	Black grape	300-7500
Malvidin	Cherry	350-4500
	Strawberry	150-750
	Red wine	200-350
	Plum	20-250
	Red cabbage	250
Hydroxybenzoic acid	Blackberry	80-270
Protocatechuic acid	Raspberry	60-100
Gallic acid	Black currant	40-130
p-Hydroxybenzoic acid	Strawberry	20-90

[A]Source: (Gharras, 2009)

2.1.2.1 Green Tea

After water, tea is one of the most consumed beverages in the world due to its refreshing, stimulant and remedial effects. The three most commonly consumed variants of tea are green, black and oolong (Peterson et al., 2005; Wang et al., 2000). Although those three tea types are obtained from same plant *Camellia sinensis*, their manufacturing processes vary. Green tea (unfermented product) is produced by steaming and drying the fresh tea leaves to inactivate the enzyme polyphenol oxidase. However, fresh tea leaves are partially fermented to produce oolong tea and fully fermented for black tea before steaming and drying (Dubeau et al., 2010; Zaveri, 2006).

The chemical composition of green tea is a complex mixture of phenolic compounds (30% dry weight), fibre (26%), protein (15-20 % dry weight, mainly enzymes), amino acids (1-4% dry weight), carbohydrates (5-7% dry weight) and other trace minerals (Cabrera et al., 2006). A comparison between the chemical composition of green tea and black tea is shown in **Table 2.2**. The main phenolic compounds in green tea are flavan 3-ols also known as catechins, a class of flavonoids. Catechins are colourless, water soluble and astringent. The catechins present in green tea include (+)-catechin (C), (-)-epicatechin (EC), (-) -epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG) (da Silva Pinto, 2013; Wang et al., 2000). The structure of EGCG was presented in **Figure 2.1**. Green tea contains more catechins than oolong and black tea due to the absence of fermentation during manufacture. The majority of antioxidant activity and potential health promoting effects of green tea has been attributed to EGCG which comprises more than 50% of the catechins in green tea (Dubeau et al., 2010; Cabrera et al., 2006). Green tea is highly available around the world and a relatively inexpensive source of polyphenols. This may allow to produce cost effective food products containing green tea extract (Lorenzo and Munekata 2016; Yilmaz 2006). Consumer interest has been increasing to green tea extract

fortified products in recent years. Therefore, green tea extracts have been added to several food products (meat and poultry products, dairy products, beverages, cereals, cakes) as natural antioxidants to increase their shelf life by retarding lipid oxidation, which might improve the quality, flavour and nutritional value of the products (Senanayake, 2013; Yilmaz, 2006).

Table 2.2 Mean Composition (% dry weight) of Green tea and Black Tea (and Its Infusion).

Compound [B]	Green Tea	Black Tea	Infusion*
Proteins	15	15	trace
Aminoacids	4	4	3.5
Fibre	26	26	0
Other carbohydrates	7	7	4
Lipids	7	7	trace
Pigments	2	2	trace
Minerals	5	5	4.5
Phenolic compounds[1]	30	5	4.5
Oxidised phenolic compounds[2]	0	25	4.5

[B] (Cabrera et al., 2006)
* Black tea; infusion time: 3 min.
[1] Especially flavonoids.
[2] Especially thearubigins and theaflavins

2.1.2.2 White Grape

Grapes (*Vitis vinifera*) are one the most popular, widely consumed fruits in the word, with around 60 species. This fruit is used to produce various economically important products such as wine, juice, vinegars, jams and raisins (Kammerer et al., 2004). The main component

of grapes is phenolic compounds after carbohydrates and acids. Juice, pulp, skins, and seeds contain 5%, 1%, 30%, and 64% of total phenolics, respectively (Yang and Xiao, 2013). The most abundant polyphenols in white grapes are flavanols including both monomers (epicatechin, catechin, gallocatechin, epigallocatechin, and epicatechin 3-gallate) and oligomers or polymers (procyanidins known as condensed tannins) (Yilmaz and Toledo, 2004). The flavanols are responsible for astringent and bitter tastes in grapes. White grape also contains phenolic acids, such as gallic acid, and stilbenes, such as resveratrol (**Figure 2.1**). However, white grapes lack anthocyanins which are responsible for the colour of red grapes. Grape extracts are accepted as generally recognized as safe (GRAS) by US Food and Drug Administration since 2003 (da Silva et al., 2015). The extracts of grape byproducts such as pomaces, seeds, skins, stems and callus have been added into several food products (Sanchez-Alonso et al., 2008; Karaaslan et al., 2011; da Silva et al., 2015) due to their rich antioxidant capacities (Anastasiadi et al., 2010) and the potential health benefits of grape phenolics (Yang and Xiao, 2013).

2.1.3 Health Benefits

Epidemiological studies suggested that regular dietary intake of polyphenols has been linked to a reduction in the risk of several chronic diseases (McDougall, 2017; Vauzour et al., 2010), including cardiovascular disease, cancer, Alzheimer's (Hartman et al., 2006) diabetes and osteoporosis (Scalbert et al., 2005). Moreover, studies have reported that polyphenols have strong antioxidant capacities (Han et al., 2007), which enable the quenching of free radicals produced constantly by the human body during mitochondrial electron transfer (Wootton-Beard and Ryan, 2011). Many of the health benefits of polyphenols have been attributed to the antioxidant capacity of phenolic compounds. However, others claim that there have not been adequate human studies showing the relationship between polyphenol antioxidant properties and their positive effect on diseases

(EFSA, 2010; Azzi et al., 2004). The benefits of polyphenols are related to the amount consumed and their bioavailability (Manach et al., 2004). In many cases the doses or concentrations of polyphenols used in animal studies or *in vitro* were much higher than those which are typical of human dietary intake (Scalbert et al., 2005).

Green tea has been consumed as a medicinal and healthy beverage since ancient times. Green tea contains many components which contribute to human health including caffeine, theophylline and essential oils (Cabrera et al., 2006). However, interest in green tea has increased in recent years due to its content of polyphenols, which are strong antioxidants and are associated with various health promoting effects (Lorenzo and Munekata, 2016). Numerous studies have reported that the phenolic compounds of green tea have antimutagenic, antibacterial, antidiabetic, anti-inflammatory, and anti-aging properties (da Silva Pinto, 2013; Cabrera et al., 2006). Polyphenols in green tea may protect cells against cancer and slow the rate of cellular ageing. Therefore, green tea has remedial potential for life-style related diseases including cancers of lung, stomach, kidney, prostate, breast, Parkinson's disease and Alzheimer's disease (Ullah et al., 2016; Zaveri, 2006). In addition, a correlation was found between green tea consumption and reduction of mortality due to cardiovascular diseases (da Silva Pinto, 2013). The regular consumption of grape and grape products can positively affect the risk of cancers, cardiovascular disease, hypertension, cholesterol, age related cognitive decline and diabetes, these health benefits have been attributed to the flavonoid compounds in grape (Rasines-Perea and Teissedre, 2017; Yang and Xiao, 2013). In addition, grape seeds, grape juices and grape skins may protect and strengthen the immune system, improve oral health and have antiviral activity (Farahat et al., 2017; Vislocky and Fernandez, 2010). Furthermore, the health benefits of wine associated with grape polyphenols have been extensively studied in the recent years (Draijer et al., 2015; Liberale et al., 2017).

2.2 MILK

Milk is secreted by all mammals to feed their neonates and it is essential for their growth. Milk composition varies widely based on the species, breed, stage of lactation and diet (Farrell et al., 2006). Bovine milk is a colloidal suspension consisting of approximately 87.7% water, 3.4% protein, 3.7% fat, 4.8% lactose and 0.7% minerals. The protein fraction of milk consists of caseins (α_{s1}-, α_{s2}-, β- and κ-casein) and whey proteins (β-lactoglobulin, α-lactalbumin and bovine serum albumin), accounting for 80% and 20% of milk protein, respectively (Fox, 1989). Milk is a polydisperse system in which fat globules are the largest particles and relatively easy to separate by centrifugation or gravity. After the separation of fat the remaining fraction referred to as milk plasma. Milk is rich in various minerals such as potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), chlorine (CI^-) and phosphate. The pH of bovine milk is around 6.5-6.7 at 25 ºC. Lactose is also known as milk sugar and is composed of glucose and galactose (Singh et al., 2014). The functional properties of milk (such as gel formation) are of great importance for dairy processing e.g. in the production of yoghurt and cheese (Robinson, 1986).

2.2.1 Caseins

The caseins are phosphoproteins and they precipitate from milk at pH 4.6, which is the isoelectric point of caseins. There are four principal casein fractions α_{s1}-, α_{s2}-, β- and κ-casein, in approximate proportions 38%, 10%, 36% and 13%, by weight. All caseins are relatively hydrophobic. Caseins contain a high amount of proline in their primary structure, which limits the formation of its secondary structure and gives caseins an open flexible conformation. The hydrophobic and hydrophilic groups in caseins are not evenly distributed, giving the caseins an amphipathic structure. The four casein fractions have differences in hydrophobicity, proline content, degree of phosphorylation and glycosylation (Fox, 1989).

α_{s2}- casein is the most hydrophilic of the four fractions due to its negatively charged N-terminal and positively charged C-terminal. It has higher phosphorylation and lower proline than other fractions. β-casein is the most hydrophobic of the caseins. The order of hydrophobicity of caseins is $\beta > \alpha_{s1} > \kappa > \alpha_{s2}$. α and β caseins are calcium sensitive and they precipitate in the presence of calcium ions. However, κ-casein remains soluble in the presence of calcium due to the absence of phosphoseryl residue clusters and presence of a glycosidic moiety which includes galactose, galactosamine and N-acetylneuraminic acid (McSweeney and Fox, 2013; Dalgleish, 1998; Dalgleish, 2011).

Around 95% of the casein exists in milk as colloidal particles known as micelles. The mean diameter of these micelles is approximately 150 nm (range: 50-500nm). Negatively charged casein micelles contain approximately 94% protein and 6% of minerals, mainly Ca^{2+}, phosphate, Mg^{2+} and citrate which are collectively called colloidal calcium phosphate (CCP). These micelles are highly hydrated; per kilogram of protein they consist of 3.5 kg water (Fox, 1989; Dalgleish, 2011). The technological properties of milk are associated with stability and structure of the casein micelles. The structure of the casein micelle has been discussed for many years and there are many models proposed, including submicellar, nanocluster and dual binding models of the casein micelle. However, it is widely accepted that the interior of the casein micelle is relatively hydrophobic and mainly consists of α_{s1}-, α_{s2}-, β-casein. The hydrophilic surface of the micelle is composed of κ-casein with the hydrophilic C-terminal (called casein macropeptide or CMP) protruding from the micelles giving them a hairy appearance. One model proposes that the casein micelle is made up of submicelles, which are linked together by colloidal calcium phosphate (CCP) nanoclusters and hydrophobic bonds (**Figure 2.2**) (Dalgleish, 2011). CCP is responsible of the micelle stability and dissociation of micelles occurs with acidification, proteolytic enzymes or EDTA (Fox,1989; Dalgleish and Corredig, 2012; McSweeney and Fox, 2013).

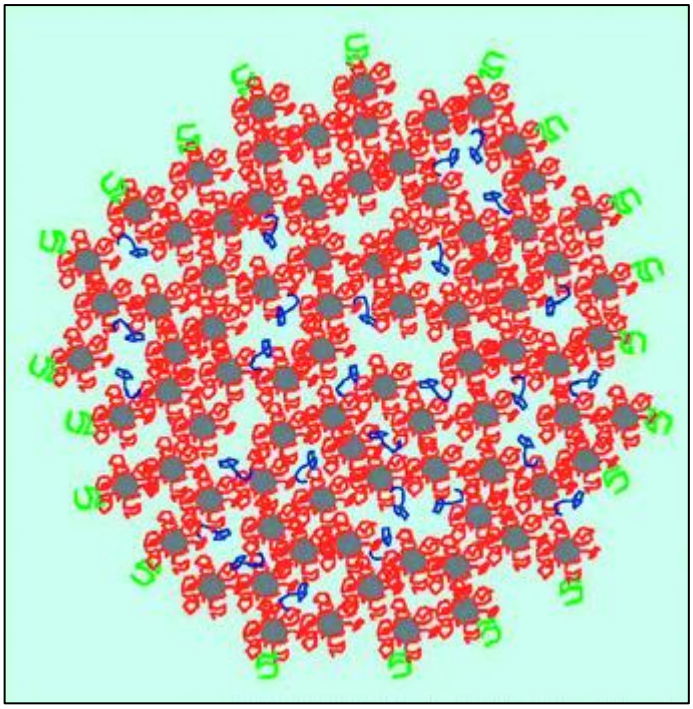

Figure 2.2 General structure of casein micelle based on the theory by (Dalgleish, 2011); calcium phosphate nanoclusters (grey) attached to caseins (red) and the surface-located κ-casein (green), ‘‘hydrophobically bound’’ β-casein in blue.

2.2.2 Whey Proteins

Whey proteins are the second major protein group in milk protein, known as serum proteins or non-casein nitrogen. There are four principle fractions of whey proteins; 50% β-lactoglobulin (β-LG), 20% α-lactoalbumin (α-LA), 3% bovine serum albumin (BSA) and 10% immunoglobulins (Ig). They are globular proteins, non-phosphorylated, insensitive to calcium ions, soluble at pH 4.6 and susceptible to heat treatment. The structure of whey proteins is stabilised by intramolecular disulphide bonds between cysteine residues. β-LG and α-LG are important whey proteins and they have technological significance (Fox, 1989). The molecular weight of β-LG is 18.3 kDa and it has an isoelectric point (pI) of 5.2. β-LG has two disulphide bonds and a free thiol group, which plays an important role in the self-aggregation and association with other proteins containing disulphide groups after heat

treatment. α-LA is second most abundant whey protein with a molecular weight of 14.2 kDa. It has four disulphide bonds and has no free thiol groups. It is a calcium metalloprotein which positively impacts its thermal stability. In general, α-LA is more heat resistance than β-LG. At temperatures above 60 ºC, whey proteins easily denature, after denaturation β-LG can interact with other cysteine containing proteins, such as κ-casein, α-lactoglobulin and bovine serum albumin (McSweeney and Fox, 2013).

2.2.3 Effect of Heat Treatment on Milk Proteins

The heat treatment of milk is a critical step in the manufacturing of most dairy products. It is applied for elimination of harmful microorganisms (pathogens), inactivation of enzymes and modification of final product quality. The temperature and holding time of heat treatment varies dependant on the product being manufactured. For instance, milk used for yoghurt production is usually heated at 80-85 ºC for 30 min or 90-95 ºC for 5 min (Tamime and Robinson, 1999). The heat treatment of milk has a considerable effect on milk proteins.

As mentioned above, heat treatment of milk above 60ºC causes denaturation of whey proteins and results in aggregation of whey proteins (Dalgleish and Corredig, 2012). The rate of whey protein denaturation is depended on various factors such as temperature, heat treatment time, pH, the structure, concentration and heat stability of the protein. Several reaction steps take places in the denaturation and aggregation of whey proteins. In the first step of denaturation, the oligomer dissociates into monomers e.g. the β-LG dimer to native β-LG monomers. In the second part, monomeric proteins can unfold. Both of these denaturation steps are reversible. In the case of more extensive denaturation, the unfolding process is followed by exposure of reactive amino side-chain groups (sulphide groups) which are otherwise buried in protein structure. The irreversible aggregation reaction occurs with the attachment of unfolded whey proteins to other unfolded whey proteins (β-LG / β-

LG or β-LG / α-LA) or casein micelles (Singh et al., 2014). Exposure of cysteine residues in unfolded whey proteins result in the formation of disulphide bonds between proteins. In terms of the dairy industry, the irreversible aggregation processes significantly affect the functional properties of dairy products (Roefs and Dekruif, 1994). Unlike whey proteins, casein micelles are relatively heat stable molecules at the normal pH of milk ~6.7, however the main effect of heat treatment on casein micelle is dissociation. The interior of casein micelle may be altered during heating of milk at pH higher than 6.9, resulting in liberation of α_s- and β-caseins into serum (Singh, 2004; Dalgleish and Corredig, 2012).

The interactions of denatured whey proteins with other milk proteins are relatively complex and numerous reaction pathways have been suggested (Vasbinder and de Kruif, 2003; Donato et al., 2007; Anema, 2008). However, the interactions between β-LG and κ-casein is similar in different model systems. β-LG must unfold to expose its sulphydryl groups and then intermolecular disulphide bonds are formed with κ-casein. It is generally accepted that β-LG / κ- casein complex is first formed then α-LA may attach to β-LG. This is possibly due to lack of a free thiol group in α-LA. Thiol-disulphide bonds, hydrophobic interactions as well as non-covalent interactions are responsible for formation of aggregates between milk proteins. (Donato and Guyomarc'h, 2009; Donovan and Mulvihill, 1987). The location of formation of the interaction between whey protein / κ-casein is still the subject of some debate. Some reports suggested that κ-casein dissociates from the micelles in the early stage of heating and denatured whey proteins then interact with κ-casein either in the serum part or on the micelles (Guyomarc'h et al., 2003; Anema, 2008). However, others reported that denatured whey protein / κ-casein interactions first occur on the surface of casein micelle and then dissociates as serum complexes (Donato and Dalgleish, 2006; Donato and Guyomarc'h, 2009).

After heat treatment, the mixture of three types of whey proteins exists in the milk matrix: native whey proteins, soluble whey protein aggregates in serum and aggregates that have formed an attachment of casein micelles (Donato and Guyomarc'h, 2009). The pH of milk during heating has a considerable effect on the attachment of denatured whey proteins to the casein micelle. Studies found that denatured whey proteins tend to associate with casein micelles when milk was heated at a pH below 6.7, whereas denatured whey proteins have a preference to form soluble aggregates in the serum at pH above 6.8 (Anema and Li, 2003a; Anema and Li, 2003b). Kethireddipalli et al. (2010) reported that milk heated at pH 6.3, 6.7 and 7.1 at 90 ºC for 10 min had 82.2%, 30% and 0-5 % of denatured proteins associated with casein micelle, respectively. The attachment of more denatured whey proteins to casein in milk heated at lower pH increased micelle size as compared to milk heated at higher pH (Anema et al., 2004). Temperature is another important factor that influences whey protein denaturation, attachment of whey protein to casein micelle and particle size. At higher temperatures, whey protein denaturation and casein micelle size increased (Vasbinder and de Kruif, 2003).

2.2.3.1 Assessment of the Denaturation of Whey Proteins in Milk and Casein Micelle Size

Whey protein nitrogen index (WPNI) is a rapid method used to measure the levels of whey protein denaturation. It was developed to measure the native whey protein levels of milk powders to check their suitability for applications in the bakery industry (Harland and Ashworth, 1947). In the WPNI, milk is saturated by salt to precipitate the denatured whey proteins and the casein. The native whey proteins in the supernatant is analysed for protein content. Hydrochloric acid (HCI) is added to supernatant to develop a turbidity depending on the concentration of native whey proteins. However, considerable variability in the degree of turbidity was observed for the samples with similar levels of whey protein denaturation

(Harland and Ashworth, 1947). After that, a dye-binding method was combined with original WPNI method to measure total protein of milk, thus giving more accurate and reliable results (Sanderson, 1970). Other methods used to determine whey protein denaturation include polyacrylamide gel electrophoresis, capillary electrophoresis, differential scanning calorimetry, and high performance liquid chromatography (HPLC). WPNI method is still commonly used in the industry to determine the level of whey protein denaturation for milk powder products (Singh et al., 2014).

There are various techniques used for particle size analysis such as dynamic light scattering (DLS), nanoparticle tracking analysis, scanning electron microscopy, size exclusion chromatography and cell electrophoresis. DLS, sometimes referred to as quasi-elastic light scattering or as photon correlation spectroscopy, is a commonly used technique to measure particle size of casein micelle. It is user-friendly method and provides accurate and consistent results for protein samples in a short period (Hristov et al., 2016). The principle of light scattering is described in **Figure 2.3**. A light is sent via a laser or monochromaric light source into sample and the scattered light is collected by a detector and transferred to a computer to analyse the signals (Alexander and Dalgleish, 2006).

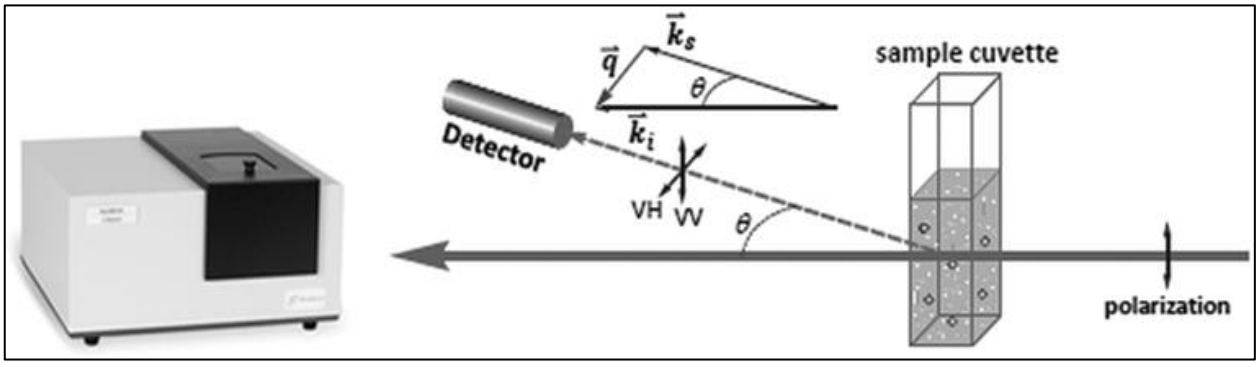

Figure 2.3 Principle of dynamic light scattering (Hristov et al., 2016).

2.2.4 Acid Gelation of Milk

The functional properties of milk, which include emulsification, foaming, solubility, water-holding capacity, rheological behaviour and gelation, have been reviewed extensively (McSweeney and Fox, 2013). Gelation of milk is utilised by the dairy industry to produce various products such as cheese, yoghurt and acidified milk products. Milk can be acidified by using bacterial cultures or addition of chemical acids such as HCI or glucono-deltalactone (GDL). GDL is accepted as a GRAS (Generally Regarded as Safe) food additive with E number E575 (Food Standards Agency, 2016). In the food industry, it is used as an acidifier, curing and leavening agent. As compared to other acidulants (such as lactic acid), GDL is preferred due to its slow hydrolysis and mild flavour in the product (Sun, 2005). GDL has been marketed for use in the production of meat products such as salami (Feiner, 2016), cheese (PMP Fermentation Products, Inc., n.d.) and tofu (Johnson et al., 2008). Acidified milk gels are produced by addition of GDL and hydrolysis of GDL to gluconic acid lowers the pH of milk to 4.6 to allow aggregation of casein (Lucey and Singh, 1997).

During acid gelation, physicochemical properties of casein micelles change considerably and those changes not only impact the surface of the micelles, but also the interior structure of caseins. As the pH drops from 6.7 to 4.6 the casein micelles are disrupted, the CCP is dissolved, and micelle integrity is lost (Dalgleish and Corredig, 2012). The changes in the casein micelles during acidification of milk can be explained through three pH regions from 6.7 to 4.6. Initially as the pH drops from 6.7 to ~6.0 the net negative charge on the casein micelle is reduced and electrostatic repulsion is also reduced. At this stage only small amounts of CCP is dissolved and the structure of casein micelles are not changed. In the range from pH ~6.0 to ~5.0 the reduction in the net negative charge and electrostatic repulsion continues. As the pH decreases, the κ-casein hairs on the micelle surface may shrink or collapse. At pH ~5.0, the CCP in the casein micelle is completely dissolved. Close

to isoelectric point of casein, the net negative charge on casein micelles declines further, which allows the formation of van der Waals forces and hydrophobic interactions. Eventually, casein micelles aggregate due to charge neutralisation and aggregated caseins link together through chains and clusters to form three dimensional networks (Lee and Lucey, 2010; Singh et al., 2014).

2.2.5 Rheological Properties of Acid Milk Gel

Food rheology is a study related to deformation and flow of food products. Acidified milk gel products have viscoelastic properties, which means that samples are not completely solid or liquid. The tests to measure the rheological properties of acid milk gels can be mainly divided into two: non-destructive (small deformation) and destructive (large deformation) tests. Non-destructive tests, including oscillatory stress and strain, are used to measure the rheological properties of acid milk gels during gel formation without damaging the gel structure. In the strain controlled version of this test the sample is exposed to a sinusoidally oscillating strain and this results in a sinusoidally oscillating stress response. Some of the main parameters generated from these responses include the elastic or storage modulus (G′), the viscous or loss modulus (G″) and the loss tangent (tan δ). G′ is a measure of the energy stored per oscillation cycle, and indicates the solid-like properties of the sample. G″ is a measure of the magnitude of energy lost per cycle of deformation and represents the liquid like properties of a sample. Tan δ is the ratio of viscous modulus to elastic modulus (G″/ G′) and reflects the type of viscoelastic properties of a sample. During gel formation, G′ gradually increases due to the formation of bonds between milk proteins and rearrangements in the protein network. In acid milk gels, G′ value is always higher than G″ indicating a solid like behaviour (Lucey and Singh, 1997).

Destructive or large deformation tests measure resistance of the gel to various processes such as stirring and some shearing operations; therefore the results of these types of test can be well correlated with the sensory attributes (Lee and Lucey, 2010). Constant shear rate and strain sweep tests are frequently used destructive tests. The rheological parameters generated from these tests are fracture stress (σ) which is the shear stress at which gel network start to break down and fracture strain (γ) which is defined as the point when shear stress begins to decrease (Lucey and Singh, 1997; Lee and Lucey, 2010). A low value for fracture stress refers to a weak or soft gel and a low value of fracture strain indicates a short or brittle gel structure. The fracture stress of the gel is dependent on protein-protein bonds, the number of bonds per cross-section of the strand, and relaxation times for the network bonds. Moreover, the heat treatment of milk before acid gelation and the degree of temperature during heat treatment are other important factors that affect the fracture stress and fracture strain (Lucey et al., 1997).

