Interactions between skimmed bovine milk and tea infusions under an acidulous environment

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A thesis

submitted in partial fulfilment

of the requirements for the Degree

of Doctor of Philosophy

at

Lincoln University

by

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Lincoln University

2019

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Ph.D.

Interactions between dairy and tea components under an acidulous environment

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Physicochemical and microstructural properties of acidified dairy gels produced with tea infusions (ADG), as well as the secondary structure of protein components, were investigated to explore the practicality of using green tea, oolong tea and black tea extracts as an additive in milk products.

Reconstituted skim milk was supplemented with different levels of green, oolong or black tea infusion (15%, 30%, 45% 60%) and acidified by glucono-δ-lactone (GDL) at 35 ± 1 °C until a pH value of 4.55 was reached. The physicochemical and textural properties were measured by texture analyser, and the antioxidant capacity of the gels was determined through four assays - Total phenolic content (TPC) by Folin-Ciocalteu assay, free radical scavenging ability by DPPH assay along with ABTS assay and FRAP assay. The cellular antioxidant activity and anti-proliferative activity were conducted on HepG2 cell lines.

The microstructure of the ADGs was observed using different microscopic techniques (SEM (scanning electron microscope) and CLSM (confocal laser scanning microscopy)). The secondary structure of proteins in ADGs were assessed by FT-IR, Circular dichroism and X-ray diffraction.

Results showed that green, oolong and black tea infusions significantly improved the antioxidant capacity of the gel system. However, lower phase stability was observed in the

ADG with a high portion of tea infusion (60%), and this appeared to be related to the water holding capacity (WHC) and microstructure. The microstructure of the ADGs observed is related to the phase stability and textural attributes.

The addition of tea extracts had no significant impact on the secondary structure of proteins, the addition of oolong tea and black tea extracts significantly changed the particle size of the ADG which could be related to the changes of textural properties.

The results suggest that tea polyphenols improved antioxidant capacity in all samples and interactions between tea polyphenols and dairy components improved the texture of ADG only at low portions of tea infusion which could due to the changes of Coulombian barrier and microstructure as well as the formation of large networks of protein/polyphenols/Ca^{2+}.

Keywords: Tea polyphenols, Dairy protein, Microstructure, texture, Antioxidant capacity, SEM, CLSM, XRD, FT-IR, Circular dichroism, Cellular antioxidant activity, anti-proliferation

Acknowledgements

I would like to express my sincere gratitude to my supervisors who provided their expertise, time, suggestions and advice throughout the project.

First and foremost, I would like to thank my primary supervisor Professor Charles Brennan for his continuous support of my Ph.D study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined how should I finish my Ph. D. study without his support.

My sincere thanks also goes to my associate supervisor Dr. Margaret Brennan for her inspiring, encouraging and personal guidance that provided a good basis of research and thesis writing. Without her precious support it would not be possible to conduct this research.

I would like to thank the staff members of Department of Wine, Food and Molecular Biosciences who provided technical assistance and helpful suggestions for my research.

I would also like to thank Dr. Xinbo Guo for his guidance for my research and help me setup the cell line test at the South China University of Technology.

I thank my Ph. D. labmates for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last four years.

Last but not the least, I would like to thank my parents for supporting me spiritually throughout writing this thesis. I would not have been able to complete my research successfully without their trust and help.

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Chapter 1

Introduction

1.1 Background

Free radicals, such as reactive oxygen species (ROS), are being generated in our body continually through normal physiological pathways. To counter this, there is an antioxidant defence system in our body, comprising of several enzymes (such as iron-dependent catalase, copper/zinc and manganese-dependent superoxide dismutase and selenium-dependent glutathione peroxidase), to detoxify these free radicals (Dinu et al., 2016; Shahreza, 2017). However, when there is an over-production of free radicals, leading to an imbalance between the generation and elimination of free radicals in the body, a situation known as oxidative stress occurs (Kontoghiorghes & Kontoghiorghe, 2019; Perrone et al., 2018). This may result in oxidative damage to cellular components and biomolecules, this marks the onset of many degenerative diseases related to ageing such as cardiovascular disease, diabetes, cancer or other neurodegenerative diseases (Pena-Bautista, Baquero, Vento, & Chafer-Pericas, 2019). As antioxidants play important roles in preventing or inhibiting oxidation of cellular components, adequate intake of these compounds is beneficial to protect against oxidative damages to the cell. In this regard, extracts of many polyphenol-rich plants or herbs, such as tea, are used more often either as additive in food industry or consumed directly as a natural source of antioxidants (Firuzi, Lacanna, Petrucci, Marrosu, & Saso, 2005).

Table 1.1 Main recognized physiological roles of free radical species.

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Adapted from Yaribeygi, Atkin, and Sahebkar (2019)

Traditionally, yoghurt has been consumed as a health food since 6000 B.C. in central Asia (Donovan & Shamir, 2014) due to its nutritional properties and taste. The health benefits of yoghurt can be further enhanced by incorporating probiotic strains of lactic acid bacteria. Acidified dairy gel (ADG), produced by glucono-δ-lactone (GDL), is commonly used in research as it has similar physicochemical properties to yoghurt but is easier to make, more stable and eliminates potential confusing issues caused by the fermentation of microbials (Mession, Roustel, & Saurel, 2017; Rimada & Abraham, 2006; Takeuchi & Cunha, 2008). Therefore, ADG was used in this study.

1.2 Research gaps

In the last decade, a large portion of research has been focused on protein or its hydrolysates from various sources about their foamability and effects on the overall texture of food matrix (Buhl, Christensen, & Hammershøj, 2019; López et al., 2019; Murray, Durga, Yusoff, & Stoyanov, 2011; Wierenga & Gruppen, 2010) or antioxidant capacity (Elias, Kellerby, & Decker, 2008; Jiang et al., 2019; Wen, Zhang, Zhang, Duan, & Ma, 2019). Some reseachers have looked at the nutritional values of tea such as anti-obesity, anti-cancer and body-weight control (Khanchemehr et al., 2019; Ren, Chen, Zhang, Lin, & Li, 2019; Sur & Panda, 2017; Suzuki, Pervin, Goto, Isemura, & Nakamura, 2016; Yang, Zhang, Zhang, Huang, & Wang, 2016). However, only a few papers have reported how the interactions between proteins and polyphenols changed the antioxidant capacity and texture of the food matrix (Foegeding, Plundrich, Schneider, Campbell, & Lila, 2017; Tang et al., 2017). The interactions between dairy and tea

components under acidulous environment have not been explored extensively and are therefore poorly understood.