2.3 INTERACTIONS BETWEEN MILK PROTEINS AND POLYPHENOLS

Polyphenols and milk proteins interact via covalent and non-covalent bonds and these interactions depend on several factors such as pH, temperature, types of protein and their concentrations. The optimal pH for precipitation of protein-polyphenol complexes was detected as close to the isoelectric point of protein (Ozdal et al., 2013). (Shpigelman et al., 2010) studied the effect of temperature on the binding affinity of EGCG to β-LG. The interactions between EGCG and β-LG were higher at 70-80 °C as compared to room temperature. Other factors that affect the interactions include the type and structure of the polyphenols (methylation, hydroxylation, hydrogenation, glycosylation) and molecular weight, whereby larger polyphenols have a higher binding affinity to proteins (Xiao et al., 2011; Dubeau et al., 2010; Spencer et al., 1988). The types and structure of the milk protein

also affects the interaction, for example, proline-rich proteins (casein) have high relative affinity for polyphenols (Spencer et al.,1988).

Studies suggest that the major interactions between milk proteins and polyphenols are hydrogen bonding and hydrophobic interactions (non-covalent interactions). Covalent bonds are also formed between polyphenols and proteins; however, they are not as extensively studied due to the lack of suitable methods available to study these interactions (Ozdal et al., 2013). Hydrogen bonds can form between peptide carbonyl and phenolic hydroxyl groups, whereas hydrophobic interactions can form between hydrophobic amino acid residues and aromatic rings of the polyphenols (Haratifar, 2012; Ozdal et al., 2013).

Several studies have been conducted recently to understand how these interactions effect proteins structure, functional properties and phenolic contents and antioxidant capacities, which are discussed below.

2.3.1 Effects of Protein-Phenolic Compounds Interactions on Proteins

Numerous studies have focused on the impact of protein-polyphenol interactions on both pure milk proteins (e.g. β-LG, alpha-casein) and milk proteins in the liquid milk. In this section, the effects of polyphenol-milk protein interactions will be examined with a focus firstly on whey protein, followed by casein proteins and concluding with liquid milk. Kanakis et al. (2011) investigated the interactions between tea polyphenols (catechin (C), epicatechin (EC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG)) and bovine β-LG. The interaction between tea polyphenols and β-LG was driven by hydrophobic interactions and hydrogen bonds. These interactions caused the alteration of the secondary structure of β-LG with an increase in β-sheet and α-helix content. They concluded that the structural changes of β-LG can be a major factor in determining the antioxidant capacity of tea polyphenols in milk. Wu et al. (2013) reported interactions between EGCG and β-LG

and binding abilities between the EGCG and β-LG can be increased by preheating treatment of protein and addition of emulsifiers. Recently, Al-Hanish et al. (2016) reported non-covalent interactions between bovine α-LA and EGCG and those interactions decreased α-helix and increased β-sheet content. Other studies have examined the effect of resveratrol, which is rich in grapes and red wine, on β-LG (Ghorbani Gorji et al., 2015; Liang et al., 2008). They found that the interaction between resveratrol-β-LG had no major effect on secondary structure of the protein but the tertiary structure was partially disrupted.

With regards to casein structure, Hasni et al. (2011) investigated the interaction of α and β-caseins with tea polyphenols (C), (EC), (EGC) and (EGCG)). It was reported that tea polyphenols weakly bind to casein by both hydrophilic and hydrophobic interactions, but predominantly hydrophobic interactions. The interactions between β-casein and polyphenols were stronger than those between α-casein and polyphenols due to the β-caseins more hydrophobic structure. The secondary structure of casein protein was modified with a major decrease in β sheets and α-helices and an increase in randomness of β sheets and α-helices resulted in more protein unfolding. Bourassa et al. (2013) studied the binding sites of resveratrol, genistein and curcumin with α and β caseins. They determined that polyphenols bond to casein through hydrophilic and hydrophobic interactions and resulted in a decrease in α-helix structure of casein with a partial destabilisation of protein.

Ye et al. (2013) studied the interactions of black and green tea polyphenols with whole milk. Casein micelles tended to bind highly polymerized polyphenols, whereas whey proteins were likely to bind smaller molecules. The interactions between tea polyphenols and milk proteins was dependent on the type of tea which affected the conformation of milk proteins. Overall, the studies indicated that polyphenol-milk protein complexation caused the change of secondary and tertiary structure of proteins and the effect of polyphenols on

the structure of milk proteins depends on the type of polyphenols and the type of milk proteins.

Milk proteins have several functional properties such as solubility, binding, gelation, emulsification and foaming. The interactions between polyphenols and milk proteins can influence the functional properties of milk. Prigent et al. (2009) found that the effects of non-covalent interactions between milk proteins and oligomeric procyanidins on the protein solubility and foam properties was dependent on pH, temperature and ionic strength. It was reported that procyanidins of an average degree of polymerization decreased the protein solubility, whereas they played a positive role in foam stability. Furthermore, caffeic acid increased the heat stability of milk at 140 ºC and had no effect on rennet coagulation time (O'Connell and Fox 1999). The thermal stability of BSA increased with binding of ferulic acid (Ojha et al., 2012). Overall, depending on the protein present and the concentration and type of polyphenol added, protein solubility can be decreased while thermal stability and foaming properties of proteins can increase.

2.3.2 Impact of Protein-Phenolic Compounds Interactions on Polyphenolic Content and Antioxidant Capacity

Numerous in vitro and in vivo studies have been conducted to determine the total antioxidant capacity of phenolic compounds in the presence of milk proteins. In recent years, some spectrophotometric assays have been applied and most popular are 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, oxygen radical absorbance capacity (ORAC), copper reducing antioxidant activity (CUPRAC), 2,2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), ferric reducing antioxidant power (FRAP) and trolox equivalence antioxidant capacity (TEAC). There is no one official standardized method, but the methods to measure antioxidant capacity can be mainly divided into two groups depending on reaction

mechanism: electron transfer (ET) based methods such as TEAC, FRAP, Folin Ciocalteu reagent (FCR) and hydrogen atom transfer based methods (HAT) such as oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP) (Prior et al., 2005; Ozdal et al., 2013). FRAP assay, which is ET based, is a simple, speedy, robust and inexpensive method. FRAP measures the reduction of the Fe(III)-complex of 2,4,6-tripyridyl-s-triazine (TPTZ) by antioxidants to the intensely coloured ferrous Fe(II)-TPTZ at a low pH (3.6), which shows maximum light absorption at 593 nm (Liang and Kitts, 2014; Ozyurek et al., 2011). The FRAP assay is a suitable method to detect antioxidant capacity of water soluble polyphenols (Biskup et al., 2013). In addition, sugars and citric acids are reported as common interferences with FRAP (Prior et al., 2005).

The effect of milk on the antioxidant capacity of polyphenols has been studied both in vitro and in vivo. However, the results have been contradictory and were classified as negative, neutral and positive effects (Rashidinejad et al., 2017). A high proportion of research has investigated the effect of milk addition on the antioxidant capacities of tea catechins. In the world, especially western countries, tea is consumed by adding milk in order to decrease the astringent taste (Ryan and Petit, 2010). Studies generally found that milk had an inhibitory effect on antioxidant capacity of tea polyphenols (Egert et al., 2013; Korir et al., 2014). Those decreasing effects were attributed to interactions between milk proteins and tea catechins as binding decreases the amount of free antioxidants (Arts et al., 2002; Moser et al., 2014). Moreover, milk had a decreasing effect on the antioxidant capacity of cocoa (Gallo et al., 2013), coffee (Liu et al., 2016) and blueberry (Serafini et al., 2009) polyphenols.

Other studies reported that the addition of milk had no significant effect on antioxidant capacities of coffee and tea (Dupas et al., 2006; Kyle et al., 2007). Milk is a good source of antioxidants and might contribute to the antioxidant activity. A neutral effect might occur

when the addition of milk weakly affected the antioxidant capacity of polyphenols (Rashidinejad et al., 2017). Interestingly, Xie et al. (2013) found that after in vitro digestion the content of catechin was higher in green tea containing milk in comparison to green tea without milk. In addition, Almajano et al. (2007) reported the antioxidant capacity of bovine serum albumin increased in the presence of EGCG after 7-day storage at 30 ºC. Despite the positive effect of milk antioxidant activities of polyphenols, the mechanisms of the results are not fully understood yet. Overall, studies indicated contradictory results regarding the effect of milk on antioxidant capacities of polyphenols, which could be related the type of polyphenol utilised. Therefore, in the current study four different sources of polyphenols were used to understand the effect of milk on the antioxidant activity.

In terms of total phenolic content (TPC) of polyphenols in milk, there are few studies which examined the effect of milk addition on the TPC of polyphenols. Sharma et al. (2008) and von Staszewski et al. (2011) reported that whey proteins and milk had a decreasing effect on the TPC of green and black tea polyphenols, respectively. The impact of dairy processing on the TPC of polyphenols will be discussed in more detail in **section 2.4**.

2.3.2.1 Extraction of Polyphenols from Dairy Matrix

In the literature, various methods have been reported to extract polyphenols from plants and fruit such as maceration, infusions and soxhlet extraction (Tiwari et al., 2013). However, there have been limited studies regarding extraction methods of polyphenols from milk-based products. Polyphenols in milk based products generate a very complex mixture due to the strong protein-polyphenol interactions (**section 2.3**). For many analytical methods, a deproteination step prior to analysis may be needed to avoid precipitation of proteins during chemical (TPC and antioxidant capacity) and chromatographic analysis (HPLC, LC-MS) (Ferruzzi and Green, 2006). The presence of proteins in extracted polyphenol samples can

result in the column clogging and interference of UV and MS signals of the target analytes (Redeuil et al., 2009). The extraction method used to separate polyphenols from the protein-containing dairy matrix is very important in order to recover the maximum level of polyphenols. Ferruzzi and Green (2006) examined the effectiveness of enzyme assisted extraction on the recovery of catechins from milk in comparison with methanol treatment and acid precipitation. They found that pepsin treated samples had a higher total catechin recovery (89-102%) in comparison with methanol deproteination (78-87%) and acid precipitation (20-74%). However, further study found this method of separating catechins from protein was not suitable for subsequent analysis of the polyphenols by LC-MS as it resulted in interference from peptides (Redeuil et al., 2009). Redeuil et al. (2009) suggested a new approach for extraction of polyphenols from dairy matrices. The authors used acidified methanol containing stable isotope labelled internal standards as the extraction solvent to precipitate proteins and this step was followed by ultrafiltration to remove remaining proteins. In addition to this, Redeuil et al. (2009) successfully applied this method to yoghurt and ice cream containing various polyphenols and the quantification of polyphenols was determined by reversed-phase liquid chromatography–tandem mass spectrometry. Based on this information about extraction methods, acidified methanol followed by filtration/centrifugation is a good way to separate polyphenols from proteins in dairy matrices in order to analyse the phenolic compounds.

2.4 IMPACT OF PROCESSING ON POLYPHENOL FORTIFIED DAIRY PRODUCTS

2.4.1 Yoghurt and Acidified Milk Gel

Yoghurt is a fermented dairy product and an important part of the human diet all over the world due to its positive health benefits (Salas-Salvado et al., 2017). In the dairy industry, synthetic additives are added to products during manufacturing processes to improve

characteristic properties of the final products. Those additives include preservatives (antioxidants, antimicrobials, antibrowning), colouring, flavouring, texturizing agents and nutritional additives (Jia et al., 2014). In recent years, consumer demand is moving towards natural products. Therefore polyphenols, as strong antioxidants, have been added to various dairy products as natural food additives and bioactive compounds (Zoidou et al., 2014; Lee et al., 2016). Baydar et al. (2007) reported that natural antioxidants from grape seed extracts showed higher antioxidant activities than that of synthetic antioxidant of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).

In recent years, several types of yoghurt products such as low fat, drinkable, probiotic have been fortified with plant and fruit based polyphenols (Pelaes Vital et al., 2015; Sun-Waterhouse et al., 2013; Muniandy et al., 2016), therefore phenolic compounds have good potential for use in dairy products. The addition of grape extracts (Karaaslan et al., 2011), green and black teas (Jaziri et al., 2009), strawberry (Oliveira et al., 2015) and hazelnut skins (Bertolino et al., 2015) improved the TPC and antioxidant capacity of yoghurts. Chouchouli et al. (2013) observed a decrease in the antioxidant activity of yoghurts containing grape seeds after 4 weeks of refrigerated storage, whereas Muniandy et al. (2016) found that the antioxidant activity of yoghurt enriched with tea extracts remained stable over the storage period. Studies investigating the effect of polyphenols on yoghurt bacteria viability during refrigerated storage (Jaziri et al., 2009; Najgebauer-Lejko et al., 2011) found that tea supplementation had no effect on the number of lactic acid bacteria during 4 weeks at 4 ºC. Moreover, Tseng and Zha (2013) found that yoghurt samples containing wine grape pomace delayed lipid oxidation during refrigerated storage, hence improving their storage stability.

As mentioned above, studies have examined the effect of polyphenol addition on the nutritional values (TPC and antioxidant activity) and microflora of yoghurts during refrigerated storage. In addition to this, studies have looked at the effect of polyphenols on

the physiochemical properties of yoghurt such as pH, textural properties, colour and syneresis. The addition of date (Trigueros et al., 2012), peanut sprout (Lee et al., 2013), hazelnut skin (Bertolino et al., 2015) and oyster mushroom (Pelaes Vital et al., 2015) extracts which are rich in phenolic compounds on the physicochemical properties of yoghurt during refrigerated storage has been investigated. Najgebauer-Lejko et al. (2014) examined the textural properties of yoghurt enriched with green and black tea infusions and reported that yoghurt containing green tea had lower firmness and was less susceptible to syneresis than yoghurt containing black tea. Recently, Donmez et al. (2017) investigated the effect of refrigerated storage on the syneresis and rheological behaviour of set yoghurt containing green tea and green coffee powders.

Sun-Waterhouse et al. (2011) looked at the impact of addition of apple polyphenols before and after fermentation on drinkable yoghurt and suggested that addition of apple polyphenols before fermentation would increase the total extractable polyphenols and growth of starter culture. Avci et al. (2010) reported that addition of green tea extract to milk before heat treatment resulted in higher firmness and water holding capacity in the yoghurt sample as compared to green tea added after heat treatment. This demonstrated that the stage of polyphenol addition could impact on physical properties of dairy products and the stage of polyphenol addition could be more interesting to study further.

There have been few studies about the effect of polyphenol addition on acidified milk gels. Harbourne et al. (2011) studied the effect of tannic and gallic acids addition on the gel formation properties. It was found that the addition of tannic and gallic acids (up to 0.8% (w/w) increased the storage modulus (G′), which is a measure of the energy stored per oscillation cycle (in Pascal), and had no effect on syneresis compared to the control gel. Furthermore, the effect of tannic and gallic acids at a higher concentration (1%) was different. The addition of 1% gallic acid resulted in a significant decrease in G′ and a

significant increase in syneresis, whereas the addition of 1% tannic acid increased G′ value. This was due to 1% gallic acid addition causing a very fast acidification (from pH 6.50 to 5.25) of milk and not allowing for proper formation of the gel network. Vega and Grover (2011) investigated the effect of cocoa flavanols on the physicochemical properties of acidified milk gels. It was reported that when the flavanol levels increased from 0 to 2.5 mg g^{-1}, syneresis and gel elasticity (tan δ) significantly increased and decreased respectively. The large deformation test results showed that cocoa flavanol addition reduced the fracture stress and no effect on fracture strain. The poor mechanical properties of acidified milk gel containing cocoa flavanols were attributed to whey proteins/flavanol interactions. These studies indicated that the effect of polyphenols on the milk gels is dependent on the sources of polyphenols and their concentrations. Therefore, it is needed to investigate the effect of various polyphenols and the stage of polyphenol addition to milk (before and after heat treatment) on the rheological and physicochemical properties of milk gels.

2.4.2 Cheese

Cheese curd is produced by coagulation of milk casein using proteolytic enzymes. Cheese is then obtained by draining whey from the curd followed by a number of additional processing steps which vary according to the cheese being produced (Smit, 2003). Various phenolic compounds were incorporated to cheese curd to understand the effect of polyphenols on cheese making properties of milk and physical properties of cheese curd. For example, Han et al. (2011b) and Han et al. (2011a) added single phenolic compounds including catechin, epigallocatechin gallate (EGCG), tannic acid, homovanillic acid, hesperetin and flavone and natural crude compounds such as grape extract, green tea extract and dehydrated cranberry powder to cheese curd at a concentration of 0.5 mg ml^{-1} to examine the effect of polyphenols on enzymatic gelation kinetics and on texture characteristics of cheese curd respectively. Han et al. (2011b) observed that addition of phenolic compounds

influenced all gel-forming parameters, primarily due to the effect of the phenolic compounds on the pH of milk. Moreover, Han et al. (2011b) found that phenolic compounds showed different levels of retention in the cheese curd depending on their molecular properties and hydrophobicity. Han et al. (2011a) reported that cheese curds containing polyphenols decreased moisture content and the structure of curds was affected with the addition of crude phenolic compounds whereas the gel strength was not affected. da Silva et al. (2015) studied the addition of grape extracts (0, 0.1, 0.2, and 0.3% wt/vol) on the kinetics of milk clotting, milk gel texture and syneresis. Increasing concentrations of grape extracts resulted in a decrease in firmness and syneresis of milk gels. Haratifar and Corredig (2014) investigated the effect of interactions between tea catechins and casein micelles on the renneting functionality of milk. The formation of catechin-casein micelles affected the rennet induced gel formation depending on the concentration of EGCG. With a different objective than the other studies, Helal et al. (2015) examined the effects of gastro intestinal digestion on release of phenolic compounds from cheese curd and the antioxidant capacity of polyphenols.

There are other studies which investigated the effect of polyphenols on the physicochemical properties and antioxidant activities of various cheese products. Giroux et al. (2013) observed that addition of 1-2 g kg^{-1} green tea extract affected the colour and textural properties of cheddar type cheese and resulted in a decrease in typical cheddar flavour and an increase in astringency of cheese. Rashidinejad et al. (2016) and Rashidinejad et al. (2013) added green tea catechin to full fat and low fat cheese, respectively. Both studies reported similar results that the addition of catechin significantly decreased the pH of cheese during cheese manufacture and during 90 days of ripening whereas no effect was observed on the protein, fat and moisture content of cheeses. Rashidinejad et al. (2013) found an increase in both TPC and antioxidant activity (AA) of cheese containing catechin during the ripening period. It was attributed to the catabolism or chemical reactions, such as proteolysis,

which may release bound phenolic compounds from milk components and expose buried amino acids. It is possible that these released phenolic compounds and amino acids are detected by the Folin-Ciocalteu and AA assays, respectively. Recently, Torri et al. (2016) measured the sensory attributes and consumer acceptability of soft cheese enriched with grape skin powders. The white colour, elastic and compact structure and the presence of lactic flavours positively affected consumer acceptability. Whereas, astringency, marbling aspect, sourness and granularity negatively affected consumer acceptability. It was concluded that cheese may not be a good vehicle for incorporation of grape skin powders. Furthermore, black cumin oil (Hassanien et al., 2014) and pomegranate rind extract (Mahajan et al., 2015) were added to cheese products to improve the quality of products during refrigerated storage due to their antimicrobial and antioxidant effects. In recent years, cheese was fortified with other polyphenol rich extracts such as the flower of Inula britannica extract (Lee et al., 2016) and peanut sprout extract (Ko et al., 2017).

2.4.3 Other dairy products

In addition to yoghurt and cheese products enriched with polyphenols, other dairy products have been fortified with phenolic compound to improve the antioxidant activity and functional properties of the final products. Hwang et al. (2009) added grape wine lees (50,100 and 150 g kg^{-1}, wet weight basis) to ice cream to improve the rheological and antioxidant properties. The addition of grape wine lees affected the rheological, functional and colour properties of ice cream depending on the added concentration. Higher concentration of grape wine lees (100, 150 g kg^{-1}) caused unpleasant effects such as the increase of particle size of fat globules and a decrease in the overrun. However, the level of 50 g kg^{-1} was reported as optimum concentration due to no adverse effects on overrun and particle size of fat globules. Moreover, the phenolic compounds of grape wine lees increased the antioxidant activity of ice cream and showed good stability during the production of ice-

cream. Cam et al. (2014) successfully incorporated microcapsules containing pomegranate peel phenolics (0.5 and 1% w/w, dry matter basis) into ice-cream and the phenolic compounds of pomegranate peels in microencapsulated form improved the antioxidant activity and functional properties of ice cream. The sensory characteristics of both polyphenol enriched ice cream products (0.5% and 1.0%) was accepted by more than 75% of the panelists, which suggested that such products as functional foods could be commercially introduced to the general public.

Colahan-Sederstrom and Peterson (2005) added epicatechin to milk before UHT processing and found that addition of epicatechin reduced the overall thermally driven formation of key aroma-active compounds and resulted in lower cooked flavour intensity than the control sample. Schamberger and Labuza (2007) also demonstrated that addition of tea flavonoids epicatechin and EGCG reduced the formation of Maillard reaction derived compounds and overall total colour difference with UHT processing. Therefore, green tea flavonoids were suggested as a method that the food industry could use to control Maillard browning.

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CHAPTER 3

IMPACT OF HEAT TREATMENT AND ACID GELATION ON POLYPHENOLS ENRICHED DAIRY MATRICES

3.1 INTRODUCTION

Polyphenols are phytochemicals synthesised by plants. There are around 8000 known phenolic structures which consist of a hydroxyl group linked to an aromatic ring (Bravo, 1998). Polyphenols are classified according to their carbon skeleton: phenolic acids, flavonoids, stilbenes and lignans (Bravo, 1998). In recent years, there has been increased interest in the study of polyphenols due to their possible health benefits. Previous studies have suggested that they have strong antioxidant capacities, decrease the risk of cancers and prevent cardiovascular disease (Scalbert et al., 2005).

Milk proteins are natural delivery vehicles of bioactives due to their physicochemical and functional properties (Livney, 2010). Therefore, polyphenols could be delivered by dairy products and the nutritional value of the final product might be improved. However, polyphenols may lead to changes in the structural and functional properties of milk proteins as polyphenols interact with milk proteins via hydrogen bonding, hydrophobic interactions and covalent bonds, leading to the formation of soluble and insoluble complexes (O'Connell and Fox, 2001). These interactions depend on pH, temperature, type and structure of both proteins and polyphenols (Bandyopadhyay et al, 2012).

The enrichment of various dairy products with polyphenols has been widely studied in recent years (Chouchouli et al., 2013; Giroux et al., 2013). Past studies reported contradictory results related to the effect of milk on the antioxidant capacity of polyphenols (Keogh et al., 2007; Korir et al., 2014) which is possibly a consequence of either various methods used for the measurement of antioxidant capacity, or the phenolic types utilised. Therefore, in this study a phenolic acid (gallic acid), tannin (tannic acid) and polyphenol rich

extracts from green tea and white grape were used to try and understand the effect of phenolic types on the antioxidant activity and dairy product stability.

There is still a lack of information regarding the effect of dairy processing conditions on polyphenols and milk proteins in a dairy matrix. Acidified milk gel products (e.g. yoghurt) are widely consumed. Thermalization of milk (85 $^{\circ}$C for 30 min) is one of the critical steps in the manufacture of this products as it is required to deliver a product of suitable textural quality and water holding capacity (Lee and Lucey, 2010). Therefore, it is necessary to understand the impact of this thermal process on the polyphenol content, antioxidant capacity and stability of proteins in polyphenol enriched dairy products. Furthermore, these properties may also be affected by polyphenol addition before or after the thermal treatment. The objective of the present study was to determine the impact of polyphenol addition, of various types, before or after heat treatment on the total phenolic content, antioxidant capacity, casein micelle size (CMS) & whey protein denaturation of milk and acidified gel samples enriched with polyphenols.

3.2 MATERIALS AND METHODS

3.2.1 Experimental Design

Polyphenol solutions were prepared by dissolving polyphenol powders in distilled water (1mg ml^{-1}) without (P) and with heat treatment (P_h) at 85 °C for 30 minutes to understand the impact of heat treatment on the reconstituted polyphenol powders. To investigate the effect of heating on milk-polyphenol samples a fully randomised experiment was carried out in triplicate based on a 4 x 3 factorial design. 4 polyphenols: green tea, white grape, tannic and gallic acids and 3 sample preparations: pasteurized skim milk-polyphenol mixtures (MP), pasteurized skim milk heated before polyphenols addition (M_hP) and milk heated after polyphenols addition (M_hP_h) (**Table 3.1**). For each treatment, a control sample which has no

polyphenols was prepared by adding distilled water in place of the polyphenols. Acid milk gels were prepared with M_hP_h to determine the effect of acid gelation on the milk-polyphenol mixtures.

Table 3.1 Preparation of polyphenols with or without milk.

Samples	Preparation
P (polyphenol solutions)	Polyphenol powders in distilled water (1 mg ml^{-1}) without heat treatment.
P_h (heated polyphenol solutions)	Polyphenol powders in distilled water (1 mg ml^{-1}) heated (85 °C, 30 min).
MP (polyphenols in milk)	5 mg ml^{-1} of stock polyphenol solutions in pasteurized skim milk (1 mg ml^{-1}).
M_hP (milk heated before polyphenol addition)	Pasteurized skim milk heated (85 °C, 30 min) then added 5 mg ml^{-1} of stock polyphenol solutions (1 mg ml^{-1}).
M_hP_h (milk heated after polyphenol addition)	Pasteurized skim milk and 5 mg ml^{-1} of stock polyphenols mixed (1 mg ml^{-1}) then heated (85 °C, 30 min) together.