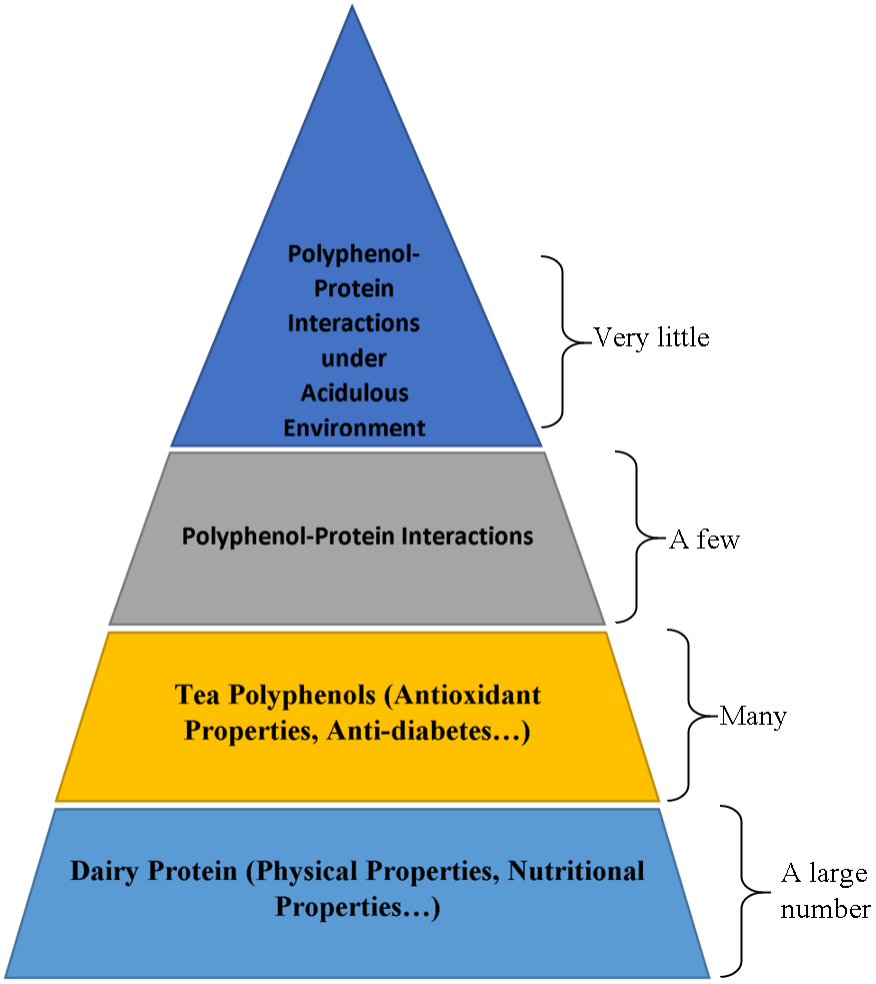


Figure 1.1 Areas covered in existing research

1.3 Aim and objectives of this research

1.3.1 Aim of this research

The aim of this study was to understand how tea extracts (rich in antioxidant materials) affected the quality of ADGs (structure, texture and antioxidant capacity), and determine the interactions between polyphenols and milk components (protein and calcium ions).

Hence, this research used ADG models containing three types of tea infusions, to determine the impacts of the interaction between tea infusion and dairy ingredients on the microstructure, texture, and antioxidant activity of the model gel through acidification. Therefore, the optimum tea infusion content was used in this research, and further research illustrated how the additions of tea infusion under an acidulous environment affect the microstructure of the acidified dairy gels. The stability of acidified dairy gels were also investigated through a 28-day cold storage (4 °C).

1.3.2 Objectives of this research

1) Explore the effects of polyphenols-rich extracts from tea on yoghurt texture;

2) Research the changes of antioxidant capacity in ADG when a tea infusion was added as an additive;

3) Understand the interaction between tea polyphenols and dairy components using an ADG system;

4) The effect of interaction between tea polyphenols and dairy components on microstructure of ADG.