3.2.2 Materials

Dried extracts: green tea (Nutraceutica, Monterenzio, BO, Italy) and white grape (Nutripy CHR-Hansen, Hørlshom, Denmark) and single phenolic compounds: tannic and gallic acids (Sigma Aldrich, Gillingham, UK) were employed in this study. Their phenolic composition is described in **Table 3.2**. Pasteurized skim milk (0.08 ± 0.01% fat; 3.36 ± 0.02% protein content; 8.07 ± 0.04% total solids, 4.75 ± 0.06% lactose) was purchased from a local retailer. Sodium carbonate (Na_2CO_3) was supplied by Thermo Fisher Scientific Ltd (Loughborough, UK). Glucono-deltalactone (GDL), hydrochloric acid (HCI, 37%), methanol, Folin-Ciocalteu reagent, sodium acetate trihydrate, acetic acid, 2, 4, 6-Tris (2-pyridyl)-s-triazine

(TPTZ), ferric chloride hexahydrate, ascorbic acid, sodium chloride (NaCI) and all the polyphenol standards that used to quantify green tea and white grape were from Sigma Aldrich (Gillingham, UK).

3.2.2.1 Individual Phenolic Detection of Green Tea and White Grape

The green tea solution (5 mg ml^{-1}) was analysed with Dionex HPLC equipment that contains P680 HPLC pump, ASI-100 automated sample injector, thermostatted column compartment TCC100, PDA-100 photodiode array detector with a Zorbax eclipse XDB-C18 column (4.6 m × 150 mm, 5 μm, 25 °C, Agilent). Separation was carried out by a gradient elution using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) with elution scheme as follows: 0-5 min 4% B; 5-40 min from 4%- 25% B; 40-55 min from 25%- 50% B, 55-60 min 50% B. The protocol used a 1 mL min^{-1} flow rate and a 50 μL injection volume. Chromatograms were recorded at 280 nm. Identification was based on retention times by comparison with HPLC grade standards and quantification of green tea solution was performed using calibration curves of epigallocatechin (range: 0-400 mg L^{-1}; R^2: 0.99), catechin (range: 0-400 mg L^{-1}; R^2: 0.99), epicatechin (range: 0-100 mg L^{-1}; R^2: 0.99), epigallocatechin-gallate (range: 0-400 mg L^{-1}; R^2: 0.99), epicatechin-gallate (range: 0-100 mg L^{-1}; R^2: 0.98), gallic acid (range: 0-100 mg L^{-1}; R^2: 0.99).

In the detection of phenolic compounds of white grape, first Dionex HPLC equipment described above was used. However, a great number of unknown peaks associated with phenolics were detected using by HPLC. Therefore, it was decided to employ LC-MS for accurate identification of phenolic compounds. The white grape solution (5 mg ml^{-1}) was analysed with a Waters UPLC-MS and Quattro Ultima mass spectrometer (Waters, Manchester, UK) with a C-18 guard column (1.7μ / 50 x 2.1 mm, Kinetex, C18 column, Phenomenex, Macclesfield, UK) was used for the analyses. Separation was carried out by a

gradient elution using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) with elution scheme as follows: B was increased from 7 to 75% (0.2 min to 8.3 min), B was decreased from 75 to 7% (9.3min to 10 min), then the column was equilibrated for 5 min at initial condition (7% B). The total run time was 15 min with flow rate of 0.1 mL min^{-1} and injection volume of 10µl. The oven temperature was set at 35°C. Detection was performed using retention time and multiple reaction monitoring transition using positive ion mode (3.35 kV). The quantification of white grape solution was estimated based on the area of 10 µM of each polyphenol standard (Resveratrol, Quercetin-3-O-glucoside, Catechin, Epicatechin, Epigallocatechin-gallate, Procyanidin B2). It was assumed that the area of estimated concentration corresponds proportionally with the area of 10 µM of each polyphenol standard. Therefore, the estimated concentrations were calculated based on the peak area given by 10 µM of each polyphenol standard.

3.2.3 Preparation of Polyphenols in Skim Milk

The stock polyphenol solutions (5 mg ml^{-1}) were freshly prepared by dissolving polyphenol powders in distilled water before each experiment. They were added to pasteurized skim milk (MP) and stirred for 30 min at room temperature. The final concentration of polyphenol powders in milk samples was 1 mg ml^{-1}. A thermalization step of 85 $^{\circ}$C for 30 minutes, typically used in acidified milk gel manufacture to denature whey proteins and enhance gel texture, was applied to pasteurized skim milk before (M_hP) and after (M_hP_h) polyphenols addition. Samples were placed (5 ml) in a shaking (90 rev/min) water bath (Grant Instrument Ltd, Cambridge, UK). After heating samples were rapidly cooled by immersion in ice-water. After preparation, all MP, M_hP and M_hP_h samples were stored at 4 ºC until analysis.

3.2.4 Preparation of Acid Milk Gels

M_hP_h samples were prepared as outlined in **section 3.2.3**. Glucono-deltalactone (GDL) (1.7% w/w) was added to M_hP_h samples and stirred for 2 min. The samples were incubated in Sanyo Gallenkamp incubator (Leicestershire, UK) at 30°C for 3 hours and 45 min until the pH reached a value of 4.6.

3.2.5 Measurement and Adjustment of pH of the Milk-Polyphenol Mixtures

The pH of each sample was determined using an Orion 3-star benchtop pH meter (Fisher Scientific Ltd, UK) fitted with a glass combination electrode. On addition of gallic acid to pasteurised skim milk, the pH decreased to 6.4. Therefore, to have a representative control for this sample, pasteurized skim milk was adjusted to 6.4 using HCI (1M) and stored at 4°C overnight to equilibrate.

3.2.6 Casein Micelle Size and Undenatured Whey Protein Content of Milk-Polyphenol Mixtures

The average CMS of samples was measured using a Zetasizer 5000 (Malvern Instruments Ltd, Worcestershire, UK) according to Chen et al. (2012). Undenatured whey protein nitrogen (WPN) of samples was determined by applying the GEA Niro method (GEA NIRO, 2009).

3.2.7 Extraction of Free Polyphenols from Milk and Acidified Milk Gel

The polyphenols were extracted from the milk and acidified milk gel according to Ye et al. (2013). Briefly, an aliquot of the milk-polyphenol mixture (5 ml) or acidified milk gel (10 g) was centrifuged (Sorvall RC 6) at 25,860 x g for 15 min at 20 °C with 10 (milk) or 15 ml (gel) of acidified methanol which included 1% HCl (12 M). TPC and FRAP (**section 3.2.8**) analysis was carried out on the supernatants. For each replicate, the extracts of milk-polyphenol mixtures were freshly prepared and were used the same day for chemical

analyses. The same treatment was applied to pasteurised skim milk without polyphenols for comparison.

3.2.8 Chemical Analyses

3.2.8.1 TPC (Total Phenolic Content)

The total phenolic content was determined according to Folin Ciocalteu method as described by (Singleton, 1985). This is a colorimetric method which measures the reduction of Folin Ciocalteu reagent by phenolic compound to a blue-coloured complex in an alkaline solution. The colour intensity of sample is measured by the absorbance readings using a spectrophotometer. Absorbance at 760 nm was determined after 2 hours incubation at 18 °C using a UV-Spectrophotometer (Perkin Elmer, Lambda 20, Norwalk, USA). The phenolic content was calculated by means of a calibration curve with standard gallic acid (**Figure 3.1A**) in the following concentrations: 0, 50, 100, 150, 250, 500, 750, 1000 mg L^{-1} (R^2 = 0.99). The results were expressed as milligrams of gallic acid equivalent (GAE) per millilitre of sample (mg GAE ml^{-1}).

3.2.8.2 FRAP (ferric ion reducing antioxidant power) Assay

The FRAP assay was performed to determine the antioxidant capacity (AC) of samples according to Benzie and Strain (1996). This assay is based on the reduction of the Fe (III)-TPTZ complex to the ferrous form which has intense blue colour at low pH. Absorbance was immediately measured using a plate reader (Tecan GENios, Geneva, Switzerland) at 595 nm. An ascorbic acid calibration curve (**Figure 3.1B)** was prepared in the following concentrations 0, 10, 50, 100, 250, 500, 750, 1000 µmol L^{-1} (R^2 = 0.99). The results were expressed as ascorbic acid equivalent (µmol AAE) per millilitre of sample (µmol AAE ml^{-1} sample).

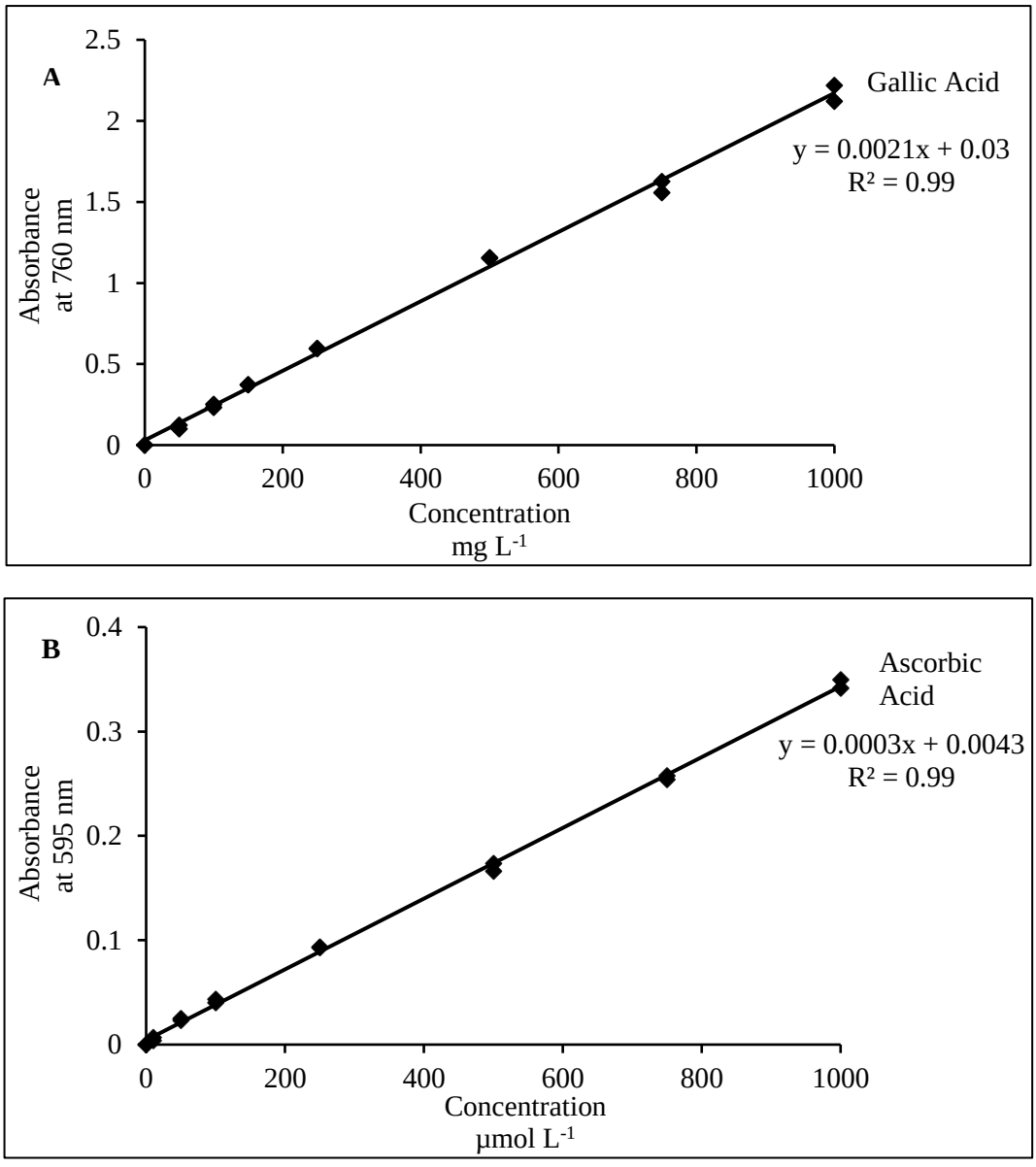


Figure 3.1 Calibration curves of gallic acid and ascorbic acid**.** Lines show the results of two replicates.

3.2.9 Statistical Analysis

Experimental and calculated TPC and FRAP results were compared to assess the impact of milk on milk-polyphenol mixtures. The calculated values are the sum of the experimental results for the milk and polyphenol solutions. If there was no significant difference between the experimental and calculated values, it was deemed milk had 'no effect'. If the

experimental values were significantly higher or lower than the calculated values, milk was deemed to have an ‘increasing’ or ‘decreasing’ effect respectively.

Results in the text are given as mean values ± 2 standard errors (SEs) and this is stated as SE in the tables and graphs. SE was used to calculate 95% confidence intervals (CI) as shown in equation 3.1 (Streiner, 1996). CI provides better understanding in the variation of population mean (M) from sample to sample as compared to standard deviation (SD). Therefore, CI should be used when comparing the means of two or more groups to find the difference among the groups (Streiner, 1996). The normality of data distribution was tested by Kolmogorov-Smirnov method. Differences between treatment methods and samples were tested by a one-way analysis of variance (ANOVA), followed by a post hoc Tukey test. A t-test was used for comparison of two means. Results with $p < 0.05$ were considered significantly different. Analyses were performed using SPSS Software for Windows (Version 21.0, Armonk, NY: IBM Corp., USA).

$$95\%\ \mathrm{CI} = M \pm (2 \times SE) \qquad (\mathbf{3.1})$$

3.3 RESULTS AND DISCUSSION

3.3.1 Selected Properties of Polyphenol Powders

The dominant compounds in green tea were epigallocatechin-gallate (41%) and catechin (37%) (**Figure 3.2**, **Table 3.2**), this is in agreement with previous studies (Jaziri et al., 2009). The phenolic compounds analysed in white grape (**Table 3.2**) were also in agreement with previous studies (Wittenauer et al., 2015). The chemical structures, equations of calibration curves and the mass spectrometry data in positive ion mode of phenolic compounds detected in green tea and white grape are shown in **Table 3.3**. The calibration curves of phenolic compounds detected in green tea are also presented in **Appendices, Figure 1**.

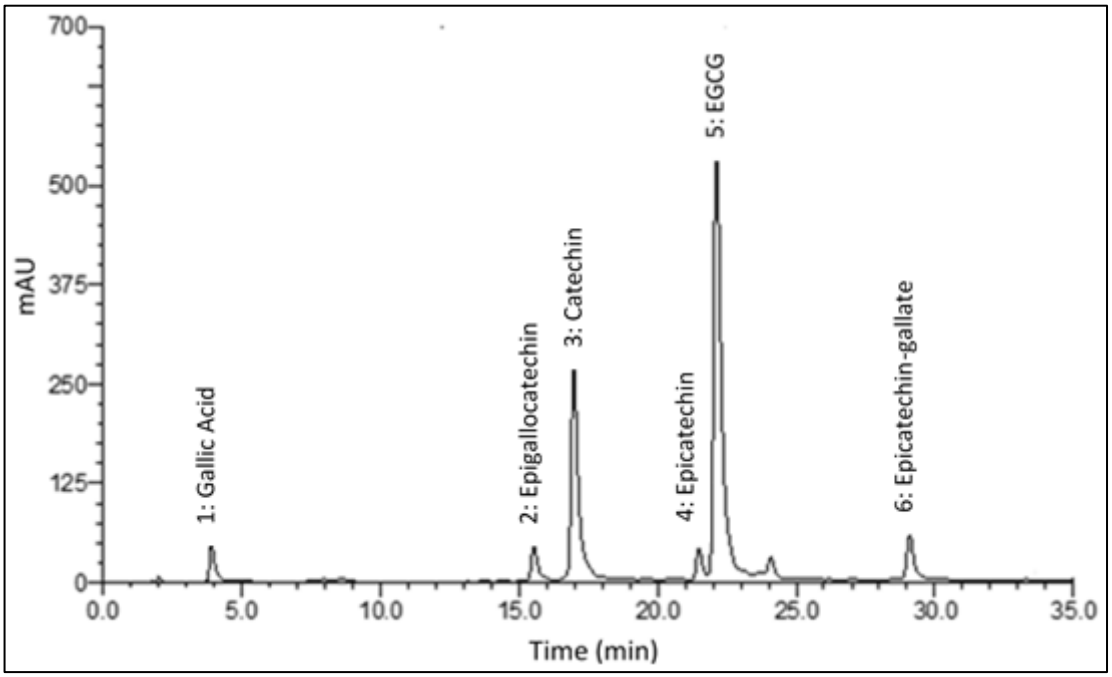


Figure 3.2 HPLC chromatogram of phenolic compounds in green tea (280 nm). EGCG: Epigallocatechin-gallate.

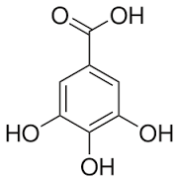
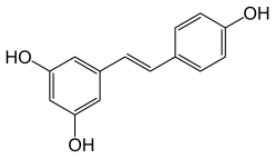
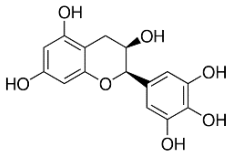
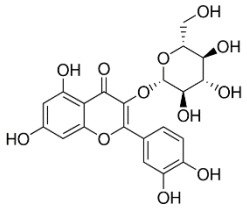
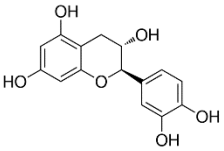
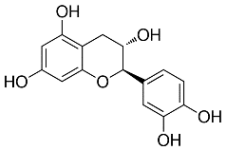
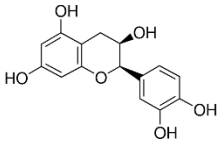
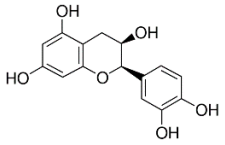
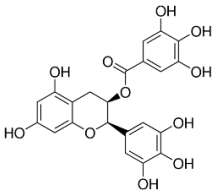
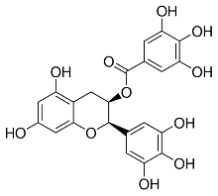
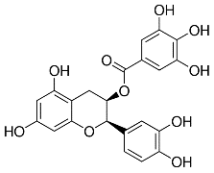
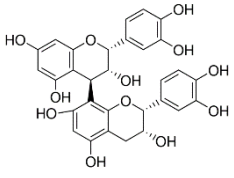
Table 3.2 Phenolic compounds of green tea and white grape powders, which were detected by HPLC and LC-MS, respectively.

Polyphenols	Phenolic compounds[A]	Concentration mg g^{-1} powder[A]
Green Tea	Gallic acid	8.24 ± 0.33
	Epigallocatechin	179.80 ± 4.32
	Catechin	373.77 ± 9.24
	Epicatechin	31.21 ± 2.28
	Epigallocatechin-gallate	413.99 ± 14.18
	Epicatechin-gallate	24.29 ± 1.52
	Total sum of phenolic compounds	1031.30
White Grape	Resveratrol	6.72 ± 1.54
	Quercetin-3-O-glucoside	30.86 ± 3.21
	Catechin	31.31 ± 6.14
	Epicatechin	53.34 ± 0.42
	Epigallocatechin-gallate	0.02 ± 0.002
	Procyanidin B2	5.58 ± 2.19
	Total sum of phenolic compounds	127.84

[A] Data represented are means of three replicates ± SE. Total sum of phenolic compounds was calculated based on the sum of concentrations of phenolic compounds detected in green tea and white grape.

Gallic acid had the highest TPC among the samples, followed by tannic acid, green tea and white grape in descending order (**Table 3.4**). There was no significant difference between the TPC of tannic acid and green tea. Similarly, the TPC of green tea and white grape were not significantly different. However, when the concentration of individual phenolic compounds were detected via HPLC and LC-MS, the concentration of the sum of all phenolic compounds in green tea was nearly 8 times more than the concentration of the sum of all phenolic compounds in white grape (**Table 3.2**). This indicated that there are some other phenolic compounds in white grape that contributed the TPC, whereas those phenolics

Table 3.3 HPLC and LC-MS data of phenolic compounds detected in green tea and white grape and their chemical structures.

	Green Tea		White Grape		
Phenolic Compounds	Equation[A]	Structure	Phenolic Compounds	m/z [M]+	Structure
Gallic acid	y=2.24x + 1.72		Resveratrol	229.0	
Epigallo catechin	y=0.14x - 0.72		Quercetin-3-O-glucoside	465.0	
Catechin	y=0.46x - 1.93		Catechin	290.8	
Epicatechin	y=0.54x - 0.25		Epicatechin	290.8	
Epigallo catechin-gallate	y=0.84x - 2.12		Epigallo catechin-gallate	459.3	
Epicatechin-gallate	y=1.76x - 1.44		Procyanidin B2	579.0	

[A] Equation of the calibration curve with y=peak area (mAU*min) and x=concentration (mg L^{-1})

Table 3.4 Total phenolic content (TPC) and ferric ion reducing antioxidant power (FRAP) results of polyphenol powders.

Samples	TPC (mg GAE g^{-1} sample)[A]	FRAP (µmol AAE g^{-1} sample)[A]
Green Tea	819.8 ± 43.7^{bc}	8772.2 ± 813.9^{b}
White Grape	753.3 ± 11.5^{c}	5508.9 ± 382.9^{c}
Tannic Acid	875.8 ± 72.3^{b}	8476.7 ± 657.9^{b}
Gallic Acid	1028.1 ± 60.9^{a}	20544.4 ± 2207.4^{a}

[a-c]: Means within a column with different superscript are significantly different at $p < 0.05$.

[A] Data are expressed as means of three replicates ± SE. TPC: total phenolic content, GAE: gallic acid equivalent, FRAP: ferric ion reducing antioxidant power, AAE: ascorbic acid equivalent.

were not quantified. The polyphenol powders used in this study have a much higher TPC than regular teas, vegetables and fruits (Bravo, 1998; Dubeau et al, 2010). In the present study, the levels of TPC in the polyphenol powders are within the expected range (da Silva et al., 2015). The pH of green tea, white grape, tannic acid and gallic acid solutions were 6.71, 6.79, 6.40, 3.62 respectively (**Table 3.5**).

The FRAP of polyphenol powders correlated with the total phenolic content results, gallic acid had the highest FRAP and it was followed by tannic acid, green tea and white grape (**Table 3.4**). The FRAP value of green tea and tannic acid were not significantly different.

3.3.2 pH of Polyphenol Enriched Skim Milk

Table 3.5 demonstrates that, as expected (Fox, 1981), the application of a heat treatment reduced the pH of all samples. The decrease in pH is probably related to the release of H^{+} due to precipitation of calcium phosphate with the heat treatment of the milk samples. The pH of the samples containing gallic acid was significantly lower than all the other samples

Table 3.5 The effects of addition of polyphenols on pH of pasteurized skim milk with three treatments.

Samples	pH[A]			
	Polyphenol solutions	MP	M_hP	M_hP_h
Control		6.77 ± 0.01^{b1}	6.71 ± 0.03^{a2}	6.72 ± 0.01^{a2}
Green Tea	6.71 ± 0.01	6.81 ± 0.02^{ab1}	6.73 ± 0.04^{a2}	6.67 ± 0.02^{cb2}
White Grape	6.79 ± 0.01	6.84 ± 0.02^{a1}	6.75 ± 0.04^{a2}	6.68 ± 0.01^{b2}
Tannic Acid	6.40 ± 0.01	6.81 ± 0.02^{ab1}	6.72 ± 0.06^{a2}	6.65 ± 0.02^{c2}
Gallic Acid	3.62 ± 0.03	6.42 ± 0.03^{c1}	6.32 ± 0.02^{b2}	6.34 ± 0.01^{d2}

[A] Data are expressed as means of three replicates ± SE. Control sample includes 20% distilled water in place of polyphenols. MP: pasteurized skim milk-phenol mixtures, M_hP: pasteurized skim milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition.

[a-d]: Letters in a column with different superscript are significantly different at $p < 0.05$ among samples.

[1-2]: Numbers in a row with different superscript are significantly different at $p < 0.05$ among three treatments for each sample.

studied. This is due to the acidic properties of gallic acid (pKa 4.41). The stage of polyphenol addition to milk had no impact on pH (M_hP versus M_hP_h).

3.3.3 Effect of Skim Milk on TPC and FRAP of Polyphenols

TPC and FRAP of milk was also established to understand the contribution of skim milk to TPC and FRAP of milk-polyphenol mixtures (**Table 3.6**). Pasteurised skim milk TPC was determined as 0.69 ± 0.01 mg GAE ml^{-1} which was in agreement with a previous study (Cebeci & Sahin-Yesilcubuk, 2014). Phenolic compounds in milk arise from ruminant digestion of forages such as breakdown of compounds as well as phenolic content of feed (Besle et al., 2010). Additionally, milk casein or whey proteins and some reducing compounds might contribute the total phenolic content of milk (Chouchouli et al., 2013).

The antioxidant capacity of the milk as determined by FRAP was 0.22 ± 0.02 µmol AAE ml^{-1} which is in line with previous studies (Dubeau et al., 2010).

Table 3.6 The effects of pasteurized skim milk on total phenolic content (TPC) and ferric reducing antioxidant power (FRAP) of polyphenols (MP samples).

Samples [A]	TPC[a] (mg GAE ml^{-1})	*p* value[b]	FRAP[a] (µmol AAE ml^{-1})	*p* value[b]
Skim Milk				
Ex. value	0.69 ± 0.02		0.22 ± 0.03	
Green Tea		NS		NS
Ca. value	1.38 ± 0.04		8.95 ± 0.82	
Ex. value	1.51 ± 0.11		7.89 ± 1.01	
White Grape		NS		NS
Ca. value	1.31 ± 0.02		5.67 ± 0.41	
Ex. value	1.35 ± 0.15		5.48 ± 0.57	
Tannic Acid		NS		**
Ca. value	1.43 ± 0.08		8.98 ± 0.19	
Ex. value	1.52 ± 0.09		4.86 ± 0.98	
Gallic Acid		*		NS
Ca. value	1.59 ± 0.06		20.72 ± 2.18	
Ex. value	1.75 ± 0.05		17.77 ± 1.50	

[A] Data were examined for a significant difference between Ca. and Ex. values. Ca.value: Calculated value; Ex. value: Experimental value. Calculated value: TPC or FRAP value of milk + TPC or FRAP value of each stock polyphenol solution. Skim milk does not include distilled water.

[a] Data are expressed as means of three replicates ± SE. TPC: total phenolic content, GAE: gallic acid equivalent, FRAP: ferric ion reducing antioxidant power, AAE: ascorbic acid equivalent.

[b] NS: indicated no significant difference between calculated and experimental values. *: $p < 0.05$, **: $p < 0.01$.

For all phenolic enriched samples, the experimental TPC values were slightly higher than the calculated TPC values, however there was only a significant difference between the experimental and calculated TPC result for gallic acid enriched milk (p =0.026), (**Table 3.6**). This can be explained by the lower pH of samples containing gallic acid (**Table 3.5**). Dalgleish and Law (1988) reported that when milk pH decreased from 6.8 to 6.3 at 4 ºC the dissociation of total casein, which was predominantly β-casein, to serum increased. It is likely that the lower pH (i.e. 6.4) of the gallic acid sample caused more dissociation of casein from the micelle to the serum phase as compared to pH 6.7-6.8 at 4 ºC, which was the storage temperature for all milk-polyphenol mixtures. Therefore, a higher content of casein protein in the serum phase may have been detected by the Folin-Ciocalteu method resulting in higher TPC.

There was no significant difference between calculated and experimental FRAP values for milk enriched with green tea, white grape or gallic acid. However, the experimental FRAP values of the tannic acid enriched sample was lower than the calculated value. This could be related to the reaction time of the FRAP assay. FRAP has quite a short reaction time around (~4 min) and this is not enough for some phenolic compounds to reduce the ferric tripyridyltriazine complex (Fe3+-TPTZ) to a ferrous (Fe2+-TPTZ) form (Prior et al., 2005). Pulido et al. (2000) detected that the absorption of tannic acid, caffeic acid and ferulic acid slightly increased after several hours of reaction time. This does not seem the case for tannic acid solution in water in this study, whereas in the presence of milk tannic acid might require longer time to complete the reaction.

3.3.4 Effect of Heat Treatment on Polyphenol Enriched Skim Milk

3.3.4.1 Total Phenolic Content and FRAP

Before examining the impact of heat treatment on the polyphenol enriched milks the effect of heat treatment (85°C for 30 min) on TPC and FRAP of the polyphenol powders reconstituted in water (1 mg ml^{-1}) was determined. Heating the polyphenol solutions had no significant effect on the FRAP of the solutions. Furthermore, there were no significant differences between the TPC of green tea and white grape solutions after heating. However,

Table 3.7 The effect of heat treatment on TPC and FRAP values of the polyphenol powders reconstituted in water.

Samples[A]	TPC[a] (mg GAE ml^{-1})	*p* value[b]	FRAP[a] (µmol AAE ml^{-1})	*p* value[b]
Green Tea		NS		NS
P	0.71 ± 0.05		6.95 ± 0.77	
P_h	0.68 ± 0.02		6.41 ± 0.62	
White Grape		NS		NS
P	0.71 ± 0.02		5.32 ± 0.82	
P_h	0.72 ± 0.02		4.30 ± 0.44	
Tannic Acid		*		NS
P	1.22 ± 0.15		7.91 ± 0.84	
P_h	1.02 ± 0.05		6.82 ± 0.83	
Gallic Acid		*		NS
P	1.38 ± 0.03		20.79 ± 1.18	
P_h	1.29 ± 0.06		20.51 ± 1.68	

[A] Data were examined for a significant difference between P and P_h values. P: polyphenol solutions, P_h: heated polyphenol solutions.

[a] Data are expressed as means of three replicates ± SE. TPC: total phenolic content, GAE: gallic acid equivalent, FRAP: ferric ion reducing antioxidant power, AAE: ascorbic acid equivalent.

[b] NS: indicated no significant difference between calculated and experimental values. *: $p < 0.05$.

the TPC of both tannic and gallic acid solutions significantly decreased by 16% and 7% respectively after heating (p = 0.038, p = 0.033) (**Table 3.7**). This suggests that multiple phenolic compounds present in a solution may combine to have a protective effect in comparison with solutions with individual compounds. This is supported by the results of previous studies (Sari et al., 2012; Volf et al., 2014).

Figure 3.3 represents the TPC of samples subjected to MP, M_hP and M_hP_h treatments. Adding polyphenols significantly increased the TPC of all the samples as expected. The TPC of control pasteurized skim milk (MP) was detected as 0.57 ± 0.06 mg GAE ml^{-1}. This was lower than TPC of pasteurized skim milk (**Table 3.6**) mainly due to the fact that control pasteurized skim milk had 20% water addition in place of the polyphenols. The TPC of control pasteurized skim milk is not affected by heat treatment. Pasteurized skim milk heated before polyphenols addition (M_hP) resulted in a significant decrease in the TPC of milk containing tea, grape and tannic acid as compared to MP samples. This is probably due to increased interactions between milk proteins and polyphenols (Wu et al., 2013). Heating results in denaturation of whey proteins revealing hydrophobic and sulphur containing groups (Anema and Li, 2003). This increases the probability that polyphenols will bind to the protein. It was previously observed that the binding of epigallocatechin gallate (EGCG) was higher with preheated beta lactoglobulin (β-LG) at 75-85 ºC for 20 min as compared to native protein at room temperature (Shpigelman et al., 2010). This was attributed to hydrophobic interactions and hydrogen bonding between EGCG and β-LG. It has also been shown that heat-induced denatured whey proteins played a role in strengthening casein-polyphenol interactions (Yazdi and Corredig, 2012).

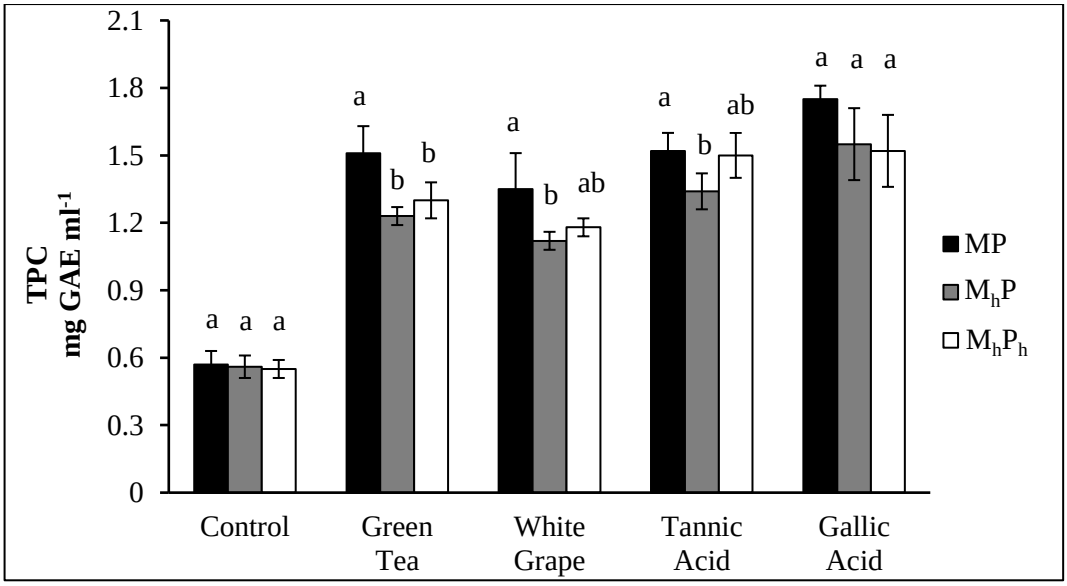


Figure 3.3 Total phenolic content (TPC) of milk-polyphenol mixtures following three different treatments (MP, M_hP, M_hP_h). Control sample includes 20% distilled water in place of the polyphenols. GAE: gallic acid equivalent, MP: pasteurized skim milk-polyphenol mixtures, M_hP: pasteurized skim milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition. For each sample, different letters, namely a and b, indicate significant differences at $p < 0.05$ and means that share the same letter, namely ab and a or ab and b, are not significantly different ($p > 0.05$) among three treatments. Error bars represent means of three replicates ± SE.

With the exception of samples containing tannic acid, a similar decrease in TPC was evident when pasteurized skim milk heated after polyphenols addition (M_hP_h) was compared to MP (**Figure 3.3**). However, the TPC was only significantly lower for green tea samples. As mentioned above, when the tannic acid solution was heated the TPC decreased by 16%. However, no significant decrease was evident when tannic acid was heated with milk (M_hP_h V MP). This suggests that milk may have a protective effect on the phenolic compound in the sample. This has been previously demonstrated for anthocyanins extracted from corncob (Jing and Giusti, 2005). Overall, heat treatment had a significant effect on the TPC of milk-

polyphenol mixtures. With the exception of tannic acid, the stage of polyphenol addition (M_hP v M_hP_h) had no significant effect on the TPC of milk-polyphenols.

The addition of polyphenols to milk significantly increased the antioxidant capacity as measured by FRAP for all samples (**Figure 3.4**). Additionally, heat treatment had no significant effect on FRAP of control sample. Heat treatment, regardless of stage of polyphenol addition, either decreased or had no impact on the FRAP of the samples. These results are inline with the the TPC results in **Figure 3.3**, indicating that in general increased interaction between milk proteins and the polyphenols occured. This is most likely due to

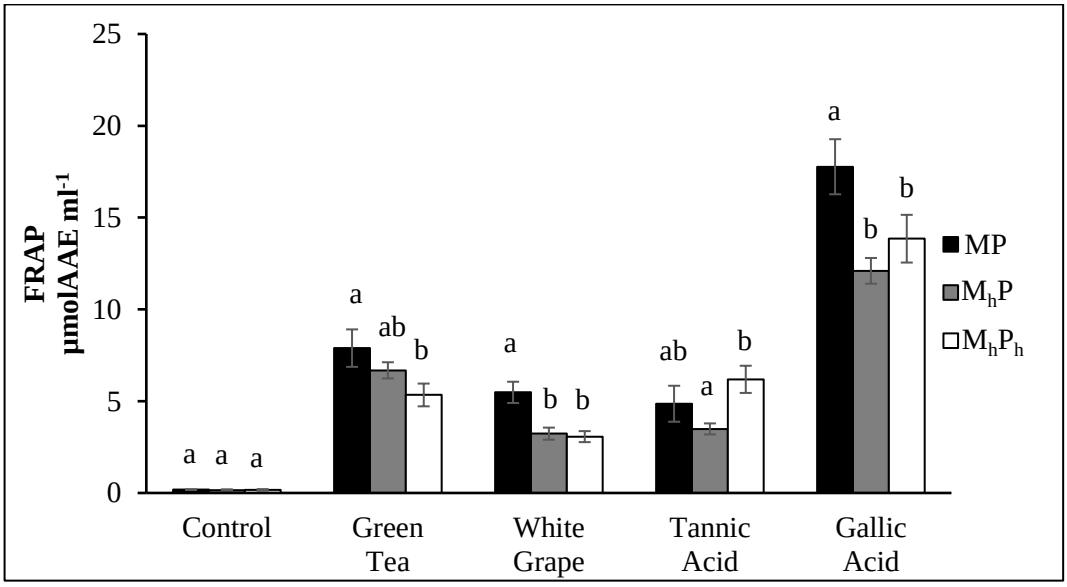


Figure 3.4 Ferric ion reducing antioxidant power (FRAP) of milk-polyphenol mixtures following three different treatments (MP, M_hP, M_hP_h). Control sample includes 20% distilled water in place of the polyphenols. AAE: ascorbic acid equivalent, MP: pasteurized skim milk-polyphenol mixtures, M_hP: milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition. For each sample, different letters, namely a and b, indicate significant differences at $p < 0.05$ and means that share the same letter, namely ab and a or ab and b, are not significantly different ($p > 0.05$) among three treatments. Error bars represent mean ± SE.

the interaction between polyphenols and milk proteins which have been denatured, as previously mentioned. The FRAP of tannic acid added to pasteurized skim milk before heat treatment (M_hP_h) was significantly higher than tannic acid added after heat treatment (M_hP). The stage of polyphenol addition had no effect on FRAP of any other sample.

Overall, with the exception of tannic acid, while heat treatment reduces the TPC and FRAP of polyphenol enriched samples, polyphenol addition before or after heat treatment did not significantly impact on TPC and FRAP values.

3.3.4.2 Casein Micelle Size and Undenatured Whey Protein Amount of Polyphenol Enriched Skim Milk

Figure 3.5 presents the CMS of MP, M_hP and M_hP_h samples. Regardless of the sample the CMS increased after heat treatment for samples where polyphenols were added after the milk was heat treated (M_hP), with the exception of gallic acid, these increases were significant. This is in agreement with previous studies that show whey proteins become denatured following heat treatment. The denatured whey proteins, mainly β-LG and α-LA, intreact with κ-casein by forming disulfide bonds on the surface of micelle and this attachment leads to ultimately increasing the micelles average diameter (Dalgleish and Corredig, 2012; Martin et al., 2007). However, when polyphenols were added prior to heat

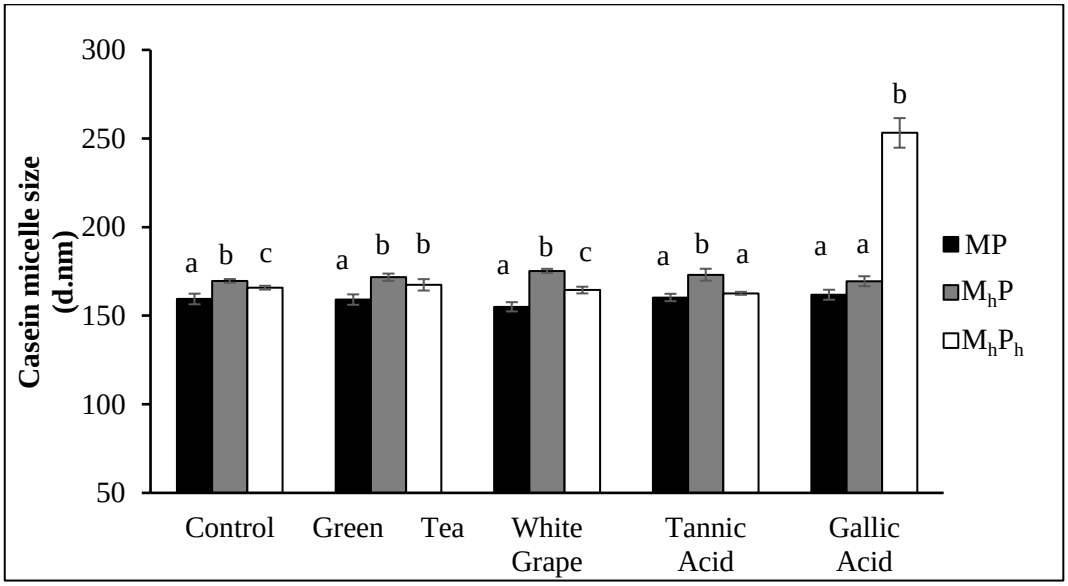


Figure 3.5 Casein micelle size of milk-polyphenol mixtures shown in three different treatments (MP, M_hP, M_hP_h). Control sample includes 20% distilled water in place of the polyphenols. MP: pasteurized skim milk-polyphenol mixtures, M_hP: pasteurized skim milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition. Different letters indicate significant differences at $p < 0.05$ among treatments for each sample. Error bars represent means of three replicates ± SE.

treatment (M_hP_h) in all cases, except gallic acid, the CMS tended to be smaller than for the M_hP sample, albeit this decrease was insignificant for green tea. This is possibly due to concentration of milk proteins in the sample during heat treatment. M_hP_h samples contained either 20% polyphenol solutions or distilled water before heat treatment. However, for M_hP samples, polyphenol solutions or distilled water were added to pasteurized skim milk after heat treatment. Therefore, lower milk protein concentration in the M_hP_h samples during heat treatment leads to lower levels of whey protein denaturation and hence smaller CMS. This

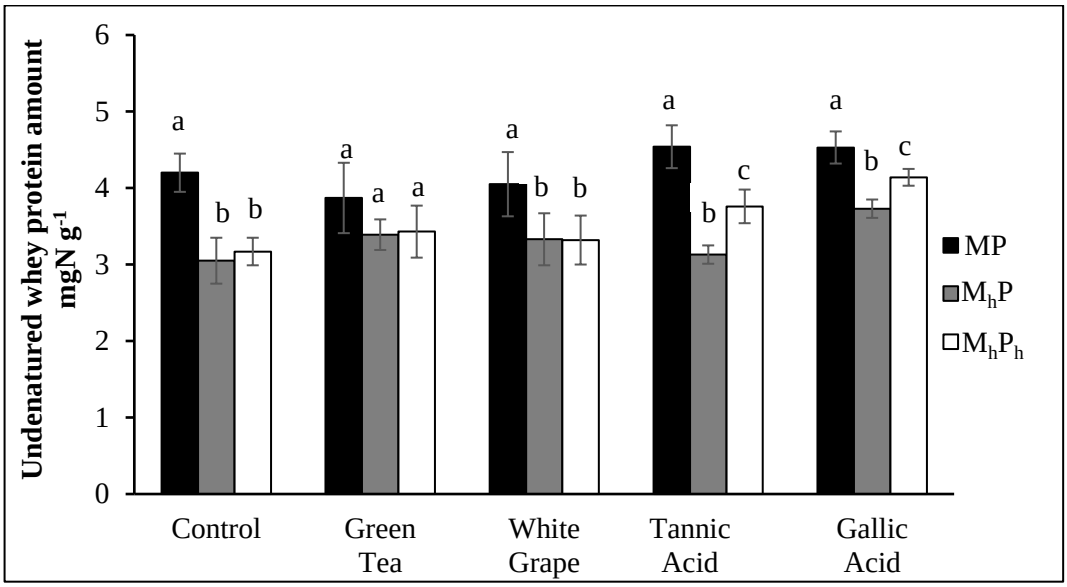


Figure 3.6 Undenatured whey protein amount of milk-polyphenol mixtures following three different treatments (MP, M_hP, M_hP_h). Control sample includes 20% distilled water in place of the polyphenols. MP: pasteurized skim milk-polyphenol mixtures, M_hP: pasteurized skim milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition. Different letters indicate significant differences at $p < 0.05$ among treatments for each sample. Error bars represent means of three replicates ± SE.

is supported by the levels of undenatured whey protein that were detected in the samples (**Figure 3.6**). In general, M_hP_h samples showed higher levels of undenatured whey protein in comparison to M_hP sample, albeit the differences were not statistically significant for control, green tea or white grape. The trend for the gallic samples was different. Specifically, when gallic acid was heated together with the milk (M_hP_h) the CMS was larger than all other samples. This is probably related to the heat treatment of the sample at a lower pH than other samples (**Table 3.5**). Heating milk proteins at lower pH values results in a higher attachment of denatured whey proteins to casein micelles (Taterka and Castillo, 2015). To understand the effect of the pH of pasteurized skim milk on the CMS and undenatured whey protein amount, the pH of pasteurized skim milk was adjusted to 6.41 and heated at 85ºC for 30 min. The CMS and undenatured whey level of the pH adjusted sample was not significantly different to the M_hP_h gallic acid sample (**Appendices**, **Table 1**).

3.3.5 Effect of Acid Gel Formation on TPC and FRAP

The effect of polyphenol addition on the TPC of M_hP_h milk samples was compared to acidified milk gels prepared with M_hP_h (**Figure 3.7A**). As expected the acidified milk gels containing polyphenols had a significantly higher TPC than the control gel (Chouchouli et al., 2013). There was a significant decrease in the TPC of acidified gel samples in comparison to M_hP_h milk samples for all types of polyphenols studied. The decrease in the level of extractable polyphenols in the acidified milk gels is probably because they are very tightly bound to the casein.

There was no significant difference in FRAP values between M_hP_h milk and acidified milk gel samples, with the exception of acid milk gels containing green tea which had a significantly higher FRAP value than milk containing green tea (**Figure 3.7B**). Overall, there is poor correlation between the total phenolic content and antioxidant capacity in polyphenol

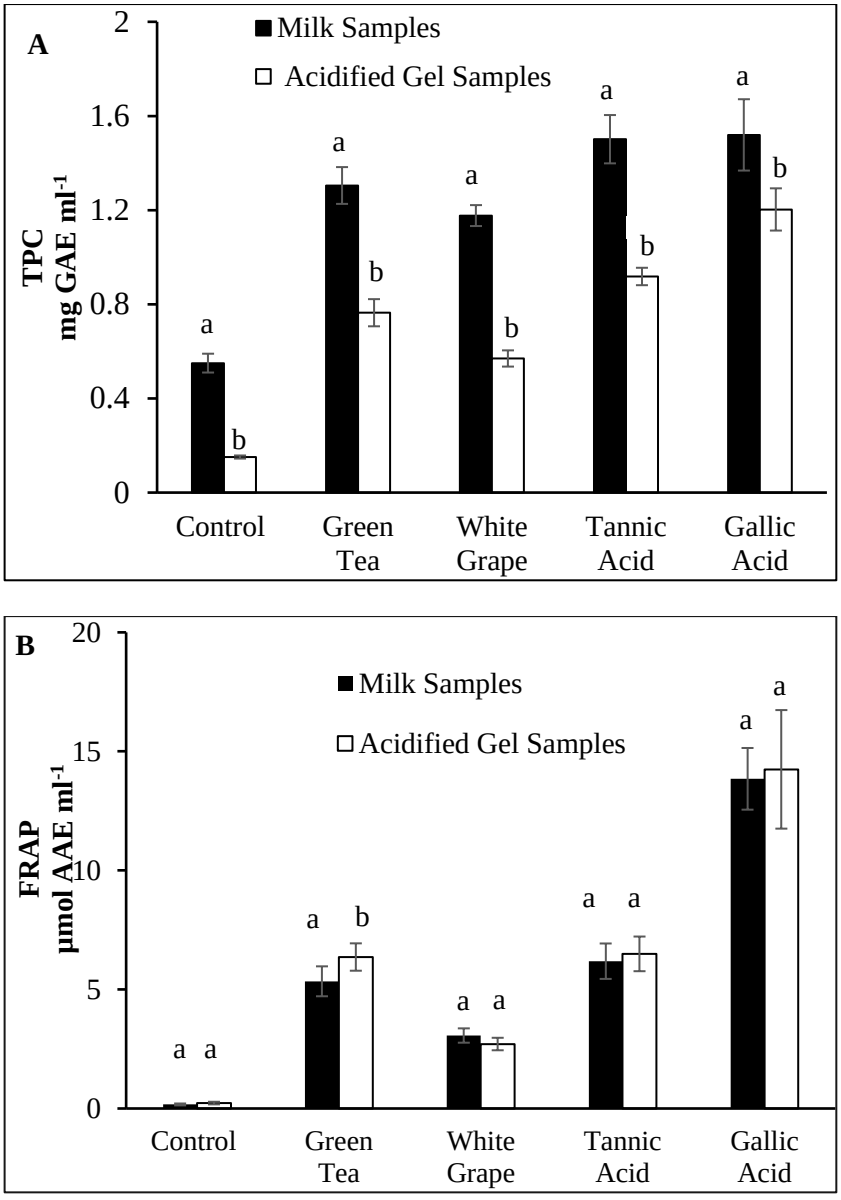


Figure 3.7 The effect of heat treatment and acid gelation on total phenolic content (TPC) and ferric ion reducing antioxidant power (FRAP) of milk-polyphenol mixtures. Control sample includes 20% distilled water in place of the polyphenols. GAE: gallic acid equivalent, AAE: ascorbic acid equivalent. Different letters indicate significant differences at $p < 0.05$ between M_hP_h milk and M_hP_h acidified milk gel for each sample. Error bars represent means of three replicates ± SE.

enriched acidified milk gels, which is in agreement with previous studies on yoghurt (Trigueros et al., 2014). They attributed this poor correlation to the complex nature of yoghurt. It is possible that whey protein and polyphenol complexes in the supernatant

exhibited antioxidant capacity, which may explain the reduction in TPC without an impact on FRAP. (Almajano et al., 2007)) found that mixing whey proteins (BSA, β-LG, α-LA) with EGCG (antioxidant from green tea) resulted in the formation of a complex with antioxidant capacity. The antioxidant of the complex formed increased during 7-day storage at 30 ºC possibly due to increased interactions between the polyphenol and protein. In the present study, it is possible that the acidification conditions may have resulted in increased interactions between the whey proteins in the supernatant and polyphenols resulting in complexes with antioxidant capacity.

3.4 CONCLUSIONS

Commercial polyphenol extracts and single phenolic compounds can be successfully incorporated into milk which could be used to produce acidified milk gel products such as yoghurt. In general heat treatment at 85ºC for 30 minutes reduced TPC and FRAP of the milk samples enriched with polyphenols, regardless of whether the polyphenols were added before or after heat treatment. However, the acid dissociation constant of a polyphenol needs to be considered when enriching milk samples as this may impact on selected properties of the samples. For example, the lower pH of gallic acid resulted in a significant increase in the CMS of milk when the gallic acid was added before heat treatment due to higher attachment of denatured whey proteins to casein micelles at low pH. Acid gel processing decreased the extractable polyphenols as compared to M_hP_h milk samples, however there was no significant decrease in FRAP of polyphenol enriched milk.

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CHAPTER 4

EFFECT OF POLYPHENOL ADDITION AND HEAT TREATMENT ON THE RHEOLOGICAL PROPERTIES OF ACIDIFIED MILK GELS

4.1 INTRODUCTION

Polyphenols are plant based secondary metabolites which have many health benefits associated with them such as antioxidant activity, antidiabetic & anticarcinogenic properties (McDougall, 2017; Vauzour et al., 2010). Consumers are currently demanding health promoting foods and in this context polyphenols have attracted much attention (Wootton-Beard and Ryan, 2011). Dairy products, such as yoghurt and dairy dessert, are potential delivery matrices for bioactive compounds (Oliveira et al., 2015; Szwajgier and Gustaw, 2015). The addition of polyphenols to dairy products (e.g. acidified milk gels) has the potential to improve the nutritional value of the final product, however it may also impact the technological functionality (Foegeding et al., 2017).