1.4 Thesis structure

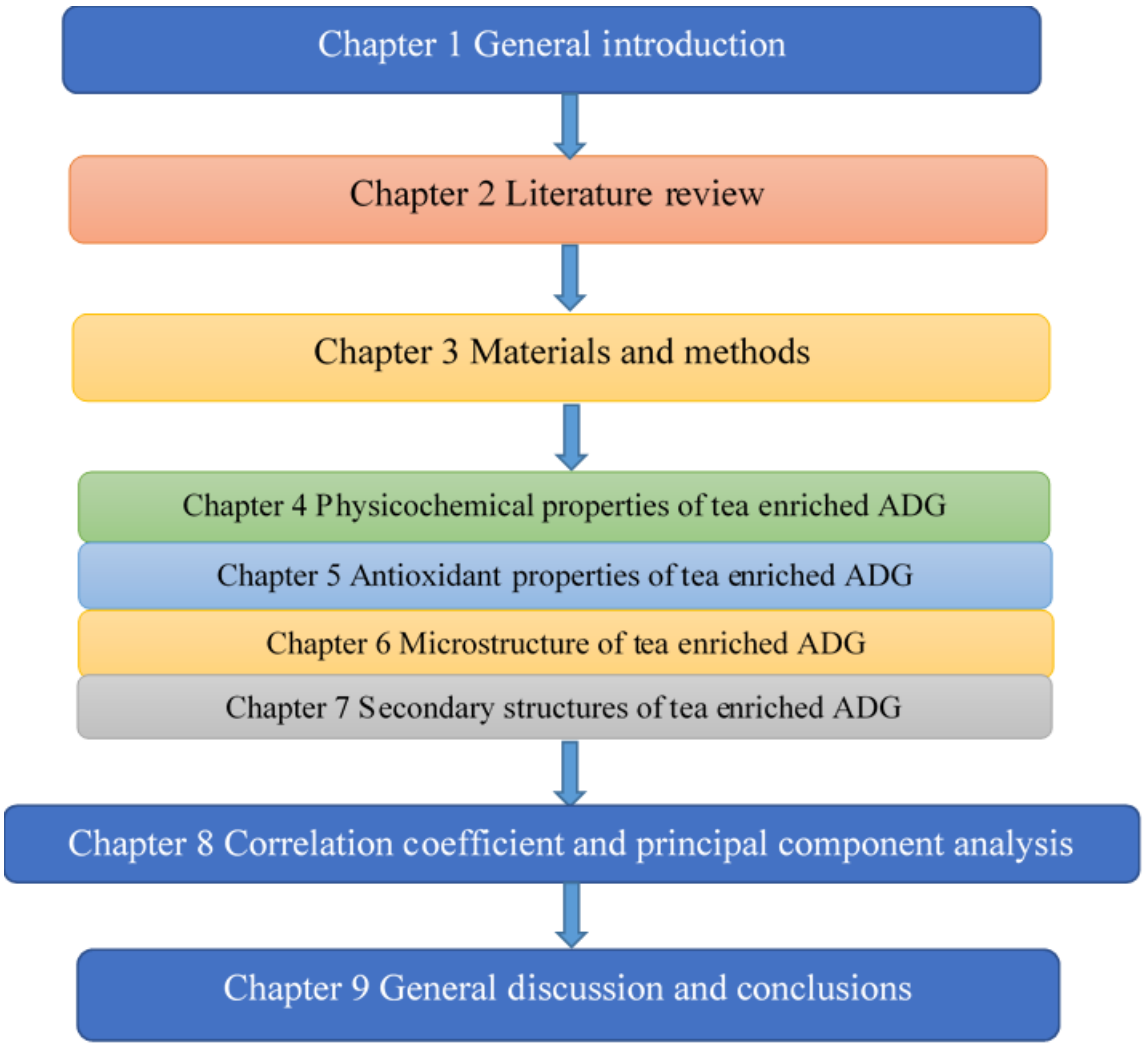


Figure 1.2 Thesis overview

Chapter 2

Literature review

2.1 Free radicals and antioxidants

2.1.1 What are free radicals

The theory of free radicals being responsible for ageing was first proposed by Denham Harman in the 1950s which presumed that free radical compounds, and related oxidants, from the environment or internal metabolism, can damage the constituents of the constituents, and over time result in an accumulation of structural and functional damage (Pomatto & Davies, 2018). A free radical can refer to any molecular species which exists independently and contains an unpaired electron in an atomic orbital (Lobo, Patil, Phatak, & Chandra, 2010). Free radicals are generally unstable and reactive due to the unpaired electron. They can act as either oxidants or reductants depending on whether they donate or accept an electron from other molecules.

Hydroxyl radical (·OH), superoxide anion ($O_2{\cdot}^-$), hydroperoxyl radical ($HO_2{\cdot}$), singlet oxygen ($^1O_2{\cdot}$), nitric oxide radical (·NO), and nitrogen dioxide radical ($\cdot NO_2$), and peroxynitrite radical ($\cdot ONO_2$) are the most important oxygen-containing free radicals relating to many disease states (Zukowski, Maciejczyk, & Waszkiel, 2018). These are highly reactive molecules which might cause biological damage to DNA, proteins, carbohydrates, and lipids due to their attack onto all kinds of important macromolecules in the body

leading to cell damage and homeostatic disruption. Nucleic acids, lipids, and proteins are the major targets among all objectives (Naik, Prakash, & Thomas, 2018).