The effect of thermal processing and acid gelation on the total phenolic content (TPC) and antioxidant capacity (FRAP) of polyphenol enriched dairy matrices was presented in **Chapter 3**. Polyphenols were successfully incorporated into skim milk and acidified milk gel samples, thus improving their nutritional value. However, heat treatment reduced the TPC and FRAP of polyphenol-milk samples. Furthermore, acid gelation decreased the extractable polyphenols in the enriched milk possibly due to interactions between the polyphenols and the casein. These interactions between polyphenols and proteins may influence the technological functionality of the product such their gelation kinetics and rheological properties (Harbourne et al., 2011).

Textural and rheological properties of acidified milk gel can impact the sensory attributes, which are important factors determining consumer preference (Jaworska et al., 2005; Skriver et al., 1999). Previous studies have shown that the addition of polyphenols into acidified

milk gels influenced the gel formation properties (Harbourne et al., 2011) and rheological properties of the final gel (Vega and Grover, 2011). In these studies, polyphenols were added either before or after the milk was heat treated. However, it would be interesting to assess both methods in order to understand the effect of added polyphenols before or after heat treatment on the rheological properties of the gel. While typically polyphenols would be added before heat treatment in an industrial process, it is hypothesised that addition of polyphenols after heat treatment will have a different impact on product quality. Therefore, if technological improvements were found, manufacture of the polyphenol extracts and dairy products could be modified to achieve addition of polyphenols after heat treatment. The results of **Chapter 3** showed that heating the milk either before or after polyphenols addition did not influence the total phenolic content (TPC) of milk containing polyphenols. Therefore, the objective of the present study is to determine the impact of (a) four sources of polyphenols and (b) stage of addition of the polyphenols to milk (i.e. before or after it is heat-treated) on the rheological properties of acidified milk gels.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Design

A fully randomised factorial experiment was carried out in triplicate. The factors were polyphenol type (green tea, white grape, tannic or gallic acids) and stage of polyphenol addition i.e. polyphenols added before heat-treatment (M_hP_h) or after heat treatment (M_hP). A control sample which has no polyphenols was prepared by adding distilled water in place of the polyphenols.

4.2.2 Materials

Green tea (Nutraceutica, Monterenzio, BO, Italy), white grape (Nutripy CH- Hansen (Hørlshom, Denmark), tannic and gallic acids (Sigma Aldrich, Gillingham,UK) were

employed in this study. Pasteurized skim milk (0.08 ± 0.01% fat; 3.36 ± 0.02% protein content; 8.07 ± 0.04% total solids, 4.75 ± 0.06% lactose) was purchased from a local retailer. Glucono-deltalactone (GDL), sodium hydroxide (NaOH) were from Sigma Aldrich (Gillingham, UK).

4.2.3 Preparation of Milk-Polyphenol Samples for Acidification

Polyphenol solutions (5 mg ml^{-1}) were prepared by dissolving polyphenol powders in distilled water. This was added to milk, at the required addition stage according to the experimental design, to achieve a final polyphenol concentration of 1 mg ml^{-1} in the milk. When polyphenols were added to the milk, the samples were stirred for 30 min at room temperature.

The heat treatment typically used in yoghurt manufacture (85 °C for 30 minutes) was used in this experiment. It was applied to the M_hP_h and M_hP samples using a shaking water bath at 90 rev/min (Grant Instrument Ltd, Cambridge, UK). After heating the samples were rapidly cooled by immersion in ice-water. Heating and cooling profiles of the samples were monitored with a temperature logger which has type K thermocouple attached (ExTech SD200, Massachusetts, United States). After preparation of all samples, they were stored at 4 ºC overnight.

4.2.4 Acid Gel Preparation and Formation

The day after milk-polyphenol sample preparation, the temperature of the samples were adjusted to 30 °C in a water bath. Increasing the temperature from 4 ºC to 30 °C took on average 7 ± 0.1 min. The pH values of the milk samples were recorded using an Orion 3-star benchtop pH meter (Fisher Scientific Ltd, UK) and a glass combination electrode at 30 °C prior to acidification. The addition of polyphenols, except gallic acid, to pasteurized skim milk had no significant effect on the pH. However, addition of gallic acid lowered the pH of

both M_hP_h and M_hP samples from 6.7 to 6.4. Therefore, the pH of the gallic acid samples were adjusted back to pH 6.7, prior to acidification, by addition of 0.1 ml NaOH (1M). Glucono-deltalactone (GDL) was added (1.7%, w/w) to the milk and stirred for 2 minutes to produce acidified milk gels. Following GDL addition samples were incubated in a water bath at 30 °C. The pH values of the samples were measured every 10 minutes during gel formation using the pH meter and glass combination electrode. The samples were incubated for 3 hours 45 minutes until the pH of the samples dropped to at least pH 4.6.

4.2.5 Rheological Measurement

Rheological properties of acidified milk gels were measured using a C-VOR controlled stress rheometer (Bohlin Instruments Ltd., Gloucestershire, UK). An oscillation test was performed with a cup and bob geometry (C25DIN53019). All samples (M_hP and M_hP_h) were transferred to the rheometer 3 minutes after adding GDL and were covered with a layer of vegetable oil to prevent evaporation of the sample. A single frequency test to monitor gelation kinetics was performed with a constant strain of 1% and frequency of 0.1 Hz at 30 ºC (Lucey et al., 1997). Measurements were taken every 32 s for 3 hours and 45 minutes. Gelation time was defined as time taken to reach a storage modulus (G′) of 1 Pa, which was referred to start of aggregation of micelle. Gelation pH was defined as the pH at the gelation time (Harbourne et al., 2011; Anema et al., 2004). Final G′ was obtained from the rheometer after 3 hours and 45 minutes.

The gel formed after 3 hours and 45 minutes was subjected to a frequency sweep test from 0.001 to 1 Hz at 30ºC. Finally, a strain sweep from 0.5% to 300% (Oh et al., 2007) was also applied to the final gel at 30 ºC. Fracture stress was determined as maximum stress in which the gel started to break and the related strain was defined as fracture strain.

4.2.6 Statistical Analysis

Results in the text are given as mean values ± 2 standard errors (SEs) and this is stated as SE in the tables and graphs. The normality of data distribution was tested by Kolmogorov-Smirnov method. An independent t-test or a Mann Whitney test was used based on data distribution to detect differences between two treatments for each sample. Data was subjected to a one-way analysis of variance (ANOVA) or Kruskal-Wallis test to determine significant differences among samples for each treatments. Results with $p \leq 0.05$ were considered significantly different. SPSS Software was used for all analyses (Version 21.0, Armonk, NY: IBM Corp., USA).

4.3 RESULTS AND DISCUSSION

4.3.1 Effect of Addition of Phenolic Compounds on pH

The effect of polyphenol addition before or after heat treatment on the change of pH during acidification of milk is presented in **Figure 4.1a** and **4.1b**. Overall, the addition of polyphenols to pasteurised skim milk, whether before or after heat treatment, did not result in a change in the pH profile of samples during gelation. The rate of pH decrease of all samples were approximately 0.01 pH units in every 10 minutes (data not shown). In the current study, prior to addition of GDL, the pH of the gallic acid sample was adjusted back to 6.7. Therefore, during gelation the pH of all samples dropped from 6.7 to at least pH 4.6. Previous studies, which did not adjust the initial milk pH, found that addition of phenolic compounds (e.g. gallic acid, homovanillic and tannic acid) resulted in a decrease in initial pH, therefore the rate of gel formation increased (Harbourne et al., 2011; Han et al., 2011b). Similarly, Ozcan et al. (2015) found that milk heated at a lower pH value (6.2) resulted in

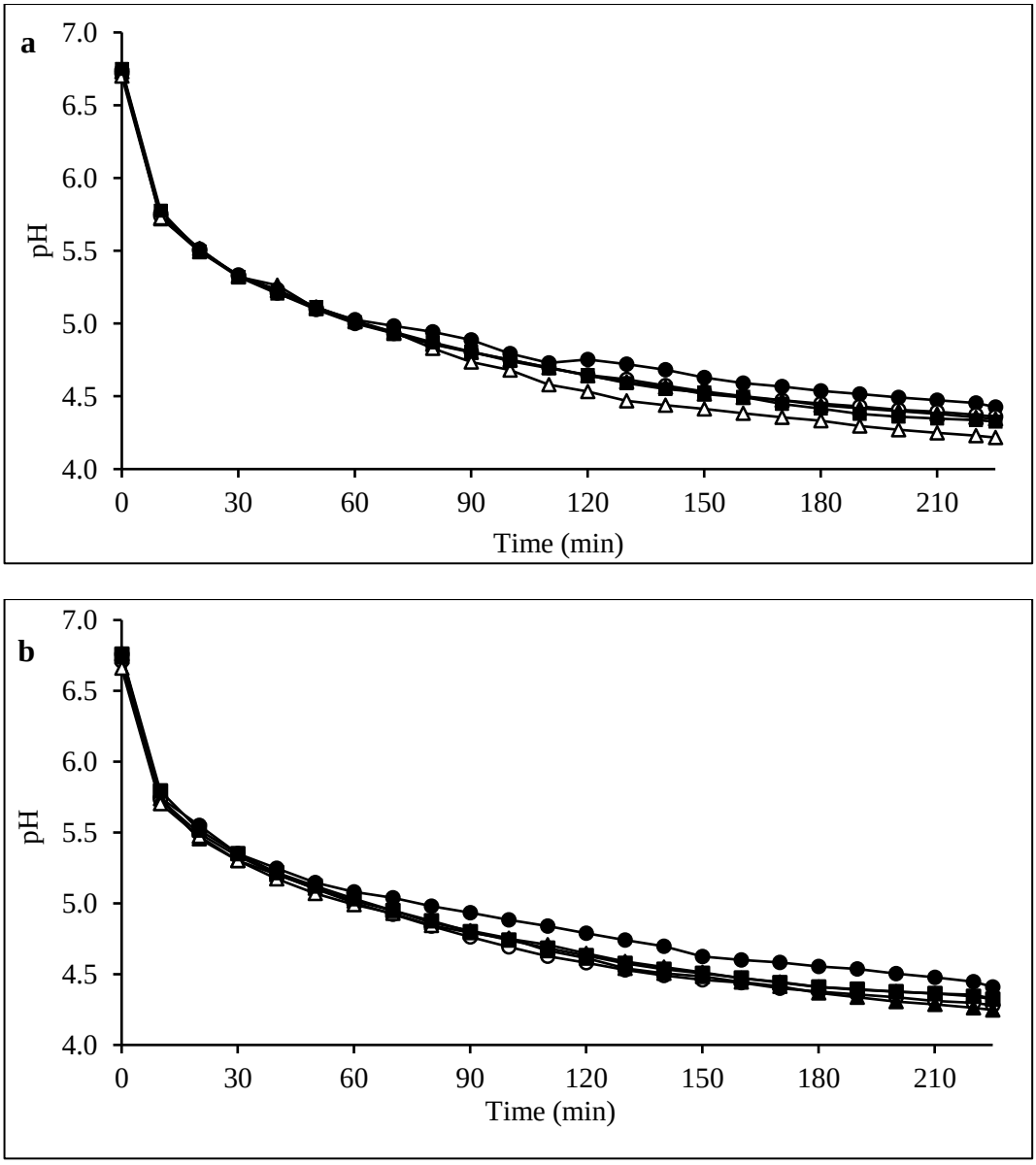

Figure 4.1 Change in pH with time after GDL addition to pasteurized skim milk heated (a) before and (b) after polyphenol addition. ●, control (no polyphenol); ○, green tea; ▲, white grape; Δ, tannic acid and ■, gallic acid. Data represented are means of three replicates.

faster fermentation rate than when milk was heated at higher pH values (6.7 and 7.2). Furthermore, pH adjustment of milk samples back to 6.7 after heat treatment showed a similar fermentation rate for all samples.

4.3.2 Gelation Kinetics

The impact of polyphenol addition on the storage modulus (G′) as a function of time after the addition of GDL is shown in **Figure 4.2**. The G′ values of all samples increased after the start of aggregation (G′ = 1 Pa) until they reached a plateau, which is in agreement with

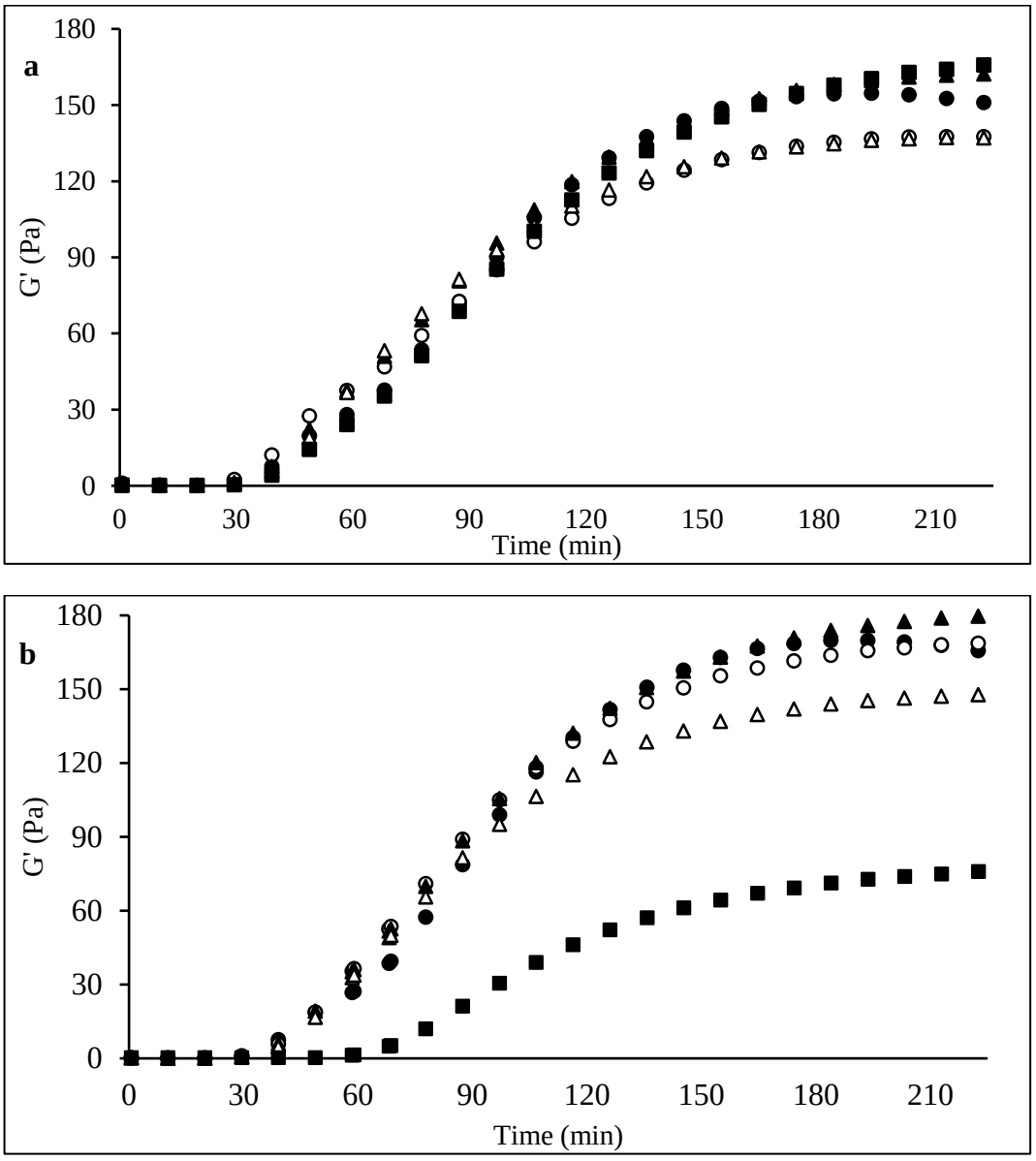

Figure 4.2 Storage modulus, G′, as a function of time for acid milk gels containing polyphenols ●, control (no polyphenol); ○, green tea; ▲, white grape; Δ, tannic acid and ■, gallic acid. Pasteurized skim milk heated (a) before and (b) after polyphenol addition. Data represented are means of three replicates.

previous studies (Lucey et al., 1997; Oh et al., 2007). After the start of aggregation, the G′ showed a rapid increase with time and reached a plateu after 200 min.

There were no significant difference between contol M_hP and control M_hP_h on gelation pH, gelation time and final G′ (**Table 4.1**). However, depending on the polyphenol employed and wheather it was added before (M_hP_h) or after (M_hP) heat treatment, the gelation kinetics of the polyphenol enriched milk samples were impacted (**Table 4.1 and Figure 4.2**). The addition of polyphenol solutions to pasteurized skim milk after heat treatment gave rise to some small differences in gelation time and gelation pH (**Table 4.1**). The final G′ of acid milk gel was not affected by the addition of polyphenol solutions to pasteurized skim milk after heat treatment (**Table 4.1 and Figure 4.2a**). Similarly, Han et al. (2011b) found that the addition of 0.5 mg ml^{-1} of green tea to milk prior to cheese manufacture did not significantly affect milk gelation kinetics. In addition to this, Harbourne et al. (2011) reported that addition of 0.1% w/w tannic acid after milk heat treated, which was the same concentration with this study, had no effect on gelation time and final G′ of acid gels.

The addition of all polyphenol solutions to pasteurized skim milk before heat treatment resulted in a significantly higher gelation time and significantly lower gelation pH as compared to the control sample (**Table 4.1**). However, the addition of gallic acid before heat treatment had the greatest impact on gelation time and gelation pH as compared to the addition of green tea, white grape and tannic acid. The addition of polyphenol solutions to pasteurized skim milk before heat treatment had a significant effect on the final G′ depending on the polyphenol employed (**Figure 4.2b**). The addition of white grape to pasteurized skim milk before heat treatment resulted in a significantly higher final G′, whereas tannic acid and

Table 4.1 Effect of two different treatments on gelation properties of skim milk samples enriched with polyphenols.

Acid gel sample	Treatment	Gelation time[A] (min)	Gelation pH[A]	Final G′ (Pa)[A]
Control	M_hP	32.66 ± 0.36^{b1}	5.30 ± 0.01^{b1}	150.46 ± 16.46^{a1}
	M_hP_h	32.48 ± 0.62^{c1}	5.33 ± 0.01^{a2}	165.15 ± 5.33^{a1}
Green Tea	M_hP	29.27 ± 0.64^{c1}	5.35 ± 0.01^{a1}	137.19 ± 23.33^{a1}
	M_hP_h	33.73 ± 0.36^{b2}	5.28 ± 0.01^{b2}	168.94 ± 3.76^{a1}
White Grape	M_hP	32.08 ± 0.51^{b1}	5.31 ± 0.03^{ab1}	162.20 ± 34.90^{a1}
	M_hP_h	34.09 ± 0.67^{b2}	5.26 ± 0.02^{b2}	179.86 ± 5.21^{b1}
Tannic Acid	M_hP	33.71 ± 1.26^{ab1}	5.29 ± 0.04^{bc1}	137.32 ± 24.19^{a1}
	M_hP_h	34.80 ± 0.71^{b1}	5.25 ± 0.02^{b1}	147.67 ± 0.96^{c1}
Gallic Acid	M_hP	36.05 ± 2.50^{a1}	5.25 ± 0.02^{c1}	165.91 ± 3.37^{a1}
	M_hP_h	60.72 ± 3.12^{a2}	5.02 ± 0.03^{c2}	76.97 ± 4.49^{d2}

[A] Data represented are means of three replicates ± SE. M_hP: pasteurized skim milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition. Gelation time (G′ = 1 Pa) was referred to the start of aggregation of micelle and gelation pH was defined as the pH at the gelation time.

[1-2] Numbers with different superscript in a column are significantly different at $p < 0.05$ between two treatments for same sample (M_hP versus M_hP_h).

[a-d] Letters with different superscript in a column are significantly different at $p < 0.05$ among five samples for each treatment (either M_hP or M_hP_h).

gallic acid addition gave rise to significantly lower final G′ than their relavant control sample. It can be noted that heating caused the unfolding of whey proteins (Dannenberg and Kessler, 1988) and may increase the binding affinity of polyphenols to protein surfaces (Bandyopadhyay et al., 2012). The higher final G′ may possibly be due to covalent and non-

covalent interactions between the main phenolic compounds present in the white grape flavanols, proantochyanidins and resveratrol) with milk proteins (Frazier et al., 2010; Liang et al., 2008).This occurrence during heat treatment or acid gelation could enhance the cross-linkings of proteins with grape polyphenols in the gel. Tannic acid has a relatively higher molecular weight (1,701.2 g/mol) compared to the other polyphenols used in this study. Therefore, when added prior to heat treatment it may lead to precipitation of some milk proteins (Feldman et al., 1999). This could ultimately result in the reduced gel firmness (final G′). Green tea inclusion before heat treatment had no significant effect on final G′. In particular, the additon of gallic acid (M_hP_h) had a greater effect than other polyphenols on the final G′ of acid milk gel.

The stage of gallic acid addition had a significant impact on gelation kinetics of acidified milk gels. The addition of gallic acid before heat treatment increased the gelation time by 24.7 min and decreased the gelation pH by 0.23 units as compared to its addition after heat treatment. Final G′ of the acid milk gels, with the exception of gallic acid, was not affected by the stage of addition of the polyphenol solutions. The addition of gallic acid before heat treatment significantly reduced the final G′ by over 50% compared to addition after heat treatment (**Table 4.1**). The pH adjustment of samples containing gallic acid was done after heating, therefore heating pasteurized skim milk with a lower initial pH (approximately 6.4) after addition of gallic acid (M_hP_h gallic acid) may significantly influence acid gelation kinetics of milk. The lower pH of the milk before heating markedly affected the attachment of denatured whey proteins to the casein micelle (Anema and Li, 2003). When the pH of milk is below 6.5 before heating, high levels of denatured whey proteins (~85%) attached to casein micelles. However, at high pH values (≥ 6.7) low levels of denatured whey proteins (~15%) are associated with the casein micelle and the denatured whey proteins remain in the serum phase of the milk as soluble aggregates when milk is centrifuged (Anema, 2007;

Vasbinder and de Kruif, 2003). Similar to this study's findings, Anema et al. (2004) and Schorsch et al. (2001) reported that, when milk was heated at a lower pH, the gelation of milk occurs more slowly and at a lower pH. These results suggest that denatured whey proteins that are associated with casein micelle aggregate at a lower pH than denatured whey proteins in the serum phase. This may explain the lower gelation pH of gallic M_hP_h in comparison to gallic M_hP.

Previous studies observed strong positive correlation between the denatured whey proteins in the serum phase and the gel firmness (final G′) of acid milk gel (Anema et al., 2004; del Angel and Dalgleish, 2006). Therefore, the use of milk samples in which denatured whey proteins are mostly in the serum phase resulted in higher final G′ than those in which denatured whey proteins are mostly attached to the casein micelles. The stiffness of the acid milk gels is related the number and strength of bridging or cross-linking in the gel (Lucey et al., 1998). When the pH of milk increased from 6.5 to 7.1 before heating, κ-casein dissociates from casein micelle and more κ-casein remains in the milk serum as compared to samples with lower pH values (Anema, 2007). Therefore, denatured whey proteins and κ-casein in the serum phase have more potential to interact which may be responsible for the higher G′ and increased firmness of the gel. In contrast, heating the milk at lower pH may have fewer contact points in acid gel than the milk heated at higher pH and resulted in lower final G′ (Anema, 2008).

4.3.3 Frequency Sweep

The straight lines of logarithm plots of frequency against G′ was drawn ($R^2 \geq 0.99$) and the slope of all acid gel samples regardless of the stage of polyphenol addition was approximately 0.16 (**Appendices**, **Table 2**), which is in agreement with previous studies (Lucey et al., 1997; Harbourne et al., 2011). To understand the nature of the bonds within

the gel network, tan δ (loss modulus G″/storage modulus G′) was plotted as a function of frequency for acid gel samples (**Figure 4.3**). The tan δ of all samples regardless of the stage of polyphenol addition showed a straight line against frequency and were not affected by

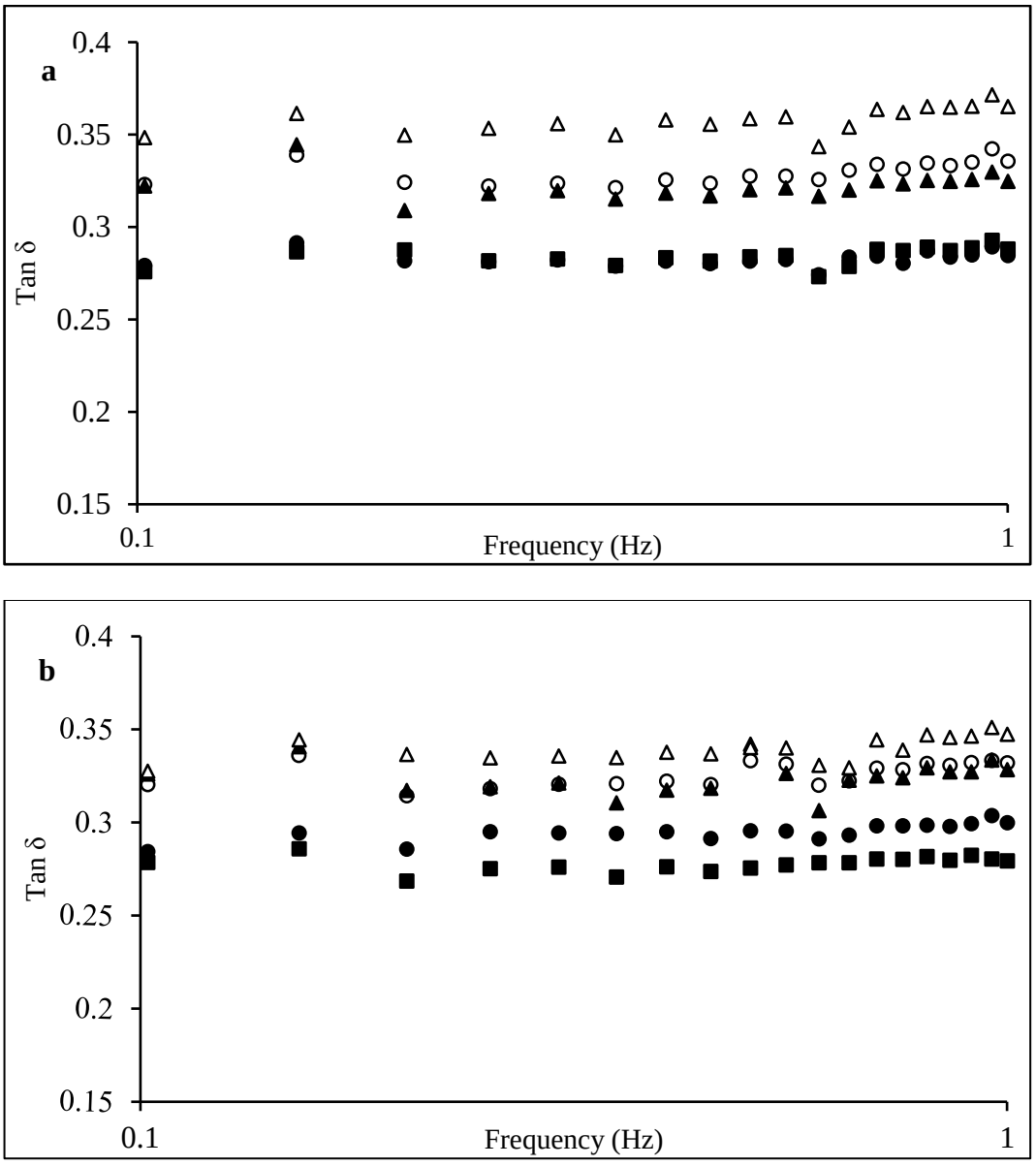

Figure 4.3 Tan δ as a function of frequency for acid milk gels containing polyphenols ●, control (no polyphenol); ○, green tea; ▲, white grape; Δ, tannic acid and ■, gallic acid. Pasteurized skim milk heated (a) before and (b) after polyphenol addition. Data represented are means of three replicates.

frequency, which is in agreement with Oh et al. (2007). In addition, all acid gel samples indicated solid-like behaviour because G′ was higher than G″. Furthermore, the difference between G′ and G″ was less than a log for all samples, which demonstrated that acid gels produced in this study could be classified as weak gels (Oh et al., 2007).

4.3.4 Large Deformation

The final polyphenol enriched acidified milk gels were subjected to a strain sweep test at 30 ºC and fracture stress was plotted against fracture strain (**Figure 4.4**). The large deformation test results demonstrate the susceptibility of strands to breakage and give an indication of types of bonds in the acid gel network (vanVliet and Walstra, 1995). The fracture stress of acidified milk gel was affected by the type of polyphenols used and the stage of polyphenol addition, whereas fracture strain did not alter with polyphenol addition (**Figure 4.4, Table 4.2**). The fracture strain is dependent on the degree of curvature strands which hold the gel network together (vanVliet and Walstra, 1995). There was no significant difference between control samples (M_hP and M_hP_h) on the fracture stress of acidified milk gels. The addition of all polyphenols to pasteurized skim milk after heat treatment (M_hP) did not show any significant effect on fracture stress and fracture strain as compared to their relevant control sample. (**Figure 4.4a, Table 4.2**). This finding correlates well with the final G′ of milk samples that enriched with polyphenols after heat treatment. Similarly, Najgebauer-Lejko et al. (2014) found that green tea addition after heat treatment had no effect on the firmness of yoghurt in comparison to a control sample. On the other hand, the addition of Pu-erh tea, which is a fermented tea like black tea, to yoghurt resulted in higher firmness than the addition of green tea. This was explained by the difference in the polyphenolic compositions of tea samples.

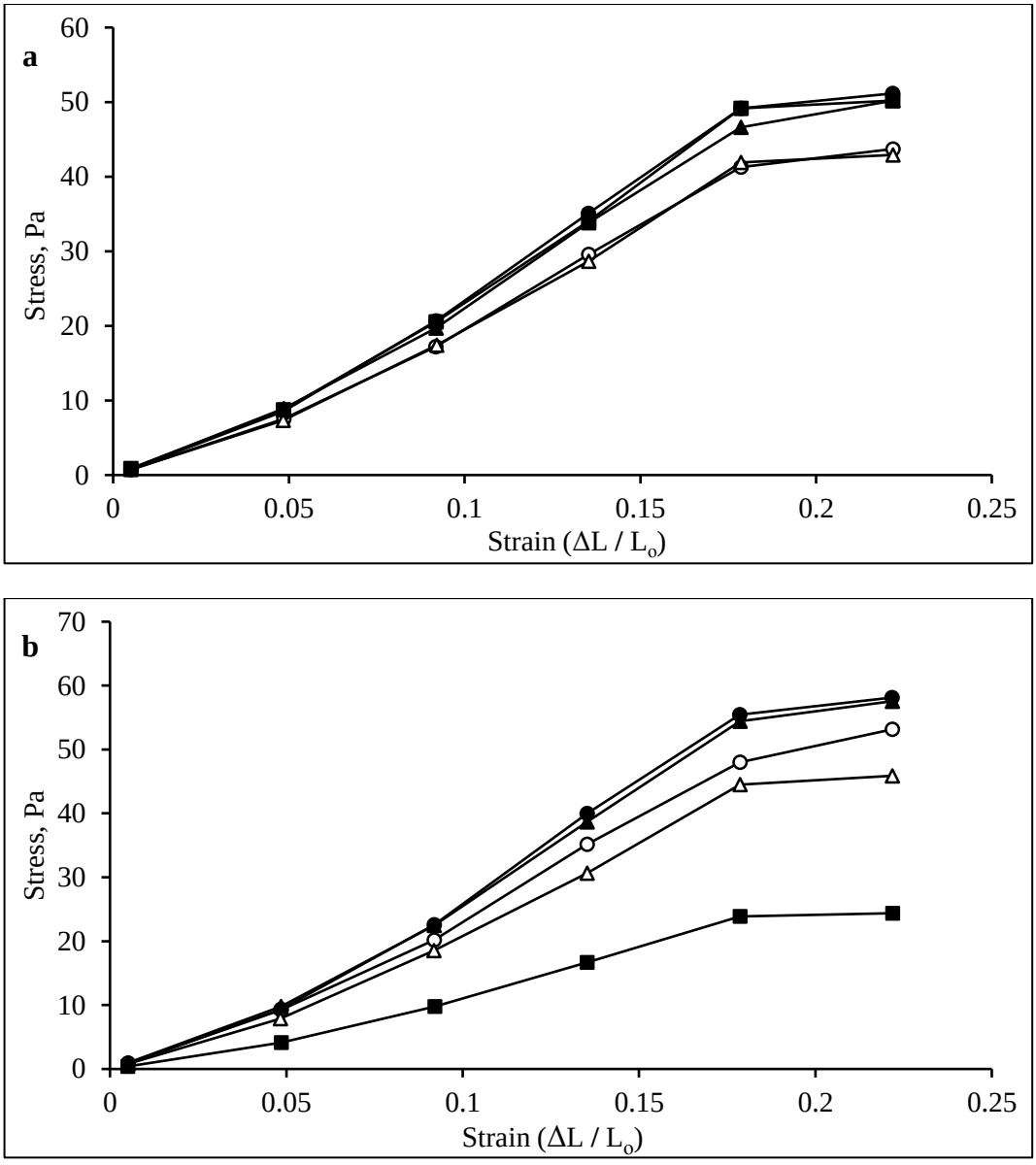

Figure 4.4 Stress as a function of strain for acid milk gels containing polyphenols ●, control (no polyphenol); ○, green tea; ▲, white grape; Δ, tannic acid and ■, gallic acid. Pasteurized skim milk heated (a) before and (b) after polyphenol addition. ΔL: change in length of sample (meter), L_o: original length of sample (meter). Maximum stress was defined as fracture stress at which the stress started to decrease. Data represented are means of three replicates.

The addition of green tea, tannic acid and gallic acid before heat treatment decreased the fracture stress in comparison with their relevant control sample. The addition of cocoa flavanols was also found to decreased the fracture stress of acidified gels (Vega and Grover,

2011). This was attributed to whey protein-flavanol interactions occurring during heating or acidification, ultimately leading to poor mechanical structure of acid milk gels. The addition of white grape to pasteurized skim milk before heat treatment resulted in the same fracture stress as its relevant control sample. Similarly, Han et al. (2011a) found that the addition of grape extract (0.5 mg ml^{-1}) to milk had no significant effect on the firmness of enzymatically

Table 4.2 Effect of two different treatments on final gel properties of skim milk samples enriched with polyphenols.

Acid gel sample	Treatment	Fracture stress (Pa)[B]	Fracture strain[B] ($\Delta L / L_o$)
Control	M_hP	51.31 ± 10.79[a1]	0.21 ± 0.027
	M_hP_h	58.12 ± 0.88[a1]	0.22 ± 0.0
Green Tea	M_hP	43.71 ± 7.24[a1]	0.22 ± 0.0
	M_hP_h	53.15 ± 1.07[b1]	0.22 ± 0.0
White Grape	M_hP	50.19 ± 13.39[a1]	0.22 ± 0.0
	M_hP_h	57.56 ± 0.13[a1]	0.22 ± 0.0
Tannic Acid	M_hP	42.92 ± 8.75[a1]	0.22 ± 0.0
	M_hPh	45.88 ± 0.37[c1]	0.22 ± 0.0
Gallic Acid	M_hP	50.38 ± 1.79[a1]	0.21 ± 0.027
	M_hP_h	24.38 ± 1.16[d2]	0.22 ± 0.0

[B] Data represented are means of three replicates ± SE. M_hP: pasteurized skim milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition. G′: storage modulus, ΔL: change in length of sample (meter), L_o: original length of sample (meter).

[1-2] Numbers with different superscript in a column are significantly different at $p < 0.05$ between two treatments for same sample (M_hP versus M_hP_h).

[a-d] Letters with different superscript in a column are significantly different at $p < 0.05$ among five samples for each treatment (either M_hP or M_hP_h).

induced milk gels. For the samples where green tea and white grape were added before heat treatment, the final G′ and fracture stress results did not correlate with that of their relevant control. This may be attributed to the large deformation test that the sample underwent which resulted in the gel network being completely broken rather than bending the structure as seen in the small deformation test (Singh et al., 2014). Covalent bonds require more energy to break than noncovalent (physical) bonds and therefore their presence will have a greater impact on the fracture behaviour of a gel in comparison to its firmness (final G′) (Anema, 2008). Therefore, adding green tea and tannic acid to milk before heat treatment could result in a reduced level of covalent bonds (such as disulphide bonds) thereby lowering their fracture stress in comparison to the control.

The fracture stress of all acid gel samples, except gallic acid, did not show any significant differences between two treatments (M_hP V M_hP_h) (**Table 4.2**, **Figure 4.4**). However, adding gallic acid before heat treatment rather than after resulted in an almost 50% decrease in fracture stress. This support the results for the final G′ of the gallic acid samples (**Table 4.1**). The low fracture stress was attributed to weak gel structure (Lucey et al., 2001). The milk heated at a lower pH caused markedly lower fracture stress of the acid milk gels than those heated at a higher pH, although heating the milk at different pH values did not show any effect on fracture strain (Anema, 2008; Lakemond and van Vliet, 2008).

4.4 CONCLUSIONS

It should be considered that the addition of polyphenols to milk before or after heat treatment could be a key parameter in modifying the gelation kinetics and fracture stress of acidified milk gel samples. However, this will depend on the sources of polyphenol. The addition of gallic acid to milk before heat treatment markedly affected the rheological properties of acid milk gel as compared to addition of gallic acid to milk after heat treatment.

This indicated that the pH of milk during heat treatment played a significant role in determining gelation time, gelation pH, final G′ and fracture stress in spite of pH adjustment of the milk samples including gallic acid, prior to acid gelation. This study suggests that the addition of polyphenols before heat treatment induced the interactions between milk proteins and polyphenols rather than polyphenol addition after heat treatment. This research can contribute the optimisation of processing conditions to improve textural properties of dairy products.

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CHAPTER 5

IMPACT OF STORAGE ON THE PHYSICOCHEMICAL PROPERTIES OF ACIDIFIED MILK GELS FORTIFIED WITH POLYPHENOLS

5.1 INTRODUCTION

In recent years, there has been increasing demand for functional foods. Yoghurts and other acidified dairy products are widely consumed, and could be an ideal dairy product to add various nutraceuticals e.g. polyphenols. Polyphenols are abundant in all plants and their primary sources are fruits and beverages such as grape, green tea and coffee. Many health benefits are attributed to polyphenols, for example, they can prevent the risk of cardiovascular diseases, cancers and neurodegenerative disorders (Cardona et al., 2013). Furthermore, polyphenols are often added to various food products to extend the shelf life due to their antioxidant and antimicrobial properties (Duangjai et al., 2016; Sayago-Ayerdi et al., 2009). For example, addition of tea catechins and grape pomace extracts increased the shelf life of meat and fish products by retarding lipid oxidation (Mitsumoto et al., 2005; Sanchez-Alonso et al., 2007). In addition, grape pomace extracts showed antimicrobial activity against foodborne pathogens in beef patties (Sagdic et al., 2011). Therefore, polyphenols may positively influence the storage stability of acidified dairy products. In **Chapter 4**, polyphenols were added to acidified milk gels and the impact of their addition of the rheological properties during gelation was established. However, to ensure the successful incorporation of polyphenols into acidified dairy products the impact of storage on technological defects (syneresis) and their physical properties (texture, colour) should be studied.

In chapter 4, polyphenols were added to acidified milk gels to improve their nutritional values. In previous years, green tea and grape extracts were also added to yoghurt formulations to investigate the effect of phenolic compounds on various parameters during

refrigerated storage. To date, most studies have focused on investigation the effect of phenolic compounds of green tea and grape extracts on the microflora of yoghurt during refrigerated storage (Jaziri et al., 2009; Najgebauer-Lejko et al., 2011). There have been limited studies regarding the effect of refrigerated storage on the physicochemical properties of yoghurt enriched with green tea and grape extracts. Tseng and Zhao (2013) reported that the enrichment of yoghurt with grape pomace during 3 weeks at 4 ºC improved the storage quality (TPC, DPPH radical scavenging activity, syneresis, viscosity, pH, peroxide value, colour, sensory attributes, lactic acid percentage) of yoghurt, thus it can be used to extend the shelf life of food products. In addition, phenolic compounds from green tea (Muniandy et al., 2016) and grape extract (Karaaslan et al., 2011) indicated good stability during refrigerated storage. In recent years, studies have investigated the effect of green tea addition on yoghurt textural properties (Najgebauer-Lejko et al., 2014) and impact of refrigerated storage on syneresis and rheological behaviours of yogurt containing green tea (Donmez et al., 2017). It can be noted that no study has investigated the effect of stage of polyphenol addition (before and after heat treatment) on the physicochemical properties of the yoghurt during storage. Therefore, the objective of the present study was to determine the effect of (a) four sources of polyphenols: green tea, white grape, tannic and gallic acids, and (b) stage of gallic acid addition on the pyhsicochemical properties of acidified milk gels during 28 days of refrigerated storage.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Design

The experiment was designed as a fully randomised two factorial (polyphenol and storage time) experiment which was carried out in triplicate. Four polyphenols (green tea, white grape, tannic and gallic acids) and 5 storage times (1, 7, 14, 21, 28 days' post manufacture) were employed. Acid milk gels supplemented with polyphenols were prepared by addition

of polyphenol solutions to skim milk before heat treatment (M_hP_h). This milk was then subjected to acidification. In addition, gallic acid was also added to milk after (M_hP) heat treatment as the results in **Chapter 4** indicated differences in the rheological properties of acidified milk gels depending on whether gallic acid was added before or after heat treatment. For each treatment, a control gel was prepared by replacing polyphenols with distilled water. The polyphenol fortified acid milk gels and control gels were stored at 4 ºC prior to analysis on days 1, 7, 14, 21, 28.

5.2.2 Materials

Dried extracts: green tea (Nutraceutica, Monterenzio, BO, Italy) and white grape (Nutripy CHR-Hansen, Hørlshom, Denmark) and single phenolic compounds: tannic and gallic acids (Sigma Aldrich, Gillingham, UK) were employed in this study. Pasteurized skim milk (0.08 ± 0.01% fat; 3.36 ± 0.02% protein content; 8.07 ± 0.04% total solids; 4.75 ± 0.06% lactose) was purchased from a local retailer. Sodium carbonate (Na_2CO_3) was supplied by Thermo Fisher Scientific Ltd (Loughborough, UK). Gluconodelta lactone (GDL), hydrochloric acid (HCI, 37%), methanol, Folin-Ciocalteu reagent, sodium acetate trihydrate, acetic acid, 2, 4, 6-Tris (2-pyridyl)-s-triazine (TPTZ), ferric chloride hexahydrate, ascorbic acid, sodium chloride (NaCI), potassium sorbate ($C_6H_7KO_2$), 0.45 µm PTFE filter and all the polyphenol standards that were used to quantify green tea and white grape were from Sigma Aldrich (Gillingham, UK).

5.2.3 Preparation of Acid Milk Gel Samples Fortified with Polyphenols

The polyphenol powders were dissolved in distilled water to prepare stock polyphenol solutions (5 mg ml^{-1}). The stock solutions were added to pasteurized skim milk to produce a final concentration of 1 mg ml^{-1} and stirred for 30 minutes at room temperature. A thermalization step of 85 °C for 30 minutes, typically used in acidified milk gel manufacture

to denature whey proteins and enhance gel texture, was applied to pasteurized skim milk using a water bath (Grant Instrument Ltd, Cambridge, UK). This heat treatment was applied to milk either after (green tea, white grape, tannic and gallic acids) or before (gallic acid) polyphenol addition. After heating, samples were rapidly cooled by immersion in ice-water and heating and cooling profiles of all samples were monitored by a temperature logger (ExTech SD200, Massachusetts, United States). All samples were stored at 4 ºC overnight. The following day, the temperature of the samples was adjusted to 30 ºC and potassium sorbate (0.01%, w/w) was added as a preservative. As the addition of gallic acid to milk reduced the milk pH from 6.7 to 6.4 as described in **Chapter 3**, the pH values of gallic acid samples were adjusted back to pH 6.7 by adding 1M NaOH prior to acidification. For acidification, glucono-deltalactone (GDL) (1.7%, w/w) was added and stirred for 2 min. The mixture was distributed into 250 ml sterile plastic containers (for texture, pH, colour, TPC and FRAP analysis), 50 ml centrifuge tubes (for syneresis by low speed centrifugation) or 250 ml volumetric flasks (for spontaneous syneresis analyses). The acidified milk samples were incubated in a temperature-controlled incubator (Sanyo Gallenkamp, Leicestershire, UK) at 30 ºC for 3 hours and 45 minutes.

5.2.4 pH Measurement

The pH values of samples were measured by an Orion 3-star benchtop pH meter (Fisher Scientific Ltd, UK) connected with a glass combination electrode at 4 ºC.

5.2.5 Syneresis

5.2.5.1 Low Speed Centrifugation

Syneresis of acid milk gels was detected using low speed centrifugation as proposed by Harbourne et al. (2011). After addition of GDL, milk samples (20 g) were poured into centrifuge tubes (30 x 115 mm) and incubated in a temperature-controlled incubator at 30

ºC for 3 hours and 45 minutes. After incubation, the samples were stored at 4°C until analysis. Then, the tubes were centrifuged (SIGMA, Laborzentrifugen, 3K10, Newtown Shropshire, UK) at 300 x g for 10 min at 4 ºC and the supernatant was removed and weighed. The syneresis index was expressed as percentage weight of supernatant over the initial weight of the acid milk gel.

5.2.5.2 Spontaneous Syneresis

Syneresis of samples was also measured using the volumetric flask method (Lucey et al., 1998). After addition of GDL, 225 g of each milk sample was poured into glass volumetric flasks (250 ml) and incubated at 30 ºC for 3 hours and 45 minutes. After the incubation, the samples were stored at 4°C until analysis. Then, the volumetric flasks were removed from the refrigerator at days 1,14, 28 and any free whey poured off and weighed. The syneresis index was expressed as percentage weight of supernatant over the initial weight of the milk.

5.2.6 Texture Analysis

The texture of acidified milk gel samples was determined in quadruplicate by a texture analyser (model TA-XT2i, Stable Micro Systems, Godalming, UK) with a 5 kg load cell. A two-cycle penetration test was performed directly on undisturbed acid gel samples in plastic containers (60 mm diameter × 60 mm height). The samples were taken refrigerated storage (4 ºC) and immediately analysed. A stainless steel cylinder probe (50.78 mm diameter) was introduced 10 mm into the samples at a constant velocity of 1 mm s^{-1}. The texture parameters: hardness, adhesiveness, springiness and cohesiveness were recorded from the generated force-time curve. Hardness is defined as the maximum force of the 1^{st} compression. Adhesiveness is measured as the negative work between the two cycles, which represents work needed to pull the probe away from the samples. Springiness is the distance of the detected height during the second compression divided by the original compression

distance. Cohesiveness is the area of work during the second compression divided by the area of work during the first compression (Bourne, 2002).

5.2.7 Colour Measurement

The colour parameters, L* (lightness), a* (redness), b* (yellowness), of samples were measured using a ColorLite spectrometer 850 (ColorLite GmbH, Germany). Acid gel samples were analysed in the sterilised plastic container (60 mm diameter × 60 mm height) with standard illumination of D65. Three measurements were taken for each sample. Hue angle (h*) expresses the colour difference and is defined as red–purple: 0º, yellow: 90º, bluish-green: 180º, and blue: 270º. Chroma (c*) is a measure of the purity or saturation of the colour. Hue angle and chroma were calculated using the following equations (McGuire, 1992). Colour difference (ΔE) of two samples were calculated based on the equations below:

$$\text{Hue angle } (h^*) = \arctan\left(\frac{b^*}{a^*}\right) \quad (\mathbf{5.1})$$

$$\text{Chroma } (c^*) = \left[(a^*)^2 + (b^*)^2\right]^{\frac{1}{2}} \quad (\mathbf{5.2})$$

$$\text{Colour difference } \Delta E^* = \sqrt{\left(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}\right)} \quad (\mathbf{5.3})$$

5.2.8 Extraction of Polyphenols from Acidified Milk Gel

The polyphenols were extracted from acidified milk gels as described in **Chapter 3**, **section 3.2.7**. After extraction, the supernatant of samples was collected for TPC, FRAP analysis and detection of individual phenolic compounds by HPLC or LC-MS.

5.2.9 TPC and FRAP Analyses

TPC and FRAP analyses were conducted as described in **Chapter 3**, **section 3.2.8**.

5.2.10 Detection of Individual Phenolic Compounds During Storage

Supernatants of green tea and white grape were filtered with 0.45 µm PTFE filter before injection on to HPLC and LC-MS respectively. The detection of green tea phenolic compounds by HPLC and white grape phenolic compounds by LC-MS were performed as described in **Chapter 3**, **section 3.2.2.1**.

5.2.11 Statistical Analysis

Results in the text are given as mean values ± 2 standard errors (SEs) and this is stated as SE in the tables and graphs. ANOVA test was used to examine the effect of polyphenol addition and storage time by using a univariate general linear model. Least Significant Difference (LSD) test was used for multiple means comparison at the significance level of $p< 0.05$. A paired t-test was used to compare the physicochemical properties of M_hP and M_hP_h gallic samples. Analyses were performed using SPSS Software for Windows (Version 21.0, Armonk, NY: IBM Corp., USA).

5.3 RESULTS AND DISCUSSION

5.3.1 TPC and FRAP Content of Polyphenol Enriched Acidified Milk Gels During Storage

As expected the addition of polyphenol rich extracts to acidified milk gel samples resulted in a significant increase in both the TPC and FRAP compared to the control (**Table 5.1**), which is in agreement with results from **Chapter 3** (**Figure 3.7**) and Najgebauer-Lejko et al. (2011). The acidified milk gels including gallic acid resulted in the highest TPC, this was followed by tannic acid, green tea and white grape respectively. The FRAP results of the polyphenol enriched milk gels followed a similar pattern (**Table 5.1**), albeit there was no significant difference between green tea and tannic acid enriched samples.

Table 5.1 TPC and FRAP values of acid milk gels fortified with polyphenols during refrigerated storage at 4 ºC.

Parameters[B]	Storage days	Samples[A]				
		Control	Green tea	White grape	Tannic acid	Gallic acid
TPC (mg GAE g^{-1})	1	0.14 ± 0.01^{a5}	0.72 ± 0.05^{a3}	0.54 ± 0.03^{a4}	0.87 ± 0.03^{ab2}	1.13 ± 0.08^{b1}
	7	0.14 ± 0.01^{a5}	0.71 ± 0.04^{a3}	0.55 ± 0.03^{a4}	0.82 ± 0.05^{b2}	1.09 ± 0.09^{b1}
	14	0.13 ± 0.01^{a5}	0.70 ± 0.04^{a3}	0.50 ± 0.03^{a4}	0.88 ± 0.03^{a2}	1.24 ± 0.04^{a1}
	21	0.13 ± 0.02^{a5}	0.70 ± 0.03^{a3}	0.54 ± 0.02^{a4}	0.85 ± 0.03^{abc2}	1.07 ± 0.06^{c1}
	28	0.11 ± 0.01^{a5}	0.67 ± 0.02^{a3}	0.50 ± 0.03^{a4}	0.79 ± 0.04^{c2}	1.09 ± 0.06^{bc1}
FRAP (µmol AAE g^{-1})	1	0.22 ± 0.04^{a4}	6.00 ± 0.54^{a2}	2.55 ± 0.25^{a3}	6.13 ± 0.69^{a2}	13.29 ± 1.68^{cd1}
	7	0.29 ± 0.04^{a4}	4.38 ± 0.49^{b2}	2.78 ± 0.59^{a3}	4.93 ± 0.82^{a2}	12.11 ± 1.66^{d1}
	14	0.26 ± 0.02^{a4}	5.07 ± 0.75^{ab2}	3.12 ± 0.58^{a3}	5.71 ± 0.56^{a2}	14.72 ± 1.86^{b1}
	21	0.23 ± 0.03^{a4}	4.52 ± 0.60^{b2}	2.28 ± 0.37^{a3}	5.46 ± 0.62^{a2}	13.66 ± 1.72^{bc1}
	28	0.29 ± 0.05^{a4}	5.17 ± 0.45^{ab2}	3.46 ± 0.57^{a3}	6.01 ± 0.82^{a2}	16.62 ± 1.68^{a1}

[A] All acid gel samples were produced by adding polyphenol solutions to milk before heat treatment.