2.1.2 Main sources in human body

Free radicals and other ROS are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals.

Sources of free radicals generated internally are:

- Mitochondria
- Xanthine oxidase
- Peroxisomes
- Inflammation
- Phagocytosis
- Phagocytosis
- Arachidonate pathways
- Exercise
- Ischaemia/reperfusion injury

sources of free radicals generated externally are:

- Cigarette smoke
- Environmental pollutants
- Radiation
- Certain drugs, pesticides
- Industrial solvents
- Ozone

(Biswas, Das, & Banerjee, 2017; Lobo et al., 2010)

2.2 Overview of tea

2.2.1 History, classification and consumption of tea

There is increasing evidence that phenolic compounds found in plants can assist in fulfilling general healthy eating recommendations. Since ancient times, tea has been considered as a traditional Chinese medicine, representing a healthful beverage which rich in specific phenolic compounds including flavonoids (Chen, Zhao, Fang, & Wang, 2007; Monteiro & Peluso, 2017).

Tea was once treated as a food resource during the “Spring and Autumn” Period (770 B.C.- 476 B.C.) in China and firstly utilized as a kind of Chinese medicine in Pre-Qin Period

(476 B.C.- 221 B.C.). Later on, some minorities in southwest China started to brew tea leaves in boiled water as tea-drinking. Tea-drinking, originated in the Three Kingdom Period (220- 280 A.D.), and spread more widely in the Tang Dynasty (618- 907 A.D.) after the Classic of Tea was published by Lu Yu (733- 804 A.D.) (Li, 1993).

The basic steps of manufacturing the various forms of teas are similar, except in the development of their aroma and in the fermentation process, which depends on the oxidation states of catechins present in tea leaves (Horanni & Engelhardt, 2013). For most common teas (black, green and oolong tea), green tea has not undergone the same withering and oxidation process used to make oolong teas and black teas which makes it non-oxidized tea. Oolong tea is defined as semi-oxidized tea with a degree of oxidation between green tea and black tea. Black tea is fully oxidized and has a stronger flavour than green tea and oolong tea. Each type of tea is classified in subclasseses in terms of growing conditions, horticultural methods, and time of harvest.

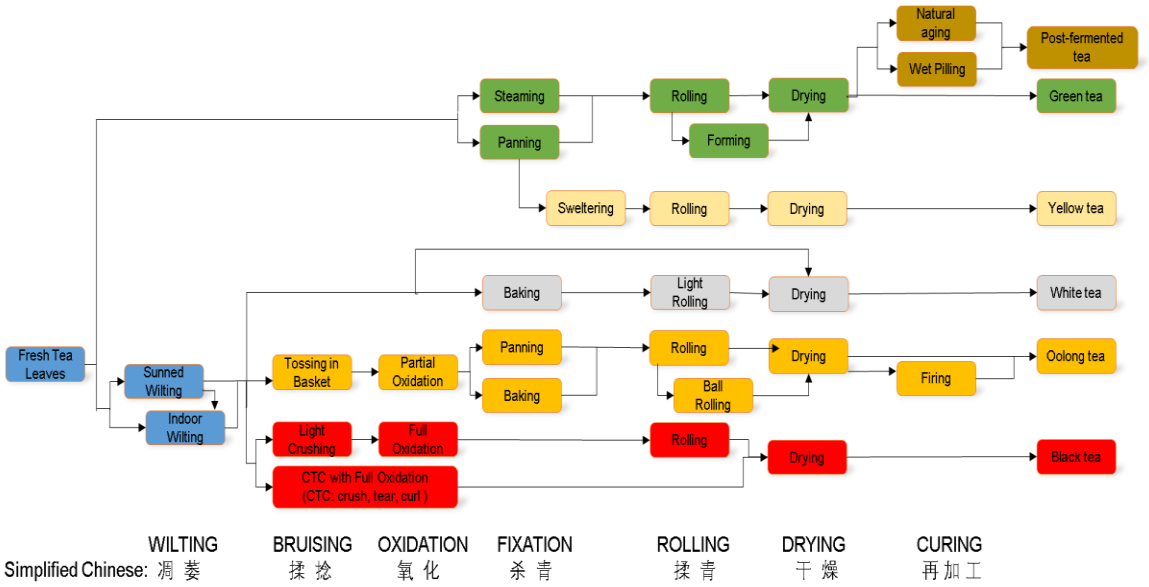


Figure 2.1 Processing of different types of tea (Feng et al., 2019; Wang et al., 2016; Zheng et al., 2018).