[B] Values represent as means of three replicates ± SE. Different superscript in a column (a-d) and row (1-5) are significant at $p < 0.05$ for each texture parameter. TPC: total phenolic content, FRAP: ferric ion reducing antioxidant power.

The TPC and FRAP values of acidified milk gel samples during 28 days of cold storage was also presented in **Table 5.1**. The TPC and FRAP values observed for the control and polyphenol enriched acid milk gels were not significantly affected by storage time. Similarly, Pelaes Vital et al. (2015) found that the TPC of control yoghurts and yoghurts supplemented with oyster mushroom, which is rich in phenolics, remained stable during 28 days of cold storage. Muniandy et al. (2016) found that the impact of storage time on the TPC and antioxidant capacity was dependent on the type of tea (green, white and black) used. The TPC of yoghurts enriched with green tea and white teas both decreased by 12% over 21 days of cold storage, whereas the TPC in yoghurts enriched with black tea were stable over the same storage period. However, antioxidant capacity of all yoghurt samples enriched with teas remained almost constant during the storage period. Contrary to the results reported in current study, Chouchouli et al. (2013) reported a decrease in the TPC and FRAP values, of grape supplemented yogurt samples, over 32 days of cold storage and Bertolino et al. (2015) reported an increase in the TPC and antioxidant capacity of hazelnut skin supplemented yoghurt samples during 3 weeks of cold storage. The reported differences in the results for TPC and antioxidant capacity of yogurt samples containing polyphenols during storage could be related to the variety of phenolic compounds used. Furthermore, the stability of TPC and FRAP values in the present study during storage might be due to acidification of milk using GDL. Sun-Waterhouse et al. (2013) reported that in yoghurts the bacteria that was used for fermentation of the milk caused the degradation of phenolic compounds and resulted in formation of small phenolic molecules (phenolic acids). The degradation of phenolics by bacteria affected the total extractable polyphenol content in the yoghurt sample.

The green tea and white grape extracts contain a mixture of phenolic compounds, therefore the effect of refrigerated storage on the individual phenolic compounds found in these extracts was determined (**Table 5.2**). The most abundant phenolic compounds detected in green tea enriched acidified milk gels were catechin, EGCG and epigallocatechin, which is in agreement with what has been previously reported (Jaziri et al., 2009). In green tea only catechin and EGCG were significantly affected by refrigerated storage time, they decreased by 4% and 6% respectively after 28 days of storage. The EGCG and catechin content significantly decreased after day 7 and 14, respectively and then remained constant during the rest of the storage time. Several studies examined the interactions of milk proteins with tea polyphenols catechin, epicatechin, epigallocatechin and epigallocatechin gallate at molecular level and reported that tea polyphenols weakly bind to caseins (α- and β-caseins) (Hasni et al., 2011) and whey proteins (β-lactoglobulin) (Kanakis et al., 2011) through hydrophobic and hydrophilic interactions. Therefore, EGCG and catechin may form weak interactions with milk proteins as total extractable catechin and EGCG decreased in small amount (4% and 6%) during storage period.

The epicatectin, catechin and resveratrol were the dominant phenolic compounds in the acidified milk gel fortified with white grape, in agreement with previous studies (Karaaslan et al., 2011; Marchiani et al., 2016). In white grape, only catechin and epicatechin were significantly affected by storage time, they increased 35% and 25% respectively after 28 days. Both catechin and epicatechin content significantly increased after day 7 and then remained constant during the rest of the storage time. Similarly, Bertolino et al. (2015) found an increase in gallic acid, protocatechuic acid and rutin of hazelnut skin supplemented yoghurt after 3 weeks of refrigerated storage at 4 ºC. This was attributed to an increase in solubilization of these compounds into yoghurt due to decrease of pH during storage. In the

Table 5.2 Phenolic compounds of green tea and white grape during refrigerated storage at 4 ºC.

		Storage Days				
Polyphenols[A]	Phenolic Compounds[B]	1	7	14	21	28
Green Tea	Gallic acid	6.08 ± 2.58[a]	7.56 ± 0.95[a]	6.72 ± 0.91[a]	7.14 ± 0.57[a]	7.32 ± 0.71[a]
(µg g^{-1} acid gel)	Epigallocatechin	134.10 ± 2.53[a]	128.65 ± 2.01[a]	125.92 ± 2.11[a]	124.63 ± 9.39[a]	123.00 ± 7.07[a]
	Catechin	431.25 ± 5.86[a]	421.43 ± 8.03[a]	410.37 ± 0.92[b]	411.35 ± 13.74[b]	415.50 ± 7.67[b]
	Epicatechin	32.07 ± 0.81[a]	31.57 ± 1.79[a]	30.88 ± 1.43[a]	27.54 ± 7.27[a]	30.27 ± 1.70[a]
	EGCG	363.74 ± 8.35[a]	349.63 ± 4.19[b]	341.49 ± 11.13[b]	335.92 ± 41.55[b]	340.31 ± 9.83[b]
	Epicatechin gallate	29.04 ± 2.32[a]	24.63 ± 1.39[a]	24.75 ± 5.08[a]	22.30 ± 7.09[a]	27.01 ± 3.73[a]
White Grape	Resveratrol	6.02 ± 0.51[a]	6.48 ± 0.60[a]	6.09 ± 0.47[a]	4.86 ± 1.22[a]	5.65 ± 0.66[a]
(µg g^{-1} acid gel)	Quercetin-3-O-glucoside	5.59 ± 0.08[a]	5.65 ± 0.12[a]	5.49 ± 0.11[a]	5.67 ± 0.15[a]	5.76 ± 0.07[a]
	Catechin	12.66 ± 0.73[b]	16.27 ± 1.56[a]	17.25 ± 1.26[a]	17.52 ± 0.46[a]	17.14 ± 1.59[a]
	Epicatechin	26.95 ± 2.24[b]	34.45 ± 2.57[a]	34.15 ± 1.54[a]	34.84 ± 1.54[a]	33.71 ± 1.28[a]

[A] All acid gel samples were produced by adding polyphenol solutions to milk before heat treatment.

[B] Values represent as mean of three replicates ± SE. Different superscript in a row (a-b) are significant at $p < 0.05$. EGCG: Epigallo catechin gallate.

present study, it is possible that catechin and epicatechin may have increased solubility with the reduction of the pH of acidified milk gel containing white grape during storage (**Table 5.3**). As mentioned above, the catechin content decreased in very small amount (4%) in green tea during storage, whereas it increased (35%) in white grape. This could be related to the different forms of catechin in green tea and white grape which may affect its binding ability to milk proteins and solubility during storage. Perumalla and Hettiarachchy (2011) reported that the majority of catechin (around 75%) in grape was found as polymeric form like dimeric, trimeric and tetrameric, whereas catechin in green tea was found in monomeric form. Xiao et al. (2011) reported that structural differences of polyphenols had an effect on the binding affinity to milk proteins. Overall, phenolic compounds detected in both white grape (with the exception of catechin and epicatechin) and green tea were very stable during 28 days of refrigerated storage.

5.3.2 The pH and Syneresis of Acidified Milk Gels Fortified with Polyphenols During Storage

The pH of acidified milk gel on the day of production (Day 0) was not affected by the addition of polyphenol solutions (**Table 5.3**). After 1 day of refrigerated storage, addition of white grape and gallic acid had resulted in a significantly lower pH value (approximately 0.16 pH unit) in comparison to the control sample. Previous studies reported that the addition of green tea (Najgebauer-Lejko et al., 2014), grape and callus extracts (Karaaslan et al., 2011) had no significant effect on the pH of yoghurt samples. However, the addition of grape pomace to yoghurt reduced the yogurt pH from 4.78 to between 4.47 and 4.60 dependent on the grape pomace concentration (Tseng and Zhao, 2013). Similar to the effect of tannic acid addition on the pH of acidified milk gel in the current study, Harbourne et al. (2011) observed that the addition of tannic acid (up to 1%) had no significant effect on the pH of acidified milk gels, whereas the addition of all concentrations of gallic acid (0.3 - 1 %) decreased the

Table 5.3 pH and syneresis of acid gels fortified with polyphenols during refrigerated storage at 4 ºC.

Parameters[B]		Samples[A]				
	Storage days	Control	Green tea	White grape	Tannic acid	Gallic acid
pH	0	4.35 ± 0.05^{a1}	4.28 ± 0.09^{a1}	4.29 ± 0.06^{a1}	4.28 ± 0.05^{a1}	4.28 ± 0.07^{a1}
	1	4.28 ± 0.07^{a1}	4.28 ± 0.14^{a1}	4.11 ± 0.04^{b2}	4.27 ± 0.15^{a1}	4.12 ± 0.09^{b2}
	7	4.02 ± 0.10^{b2}	4.05 ± 0.06^{b2}	4.04 ± 0.02^{bc2}	4.16 ± 0.08^{b1}	4.00 ± 0.05^{c2}
	14	3.94 ± 0.03^{bc2}	3.98 ± 0.09^{b12}	3.97 ± 0.11^{ce12}	4.07 ± 0.07^{bd1}	3.97 ± 0.07^{c12}
	21	3.89 ± 0.08^{c12}	3.77 ± 0.08^{c3}	3.82 ± 0.04^{d23}	3.95 ± 0.08^{c1}	3.86 ± 0.09^{d13}
	28	4.01 ± 0.05^{b1}	3.98 ± 0.08^{b1}	3.92 ± 0.07^{de1}	3.98 ± 0.05^{cd1}	3.90 ± 0.04^{cd1}
Low Speed Centrifugation (%)	1	15.52 ± 1.57^{a12}	16.82 ± 1.41^{a1}	13.68 ± 0.71^{a2}	14.75 ± 1.11^{a12}	13.59 ± 1.23^{a2}
	7	14.58 ± 1.60^{ab1}	16.07 ± 1.35^{ab1}	14.40 ± 1.15^{a1}	15.09 ± 1.11^{a1}	13.90 ± 1.58^{a1}
	14	13.38 ± 1.87^{ab12}	14.75 ± 1.32^{ac1}	14.33 ± 2.40^{a1}	13.52 ± 1.35^{a12}	12.09 ± 1.58^{a2}
	21	12.53 ± 1.25^{b1}	14.11 ± 2.22^{bc1}	13.83 ± 1.85^{a1}	13.90 ± 1.85^{a1}	12.14 ± 1.35^{a1}
	28	12.48 ± 1.82^{b1}	12.76 ± 0.61^{c1}	12.73 ± 1.68^{a1}	13.33 ± 1.97^{a1}	13.29 ± 1.01^{a1}
Spontaneous Syneresis (%)	1	0 ± 0	0 ± 0	0.22 ± 0.22	0 ± 0	0 ± 0
	14	0 ± 0	0.09 ± 0.18	0.04 ± 0.05	0 ± 0	0 ± 0
	28	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[A] All acid gel samples were produced by adding polyphenol solutions to milk before heat treatment.

[B] Values represent as mean of three replicates ± SE. Different superscript in a column (a-e) and row (1-3) are significant at $p < 0.05$ for each parameter.

pH of milk. In the present study, gallic acid addition also caused a decrease in the initial pH of the milk, however it was readjusted to pH 6.7 (**section 5.2.3**) before acidification.

The pH of control and polyphenol enriched acidified milk gel samples significantly decreased during 28 days of refrigerated storage. It is likely that the hydrolysis of GDL to gluconic acid (Lucey and Singh, 1997) continues during storage. A similar decrease in pH during refrigerated storage has been observed in control and polyphenol fortified yoghurts as fermentative metabolism of the lactic acid bacteria continues to decrease the pH during storage (Lee et al., 2013; Trigueros et al., 2011). Ultimately, on the last day of storage (Day 28) there was no significant difference in the pH of control acidified milk gels or those enriched with polyphenols.

Syneresis or whey expulsion is a quality defect which appears during storage of acidified milk gels. The syneresis index of acidified milk gel samples was measured using low speed centrifugation and also without the use of external force to determine spontaneous syneresis (Lucey et al., 1998). The syneresis index of acid milk gel samples measured by low speed centrifugation ranged between 12.09-16.82% (**Table 5.3**). The addition of polyphenol solutions to acidified milk gels after 1 day of refrigerated storage had no significant impact on the syneresis index measured by centrifugation. Previous work on acidified milk gels similarly reported that tannic acid and gallic acid (up to 0.8%) addition had no significant effect on the syneresis index measured by low speed centrifugation (Harbourne et al., 2011), however the study also reported a significant increase in syneresis index when 1% gallic acid was added to the milk gels due to a rapid decrease in pH during acidification.

The syneresis index tended to decrease during refrigerated storage for all acidified milk gel samples, however the decrease was only significant for control and green tea samples. Green tea addition had no significant effect on syneresis of acidified milk gel during storage

in comparison with the control sample. In the current study, after 28 days of refrigerated storage the addition of polyphenol solutions had no significant effect on the syneresis index measured using low speed centrifugation in comparsion with the control sample. Therefore, polyphenols can be successfully added to acidified milk gels without adversely affecting the syneresis index during storage. The results in this study are in agreement with Tseng and Zhao (2013) who reported that the syneresis of control yogurts and those containing wine grape pomace (1-2%, w/w) were stable during 3 weeks of storage at 4 ºC. Similarly, Donmez et al. (2017) found that the syneresis of control yogurt and those containing green tea (0.01-2%) did not change during 21 days of storage.

The measurement of spontaneous whey separation using the volumetric method (Lucey et al., 1998) may be a more accurate representation of syneresis from a consumer's perspective. The results for spontaneous syneresis of acidified milk gels showed that all acidified milk gels had a syneresis index of <0.2% throughout refrigerated storage for 28 days, indicating that they were very stable throughout the storage period (**Table 5.3**).

5.3.3 The Changes in Texture Parameters During Acid Gel Storage

Overall, with the exception of white grape the addition of polyphenols to acidified milk gels caused a significant decrease in their hardness after 1 day of refrigerated storage (**Table 5.4)**. This result is in agreement with the results of fracture stress of acidified milk gels containing polyphenols (large deformation test) in **Chapter 4** (**Table 4.2**, **Figure 4.4**) indicated that the addition of green tea, tannic and gallic acids before heat treatment (M_hP_h) significantly decreased the fracture stress of acidified milk gel, whereas the addition of white grape (M_hP_h) had no effect on fracture stress. The added polyphenol-protein ratio of the gel samples ranged from 0.037 to 0.027 mg polyphenol/mg protein. Such variations, albeit small, may impact on the physicochemical properties of acidified milk gels during storage.

Table 5.4 Textural parameters of acid milk gels fortified with polyphenols during refrigerated storage at 4 ºC.

Texture parameter[B]	Storage days	Samples[A]				
		Control	Green tea	White grape	Tannic acid	Gallic acid
Hardness(g)	1	338.86 ± 17.58^{b1}	285.72 ± 15.99^{c2}	300.62 ± 43.17^{c12}	288.56 ± 22.26^{a2}	290.37 ± 27.92^{a2}
	7	367.02 ± 29.41^{ab1}	306.19 ± 29.40^{bc2}	309.63 ± 37.09^{bc2}	311.41 ± 29.27^{a2}	292.69 ± 27.11^{a2}
	14	355.90 ± 35.90^{ab12}	296.29 ± 22.28^{bc3}	367.43 ± 41.41^{a1}	321.14 ± 22.27^{a23}	318.03 ± 12.89^{a23}
	21	354.12 ± 30.44^{ab1}	326.98 ± 28.00^{ab1}	339.56 ± 24.77^{ac1}	317.10 ± 40.78^{a1}	325.79 ± 13.60^{a1}
	28	388.26 ± 30.29^{a1}	364.95 ± 33.06^{a12}	373.84 ± 16.44^{a1}	320.54 ± 21.84^{a3}	326.25 ± 21.53^{a23}
Adhesiveness (g s)	1	221.58 ± 31.99^{b2}	152.97 ± 22.17^{c3}	335.08 ± 23.15^{ab1}	165.48 ± 43.53^{b3}	118.68 ± 26.78^{a3}
	7	211.33 ± 30.48^{b2}	235.27 ± 35.29^{ab12}	286.14 ± 50.15^{bc1}	246.20 ± 45.64^{a12}	154.86 ± 12.26^{a3}
	14	216.36 ± 36.04^{b2}	231.26 ± 41.24^{b2}	382.97 ± 40.30^{a1}	238.11 ± 27.09^{a2}	169.06 ± 35.71^{a3}
	21	286.89 ± 37.39^{a2}	269.49 ± 44.49^{ab2}	339.74 ± 23.65^{a1}	209.18 ± 54.85^{ab3}	161.51 ± 25.14^{a3}
	28	237.99 ± 43.38^{ab1}	285.97 ± 30.68^{a1}	278.31 ± 48.56^{c1}	247.05 ± 24.19^{a1}	164.67 ± 38.20^{a2}
Springiness	1	0.91 ± 0.05^{a1}	0.97 ± 0.01^{a1}	0.91 ± 0.03^{a1}	1.02 ± 0.14^{a1}	0.93 ± 0.03^{a1}
	7	0.89 ± 0.03^{b1}	0.91 ± 0.05^{a1}	0.99 ± 0.16^{a1}	1.02 ± 0.14^{a1}	0.92 ± 0.03^{a1}
	14	0.89 ± 0.06^{b1}	0.99 ± 0.14^{a1}	0.89 ± 0.04^{a1}	1.01 ± 0.15^{a1}	1.00 ± 0.17^{a1}
	21	0.86 ± 0.04^{b1}	0.93 ± 0.03^{a1}	0.89 ± 0.04^{a1}	0.96 ± 0.02^{a1}	0.98 ± 0.16^{a1}
	28	1.04 ± 0.23^{a1}	0.99 ± 0.16^{a1}	0.93 ± 0.03^{a1}	0.94 ± 0.04^{a1}	0.92 ± 0.02^{a1}
Cohesiveness	1	0.48 ± 0.03^{a2}	0.55 ± 0.02^{a1}	0.48 ± 0.03^{a2}	0.54 ± 0.02^{a1}	0.55 ± 0.05^{a1}
	7	0.49 ± 0.06^{a1}	0.51 ± 0.02^{ab1}	0.49 ± 0.01^{a1}	0.53 ± 0.02^{a1}	0.52 ± 0.02^{a1}
	14	0.49 ± 0.03^{a1}	0.51 ± 0.03^{ab1}	0.45 ± 0.03^{a2}	0.53 ± 0.02^{a1}	0.52 ± 0.02^{a1}
	21	0.49 ± 0.02^{a1}	0.50 ± 0.03^{b1}	0.49 ± 0.03^{a1}	0.52 ± 0.02^{a1}	0.53 ± 0.02^{a1}
	28	0.48 ± 0.03^{a2}	0.49 ± 0.03^{b12}	0.49 ± 0.01^{a12}	0.51 ± 0.03^{a12}	0.52 ± 0.03^{a1}

[A] All acid gel samples were produced by adding polyphenol solutions to milk before heat treatment.

[B] Values represent as mean of three replicates ± SE. Different superscript in a column (a-c) and row (1-3) are significant at $p < 0.05$ for each texture parameter.

White grape samples had the lowest polyphenol-protein ratio (0.027 mg polyphenol/mg protein) in comparison to green tea, tannic acid and gallic acid (0.030, 0.032, 0.037 mg polyphenol/mg protein, respectively). These higher polyphenol protein ratios may result in formation of the polyphenol-protein complexes which may cause a decrease in the gel strength. Haratifar and Corredig (2014) reported that when the ratio of EGCG increases from 0.002 to 0.02 mg EGCG /mg protein, the stiffness (G′) of rennet-induced gel decreased from 36 Pa to 21 Pa. The authors have attributed the cause of decrease in gel stiffness to formation of catechin-casein micelles complexes and EGCG-casein micelle complexes increased the gelation time as compared to control sample, so which adversely affected the rennet induced gelation of milk. In addition, Donmez et al. (2017) reported that the lower concentration of 0.01 and 0.02 % green tea addition increased yogurt consistency coefficient (Pa.s), whereas higher concentration of green tea 1 and 2 % caused a decrease in yogurt consistency.

In general, the hardness of all samples increased during storage, however there was only a significant increase for control, green tea and white grape samples. Prasanna et al. (2013) reported an increase in the firmness of yogurt during 28 days of storage, which was attributed to more protein-protein interactions in the gel matrix during storage and improved the strength of yogurt. The increase in hardness over 28 days of refrigerated storage was 15% for control samples. However, the relative increase in hardness over this time period for green tea and white grape was, 28% and 24%, respectively. This may be attributed to the interactions between the phenolic compounds of green tea and white grape with milk proteins over storage (Oliveira et al., 2015). The decrease in the content of EGCG and catechin over storage supports this hypothesis. None of the phenolics in white grape decreased during storage as an indicative of bindings to milk proteins. It is possible that some of the phenolic compounds in white grape formed interactions with milk proteins, however those phenolics were not quantified. Similarly, Donmez et al. (2017) found that consistency

coefficient (Pa.s) of control yogurt increased significantly during 21 day of cold storage and green tea addition (0.01 and 0.02%) caused a higher consistency coefficient than control sample during storage. In the current study, the hardness of acidified milk gels containing tannic and gallic acids was not affected by 28 day of refrigerated storage. In general, the addition of polyphenols and storage time had no effect on the springiness and cohesiveness of acidified milk gels.

5.3.4 Colour Changes in Acidified Milk Gels Fortified with Polyphenols During Storage

The colour of food is the first sensory attribute perceived by consumers, therefore it influences consumer preference and may modify the other perceptions such as aroma and flavour. The effect of polyphenol addition and storage time on the colour of acidified milk gels is presented in **Table 5.5**. Overall, the addition of polyphenol solutions had an effect on lightness, hue angle and chroma after 1 day of storage and the changes were dependent on the source of polyphenol. With the exception of tannic acid, the lightness of polyphenol enriched gels decreased significantly after 1 day of storage in comparison to the control sample. Similarly, other studies found that addition of green tea (Najgebauer-Lejko et al., 2014) and grape seed (Chouchouli et al., 2013) extracts had a decreasing effect on the lightness of control yogurt sample. The addition of tannic acid (Day 1) had no significant effect on hue angle of acidified milk gel in comparison to the control sample. The hue angle of the acidified milk gels containing green tea (74.18°), white grape (92.22º), gallic acid (180.56°) indicated a slightly redder, yellow-green, bluish-green colour in comparison to the control sample, respectively. The hue angle for the control gel was similar to that previously reported in literature (Chouchouli et al., 2013). Similarly, Giroux et al. (2013) observed that the addition of green tea increased the redness and yellowness in cheddar-type cheese. The acidified milk gel containing white grape and gallic acid had the highest chroma and this

Table 5.5 Colour parameters of acid milk gels fortified with polyphenols during refrigerated storage at 4 ºC.

Colour Parameters[B]		Samples[A]				
	Storage days	Control	Green tea	White grape	Tannic acid	Gallic acid
L*	1	68.87 ± 0.60^{a1}	67.16 ± 0.45^{a2}	65.43 ± 0.80^{a3}	69.74 ± 0.55^{a1}	67.12 ± 2.05^{a2}
	7	66.47 ± 0.38^{b2}	64.65 ± 0.88^{c3}	63.51 ± 0.54^{bc4}	67.35 ± 0.34^{c12}	67.56 ± 0.73^{a1}
	14	66.49 ± 0.37^{b2}	65.93 ± 0.90^{b2}	64.65 ± 0.45^{a3}	68.64 ± 0.73^{b1}	68.04 ± 0.85^{a1}
	21	66.31 ± 0.31^{b2}	64.89 ± 0.66^{bc3}	64.44 ± 0.70^{ab3}	67.81 ± 0.39^{bc1}	67.66 ± 0.75^{a1}
	28	66.10 ± 0.43^{b2}	64.32 ± 0.83^{c3}	63.28 ± 0.60^{c3}	67.43 ± 0.51^{c1}	67.18 ± 0.59^{a2}
Hue angle (h*)	1	166.11 ± 9.32^{a2}	74.18 ± 4.63^{a4}	92.22 ± 2.99^{a3}	165.92 ± 6.29^{ab2}	180.56 ± 9.98^{a1}
	7	163.36 ± 3.16^{a1}	81.76 ± 3.97^{a3}	95.41 ± 2.70^{a2}	161.36 ± 6.43^{b1}	168.58 ± 8.39^{b1}
	14	171.21 ± 3.13^{a1}	80.62 ± 4.65^{a3}	95.28 ± 2.14^{a2}	169.38 ± 4.26^{ab1}	173.49 ± 5.62^{ab1}
	21	169.08 ± 4.24^{a2}	78.01 ± 3.22^{a4}	93.42 ± 2.28^{a3}	173.81 ± 6.06^{a12}	180.49 ± 10.78^{a1}
	28	170.90 ± 2.49^{a1}	78.59 ± 3.35^{a3}	93.44 ± 2.66^{a2}	171.24 ± 5.29^{a1}	176.32 ± 11.75^{ab1}
Chroma (c*)	1	2.23 ± 0.05^{a3}	3.04 ± 0.14^{a2}	4.12 ± 0.29^{a1}	1.49 ± 0.07^{a4}	3.79 ± 1.53^{a1}
	7	2.65 ± 0.20^{a3}	3.16 ± 0.28^{a2}	4.15 ± 0.26^{a1}	1.86 ± 0.10^{a4}	2.22 ± 0.14^{b34}
	14	2.20 ± 0.07^{a3}	3.10 ± 0.17^{a2}	4.18 ± 0.22^{a1}	1.84 ± 0.11^{a3}	2.14 ± 0.13^{b3}
	21	2.33 ± 0.10^{a3}	3.25 ± 0.25^{a2}	4.02 ± 0.21^{a1}	1.67 ± 0.07^{a4}	1.95 ± 0.10^{b34}
	28	2.39 ± 0.06^{a3}	3.22 ± 0.23^{a2}	3.98 ± 0.27^{a1}	1.63 ± 0.07^{a4}	2.03 ± 0.14^{b34}

[A] All acid gel samples were produced by adding polyphenol solutions to milk before heat treatment.