World tea production reached 5.73 million tonnes in 2016. China remains the largest tea producing country with an output of 2.44 million tonnes, accounting for more than 42 percent of the world total, while production in India, the second largest producer, increased to reach 1.27 million tonnes in 2016. World tea exports reached 1.75 million tonnes and total tea consumption increased to 5.5 million tonnes in 2016 (United Nations, 2018).

Of the tea produced worldwide, 78% is black tea, which is usually consumed in the Western countries, 20% is green tea, which is commonly consumed in Asian countries, and 2% is oolong tea which is produced mainly in southern China (Naumovski et al., 2019; Yang & Landau, 2000). Tea has become the second most commonly consumed beverage worldwide, only water is drunk more frequently (Hayat, Iqbal, Malik, Bilal, & Mushtaq, 2015; Wei et al., 2018; Zhang et al., 2014).

2.2.2 Characteristic components in tea

Like most herbs, the precise composition of green tea varies with the geographic origin of the leaf, the time of harvest, climate and processing techniques (Alaerts et al., 2012). The most abundant components in dried tea leaves are polyphenols which can be up to 25%, principally flavonols (including catechins) and flavones. The leaves also contain about 4% plant alkaloids including caffeine, theobromine, and theophylline. Other components include proteins, carbohydrates and minerals (including fluoride and aluminium) (Aroyeun, 2013; Balentine, Wiseman, & Bouwens, 1997; Yang & Landau, 2000).

2.2.3 Bioactivities of tea

Tea is popular because of its aroma and taste as well as its potentially health promoting properties therefore it is considered healthy by traditional Chinese medicine (Cabrera, Artacho, & Gimenez, 2006). Various plants and their constituents have been shown to have beneficial therapeutic effects, including antioxidant, anti-inflammatory, anti-cancer, and immunomodulatory effects, with tea (*Camellia sinensis*) being one of them. According to the study of Butt and Sultan (2009), the biological activity of green tea is due to different compositions of catechins and epigallocatechin gallate (EGCG) which have been identified as the principal antioxidant contributing approximately 30% of the total antioxidant capacity of green tea and also recognized as the major and potentially effective chemopreventive agent in green tea leaves (Darvesh & Bishayee, 2013; Shimizu et al., 2015).

Paveto et al. (2004) suggested that green tea exerts anti-fungal and anti-*Trypanosoma cruzi* activities. Micronutrients such as antioxidants have shown several therapeutic potentials against various health risks such as cardiovascular disease, diabetes and cancer (Yang, Wang, & Sheridan, 2018; Yoshihara, Fujiwara, & Suzuki, 2010). Meanwhile, it has been demonstrated that EGCG, a natural compound found in abundance in many foods, especially in green tea, inhibits *in vitro* entry of the ZIKA virus and the chikungunya virus into their host cell (Carneiro, Batista, Braga, Nogueira, & Rahal, 2016; Weber, Sliva, von Rhein, Kümmerer, & Schnierle, 2015).

Green tea contains polyphenols (the major component in green tea), which represent a group of chemicals known as catechins. Catechins are reported to exhibit antioxidant, antimicrobial, anticollagenase, antimutagenic, and chemopreventive properties. Studies in cell lines have also shown that tea catechins can affect a variety of signalling and metabolic pathways. These molecular events could result in cancer cell growth inhibition, apoptosis, and inhibition of invasion, angiogenesis, and metastasis (Yang & Wang, 2010). Albuquerque et al. (2016) also demonstrated that green tea supplementation could restore the main changes to neutrophils induced by obesity in obese rats. There is growing evidence to suggest a beneficial role of green tea in oral health. It has been reported that green tea protects against bacterial-induced dental caries (Han, Hwang, & Park, 2016).