[B] Values represent as mean of three replicates ± SE. Different superscript in a column (a-c) and row (1-4) are significant at $p < 0.05$ for each colour parameter.

was followed by green tea after 1 day of storage. However, the addition of tannic acid resulted in a significant decrease in the chroma of acidified milk gel. In terms of overall colour difference (ΔE) from the control, both the green tea and white grape samples had ΔE of ~4, while the tannic and gallic acid samples had a ΔE of ~1. This suggests the tannic and gallic acid samples should be indistinguishable from the control samples unless the samples were adjacent to one another. Refrigerated storage significantly decreased the lightness of control and acidified milk gels containing polyphenols except gallic acid, albeit this decrease was very small. For example, the lightness of control sample decreased from around 69 to 66 during 28 days of refrigerated storage, which was similar for other samples. However, hue angle and chroma of control and acidified milk gels containing polyphenols were not affected by refrigerated storage with the exception of chroma of gallic acid. As reported by Sah et al. (2016), the hue angle and chroma of set-type control yoghurt was not affected by storage time. Overall, colour difference (ΔE) of polyphenol enriched acid milk gel samples from control was not affected by storage time, which would be desirable outcome for food products.

5.3.5 Physicochemical Properties of Gallic Enriched Acidified Milk Gels During Storage

The stage of gallic acid addition (before or after heating) had a significant impact on the rheological properties of acidified milk gels in **Chapter 4**, therefore the physicochemical properties of acid milk gels when gallic acid was added to milk before (M_hP_h) and after (M_hP) heat treatment were compared during storage in **Figure 5.1**. The stage of gallic acid addition had no significant effect on TPC and FRAP values of acidified milk gels during 28 days of refrigerated storage (**Appendices**, **Table 4**), which is in agreement with the results of milk-polyphenol mixtures presented in **Chapter 3** (**Figure 3.3 and Figure 3.4**). Overall, there was no significant difference in pH or lightness (**Figure 5.1**), or chroma and hue angle

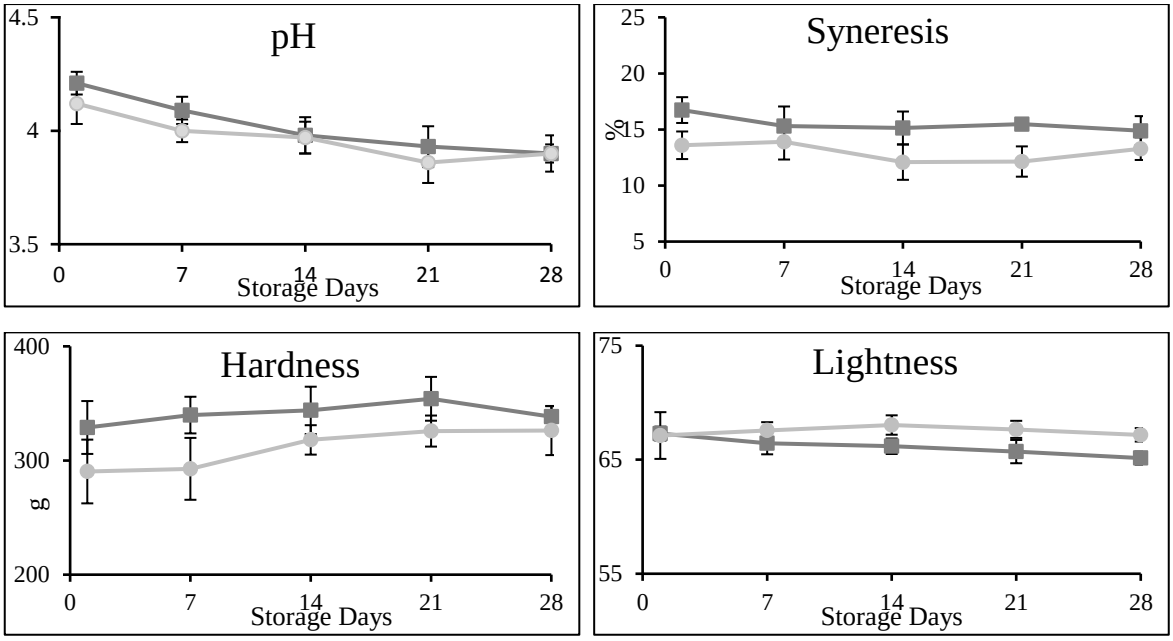

Figure 5.1 The comparion of physicochemical properties of acid milk gels when gallic acid added to milk before ● (M_hP_h) and after heat treatment ■ (M_hP) during refrigerated storage. Values represent as mean of three replicates ± SE.

(data not shown) between M_hP_h and M_hP gallic acid samples during storage. The overall colour difference (ΔE) between gallic M_hP and M_hP_h samples was ~1 at each time point. The addition of gallic acid to milk after heat treatment (M_hP) resulted in a significant increase in the syneresis index of acidified milk gel as compared to M_hP_h gallic acid sample after 1 day and 21 day of storage, whereas there was no significant difference in syneresis of gallic M_hP and gallic M_hP_h after 28 days of refrigerated storage. In general, the hardness of gallic M_hP tended to be higher than gallic M_hP_h during 28 days of refrigerated storage, which is in agreement with the results of fracture stress in **Chapter 4**. The addition of gallic acid before heat treatment (M_hP_h) resulted in a lower fracture stress than gallic M_hP. The lower pH (6.4) of gallic M_hP_h before heating increased the attachment of denatured whey proteins to casein micelles. This may result in a decrease in hardness of gallic M_hP_h in comparison with hardness of gallic M_hP. In addition, the higher hardness of gallic M_hP could be due to higher syneresis index tendency of gallic M_hP than gallic M_hP_h during storage. Therefore, the

decrease in water in the gel system may cause an increase in hardness of the gallic M_hP sample (Pelaes Vital et al.,2015). Overall, while the stage of gallic acid addition (before or after heat treatment) may not affect the nutritional properties of the product, it may impact on the syneresis and hardness of acidified milk gels during shelf life.

5.4 CONCLUSIONS

The hardness of acid milk gels containing green tea and white grape increased significantly during storage, whereas the hardness of acid milk gels containing tannic or gallic acids was not affected by refrigerated storage. This is possibly related to the interactions of phenolic compounds of green tea and white grape with milk proteins over storage. Spontaneous syneresis and colour difference (ΔE) of acid milk gels containing polyphenols were stable during 28 days of refrigerated storage. However, the pH of all samples decreased over storage, which needs to be further investigated in terms of consumer acceptance. The TPC and FRAP values of polyphenols enriched acidified milk gel samples remained stable for all samples during 28 days of refrigerated storage. This is a desirable result for consumers who demands for healthier foods with longer shelf life. The stage of gallic acid addition should be considered before fortifying such dairy products as it had an impact on syneresis and hardness of acid milk gel during 28 days of refrigerated storage. Based on the results, it is concluded that the polyphenols may be added to acidified milk gels with minimal impact on physicochemical properties during storage.

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CHAPTER 6

OVERALL CONCLUSIONS AND RECOMMENDATIONS

6.1 OVERALL CONCLUSIONS

This thesis aimed to provide the knowledge required to develop strategies that could be used to successfully incorporate polyphenols into dairy products without impacting on product quality. This included identification of appropriate inclusion points for polyphenols during processing, understanding how the chemical and physical properties of polyphenols could impact on their suitability in such an application, and exploring the quality and stability of polyphenol enriched dairy product during shelf life. In particular this thesis focused on the enrichment of acidified milk gel products using both phenolic rich extracts (green tea and white grape), and single phenolic compounds (tannic and gallic acids). In addition to phenolic source, the impact of their addition before and after a heat treatment on gelation kinetics, and nutritional, rheological and physicochemical properties of final product were determined.

The review of literature validated the research questions and design of the study. The literature review indicated that the incorporation of polyphenols into dairy products to improve their health benefits is of great interest. However, contradictory results were reported regarding the effect of milk on antioxidant capacity of polyphenols, which is possibly due to the phenolic sources utilised. In addition, the effect of dairy processing on polyphenols and milk proteins in a dairy matrix is not fully understood. Hence the requirement to evaluate the impact of the incorporation of polyphenols under typical processing conditions on the technological and physicochemical properties of acidified milk gel.

Polyphenols were successfully incorporated into skim milk and acidified milk gel samples. This thesis found that the effect of milk on the antioxidant capacity of polyphenols

is dependent on the processing conditions. Overall, the addition of polyphenols to milk at room temperature had no significant effect on total phenolic content (TPC) and antioxidant capacity of polyphenols. However, heat treatment of milk at 85ºC for 30 minutes reduced the TPC and antioxidant capacity of polyphenols regardless of whether polyphenols were added to milk before (M_hP_h) or after (M_hP) heat treatment. This suggests that thermal denaturation of whey proteins may increase the binding capacity of proteins to polyphenols. In addition, acid gelation demonstrated a lower extractable polyphenol as compared to M_hP_h milk samples. This indicated that processing conditions had a significant effect on the TPC of polyphenols in a dairy matrix. The stage of gallic acid addition significantly affected the casein micelle size (CMS) of milk, demonstrating that the addition of gallic acid before heat treatment resulted in a significant increase in CMS as compared to addition of gallic acid after heat treatment. This was attributed to higher attachment of denatured whey proteins to casein micelle due to lower pH of milk with gallic acid addition prior to heat treatment.

The effect of stage of gallic acid addition on the CMS of milk was also linked to its impact on the coagulation kinetics observed for this sample. The addition of gallic acid before heat treatment significantly affected the gel formation properties and the large deformation test results. For example, gelation time increased, gelation pH, storage modulus (G′) and fracture stress decreased. The gallic M_hP_h had the weakest gel than all other polyphenols addition. Ultimately this was suggested to be due the pH dependent nature of association of denatured whey protein during heat treatment. The stage of polyphenol addition and pH of polyphenols should be considered when enriching polyphenols into dairy products. These findings can contribute the optimisation of processing conditions to improve textural properties and nutritional content of dairy products.

The results of rheological tests demonstrated that only gallic acid showed differences with the stage of polyphenol addition rather than other polyphenols. Therefore, polyphenols, with

the exception of gallic acid, were added to milk before heat treatment (M_hP_h) and acidified milk gel samples were produced to examine the effect of refrigerated storage on physicochemical properties. The 28 days of refrigerated storage had no significant effect on TPC, FRAP values, syneresis and pH, whereas syneresis, textural properties and colour parameters of acidified milk gels were affected during storage depending on the source of polyphenols. The hardness of control, green tea and white grape significantly increased after 28 days of refrigerated storage, however the relative increase in green tea and white grape samples were higher than control sample. This can be attributed to interactions between the phenolic compounds of green tea and white grape with milk proteins over storage. The stage of gallic acid addition had no significant effect on, TPC, FRAP, pH, and colour parameters during 28 days of refrigerated storage, whereas it had an impact on hardness and syneresis of acidified milk gels.

6.2 RECOMMENDATIONS

Polyphenol-Protein Interactions

Additional research should be conducted to characterize the types of interactions between pure milk proteins and specific phenolic compounds in the dairy matrix such as covalent and non-covalent bonds by using analytical techniques. Such techniques could include circular dichroism spectroscopy, fluorescence emission spectrometry, FTIR spectroscopy, NMR and mass spectroscopy. This could provide better understanding of milk-protein interactions on the antioxidant capacity of polyphenols in the complex gel matrix and on the rheological and physicochemical properties of acidified milk gel samples.

Impact of Starter Cultures

The current study can be applied to yogurt and dairy based deserts and the effect of starter culture on the polyphenols under processing conditions could be investigated. This could include assessing the impact of polyphenols on starter culture activity, polyphenol stability and product quality.

Sensory Profiling and Consumer Tests

Sensory profiling is required to determine if polyphenols impact on the sensory quality of the final product. In addition, consumer acceptability should also be examined. This could also include the impact of providing nutritional information on the acceptability of the enriched products. Sensory analysis should be applied on either acidified milk gel or yogurt products. Sensory attributes such as appearance, texture, odour, taste and mouthfeel based on the source of polyphenols could be determined and these results could be compared with instrumental methods.

Assessing Bioavailability of the Formulated Product

The effect of milk protein-polyphenol interactions on the bioactivity and absorption of polyphenols should be further studied. Both in vitro and in vivo digestion models should be used. This would allow for a true assessment of polyphenol and protein bioavailability and digestibility. This could lead to further optimization of dairy processing to ensure maximum nutritional benefit.

1. Additional results which are related Chapter 3

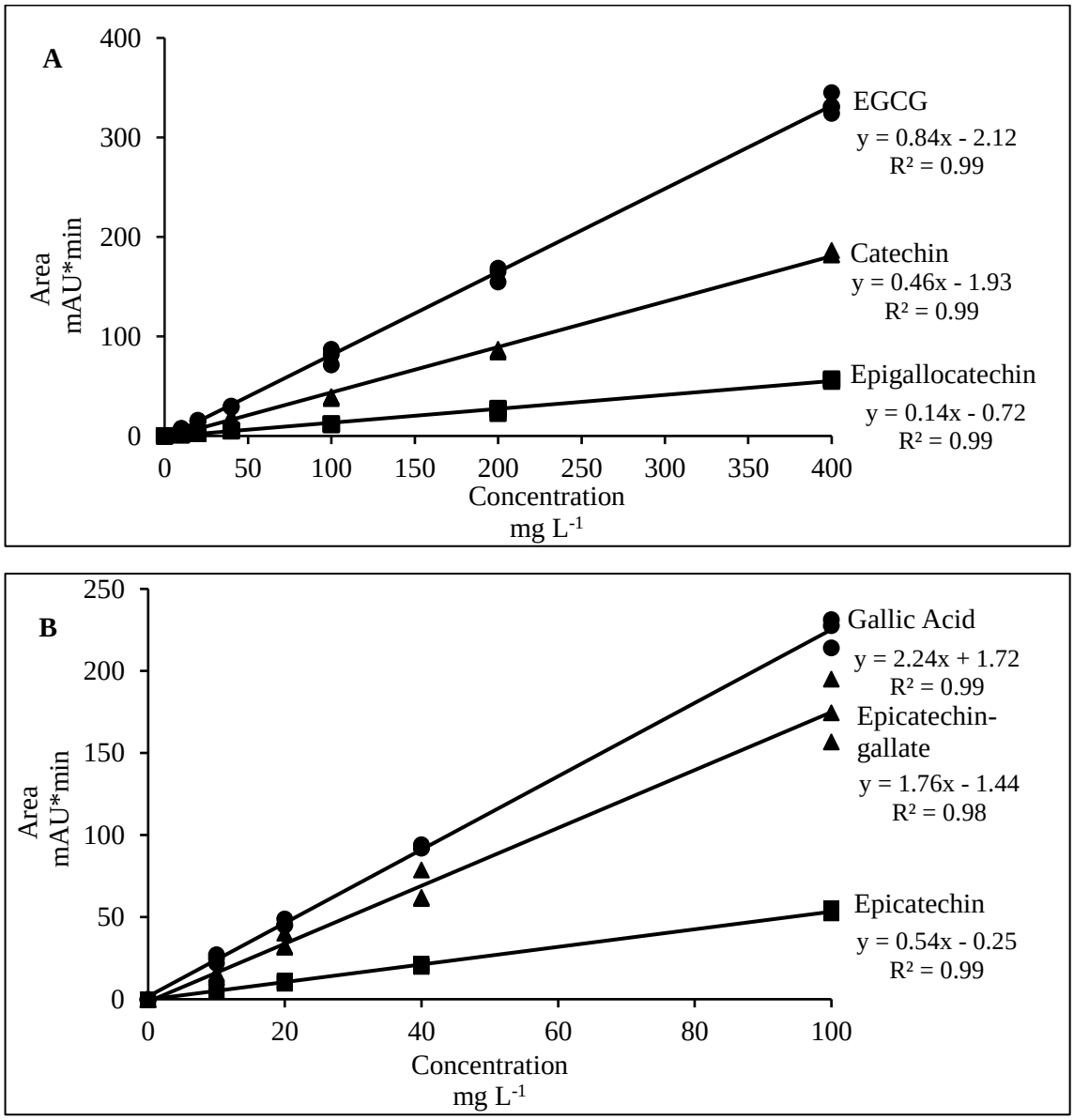

Figure 1 Calibration curves of phenolic compounds detected in green tea. Lines show the results of three replicates. EGCG: Epigallocatechin-gallate.

Table 1 The comparison of the effect of pH adjusted pasteurized skim milk and gallic M_hP_h on CMS and undenatured whey protein level physical properties of milk proteins.

Samples[A]	pH	CMS (d. nm)	Undenatured-whey protein level (mgN g^{-1})
pH adjusted milk sample	6.41 ± 0.02[a]	255.29 ± 5.67[a]	3.92 ± 0.17[a]
Gallic M_hP_h	6.40 ± 0.01[a]	253.17 ± 8.35[a]	4.14 ± 0.11[a]

[A] Data are expressed as means of three replicates ± SE. CMS: casein micelle size.

[a] Means within a column with different superscript are significantly different at $p < 0.05$.

2. Additional results which are related Chapter 4

Table 2 Effect of two treatments on the pH of skim milk and acid gel samples enriched with polyphenols and final gel properties.

Acid gel[A] sample	Treatment	Initial pH of skim milk at 30 ºC	Final pH of acid milk gels	Slope of logarithm plots of frequency against G′
Control	M_hP	6.74 ± 0.01^{a1}	4.43 ± 0.11^{a1}	0.158 ± 0.002^{c1}
	M_hP_h	6.76 ± 0.02^{a1}	4.41 ± 0.01^{a1}	0.160 ± 0.002^{c1}
Green Tea	M_hP	6.72 ± 0.01^{b1}	4.36 ± 0.04^{a1}	0.177 ± 0.002^{b1}
	M_hP_h	6.71 ± 0.01^{b2}	4.28 ± 0.05^{b1}	0.177 ± 0.002^{b1}
White Grape	M_hP	6.73 ± 0.01^{b1}	4.35 ± 0.03^{a1}	0.177 ± 0.002^{b1}
	M_hP_h	6.74 ± 0.01^{ab1}	4.25 ± 0.05^{b2}	0.178 ± 0.001^{b1}
Tannic Acid	M_hP	6.70 ± 0.02^{b1}	4.22 ± 0.02^{b1}	0.191 ± 0.001^{a1}
	M_hPh	6.66 ± 0.01^{c2}	4.33 ± 0.05^{ab2}	0.184 ± 0.001^{a1}
Gallic Acid	M_hP	6.37 ± 0.04^{c1}	4.33 ± 0.04^{ab1}	0.160 ± 0.001^{c1}
	M_hP_h	6.40 ± 0.02^{d2}	4.32 ± 0.03^{ab1}	0.163 ± 0.001^{c1}

[A] Data represented are means of three replicates ± SE. M_hP: pasteurized skim milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition. G′: storage modulus.

[1-2] Numbers with different superscript in a column are significantly different at $p < 0.05$ between two treatments.

[a-d] Letters with different superscript in a column are significantly different at $p < 0.05$ among samples for each treatment.

3. Additional results which are related Chapter 5

3.1. Protein Analysis by HPLC

Acid milk gel samples were centrifuged (SIGMA, Laborzentrifugen, 3K10, Newtown Shropshire, UK) at 4000 G at 4 ºC for 15 min to seperate casein and whey proteins. The precipitated casein was washed twice with distilled water and solubilised by increasing the pH to 7 by adding 0.1 M NaOH. The solutions containing whey and casein proteins were filtered using 0.45 µm PVDF filter and injected to a Dionex HPLC with a P680 HPLC pump, ASI-100 automated sample injector, thermostated column compartment TCC-100 and PDA-100 photodiode array detector. A C18 column (5 µm/ 250 x 4.6 mm, Jupiter 5u, Phenomenex, Macclesfield, UK) was used at 40 ºC. Separation was carried out by gradient elution using 0.1% trifluroacetic acid (TFA) in water (mobile phase A) and 80% acetonitrile and 0.555% TFA in water (mobile phase B). The following gradient was used: 0-8 min 43-47 B; 8-16 min 47-52% B; 16-22 min 52-57% B, 22-23 min 57-58% B; 23-28 min 58-100% B. Analysis was carried using an injection volume of 50 µL, flow rate of 1 mL min^{-1}, and the UV detection was at 220 nm. Standard curves of bovine milk proteins (β-LG, α-LA, α-CN, β-CN and κ-CN) were prepared in triplicate for quantification of acid milk gel proteins.

Table 3 Protein content of acid milk gels fortified with polyphenols during refrigerated storage at 4 ºC.

		Samples[A]				
Proteins[B]	Storage Days	Control	Green tea	White grape	Tannic acid	Gallic acid
κ-casein (mg g^{-1})	1	17.36 ± 4.33^{a1}	15.25 ± 4.44^{a1}	13.48 ± 5.00^{a1}	12.28 ± 4.94^{a1}	12.34 ± 3.90^{a1}
	28	18.63 ± 3.42^{a1}	15.63 ± 2.03^{a1}	15.52 ± 3.15^{a1}	13.56 ± 3.09^{a1}	13.62 ± 2.71^{a1}
α-casein (mg g^{-1})	1	73.03 ± 11.80^{a12}	73.26 ± 17.40^{a12}	75.88 ± 10.09^{a1}	65.76 ± 8.57^{a12}	56.38 ± 7.24^{a2}
	28	64.49 ± 7.58^{a2}	86.17 ± 22.43^{a1}	59.69 ± 5.54^{a2}	62.65 ± 6.23^{a2}	63.37 ± 8.06^{a2}
β-casein (mg g^{-1})	1	86.33 ± 12.86^{a1}	85.94 ± 19.85^{a1}	83.65 ± 11.62^{a1}	75.19 ± 9.25^{a1}	71.56 ± 10.01^{a1}
	28	80.06 ± 8.43^{a1}	75.75 ± 10.36^{a1}	66.58 ± 7.96^{b1}	68.67 ± 15.12^{a1}	75.43 ± 10.00^{a1}
α-LA (mg ml^{-1})	1	0.14 ± 0.04^{a1}	0.31 ± 0.06^{a1}	0.20 ± 0.06^{a1}	0.21 ± 0.11^{a1}	0.28 ± 0.23^{a1}
	28	0.16 ± 0.07^{a1}	0.33 ± 0.07^{a1}	0.20 ± 0.06^{a1}	0.23 ± 0.12^{a1}	0.31 ± 0.27^{a1}

[A] All acid gel samples were produced by adding polyphenol solutions to milk before heat treatment.

[B] Values represent as means of three replicates ± SE. Different superscript in a column (a-b) and row (1-2) are significant at $p < 0.05$.

Table 4 TPC and FRAP values of acid milk gels when gallic acid added to milk before (M_hP_h) and after heat treatment (M_hP) during refrigerated storage at 4 ºC.

		Storage Days[A]				
Paramaters	Samples	1	7	14	21	28
TPC (mg GAE g^{-1})	Gallic M_hP	1.20 ± 0.03	1.14 ± 0.06	1.15 ± 0.05	1.15 ± 0.03	1.19 ± 0.02
	Gallic M_hP_h	1.13 ± 0.08	1.09 ± 0.09	1.24 ± 0.04	1.07 ± 0.06	1.09 ± 0.06
FRAP (µmol AAE g^{-1})	Gallic M_hP	11.27 ± 2.01	14.14 ± 0.58	14.49 ± 0.42	13.47 ± 0.28	13.46 ± 2.92
	Gallic M_hP_h	13.29 ± 1.68	12.11 ± 1.66	14.72 ± 1.86	13.66 ± 1.72	16.62 ± 1.68

[A] Data represented are means of three replicates ± SE. M_hP: pasteurized skim milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition.

4. Scientific Publications

- **Poster Presentations**

Kilic Bayraktar, M., C.C. Fagan, and N.B. Harbourne. 2015. Effect of heat treatment on milk-polyhenol mixtures. Presented at Institute of Food Technologist (IFT)15: Where Science Feeds Innovation, 11-14 July, 2015, Chicago, Illinois, United States.

Kilic Bayraktar, M., C.C. Fagan, and N.B. Harbourne. 2015. Impact of polyphenol addition on the total phenolic content and antioxidant capacity of acidified milk gels. Presented at 4th International Conference and Exhibition on Food Processing & Technology, 10-12 August, 2015, London, United Kingdom.

- **Oral Presentations**

Kilic Bayraktar, M., C.C. Fagan, and N.B. Harbourne. 2016. Impact of Storage on the Physicochemical Properties of Acidified Milk Gels Fortified with Polyphenols. Presented at 10th World Congress on Polyphenols Applications, 29 June - 1 July, 2016, Porto, Portugal.

Kilic Bayraktar, M., C.C. Fagan, and N.B. Harbourne. 2016. Rheological properties of acidified skim milk gels enriched with polyphenols. IUFOST 2016: 18th World Congress of Food Science and Technology, 21-15 August, 2016, Dublin, Ireland.

- **Journal Publications**

Kilic Bayraktar, M., C.C. Fagan, and N.B. Harbourne. 2016. Impact of heat treatment and acid gelation on polyphenol enriched dairy matrices. LWT- Food Science and Technology. (submitted and under review).