2.3 Introduction of yoghurt

2.3.1 Definition of yoghurt

Yoghurt has long been known in human history as a way of preserving milk. According to the Food Standards Australia New Zealand (FSANZ) (2002) Standard 2.5.3, yoghurt is defined as "fermented milk where the fermentation has been carried out with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* with or without lactic acid producing micro-organisms where they have to be viable during shelf life at pH 4.5 with minimum protein content (measured as crude protein) of 30 g/kg". The addition of probiotics is a common practice in yoghurt manufacture to improve the nutritional status of the product and the health promoting effects on consumers (Heydari et al., 2018; Mousa, Liu, Chen, Zhang, & Chen, 2014).

Fermented dairy products containing probiotic lactic acid bacteria and *Bifidobacteria* are one of the best-known examples of functional foods (Yadav, Jain, & Sinha, 2007). Despite the distinctive acidity of natural yoghurt, the consumption of yoghurt or other cultured milk products is believed to have additional health promoting benefits to the host (Kosasih, 2011).

The microstructure of yoghurt gels has been observed using confocal scanning laser microscopy (CSLM) (Skytte et al., 2015). CSLM studies on acid gels have shown that these gels consist of a coarse particulate network of casein particles linked together in clusters, chains and strands.

2.3.2 Bioactivities of yoghurt

Probiotic yoghurt enriched with *B. lactis Bb12* and *L. acidophilus La5* has shown the ability of modulating total cholesterol and low-density lipoprotein cholesterol concentrations in type 2 diabetic people and may contribute to the improvement of cardiovascular disease risk factors (Ejtahed et al., 2011). The research of Jin et al. (2016) revealed that κ-CN and β-CN improved the diversity of peptides during the fermentation process and gastrointestinal digestion.

A recent study, attributed "glow of health" to the yoghurt, found that feeding of probiotic bacteria to aged mice induced integumentary changes mimicking peak health and reproductive fitness characteristic of much younger animals. Eating probiotic yoghurt triggered epithelial follicular anagen-phase shift with sebocytogenesis resulting in thick lustrous fur due to a bacteria-triggered interleukin-10-dependent mechanism. Aged female animals eating probiotics exhibited increased subcuticular folliculogenesis when compared with matched controls. Female animals also displayed probiotic-induced hyperacidity coinciding with shinier hair, a feature that aligns with fertility in human females (Levkovich et al., 2013).

Other benefits of yoghurt include improvement of nutritional status (Clerfeuille et al., 2013), maintenance of health (Sanders, 2008), the prevention and treatment of acute diarrheal disease (Eren, Dinleyici, & Vandenplas, 2010; Morelli, 2014), and the prevention or treatment of chronic diseases such as elevated blood pressure, weight gain, and metabolic diseases (Garcia et al., 2017; Ivey et al., 2011; Park & Cifelli, 2013; Szulinska et

al., 2017; Vergnaud et al., 2008; Wang, Livingston, Fox, Meigs, & Jacques, 2013; Yang et al., 2018).

2.3.3 Overview of probiotics

Probiotics are live micro-organisms that when administered in quantities sufficient confer a health benefit (FAO/WHO, 2001). As a whole, the world population is becoming more conscious of the relationship between nutrition and good health and as a result throughout the world the consumption of probiotic foods has increased greatly in recent years. In Europe the probiotic sector involves a total of 1.4 billion euros, led by yoghurts and desserts, accounting for approximately 72% of the total probiotics consumed (Saxelin, 2008). This has stimulated an increase in research focussing on the identification of food and food components that have health benefits to the consumer. As a result of these efforts, probiotic products have come into the market, which are identified as functional foods. These include foods containing phytochemicals, dietary fibre, structural lipids, bioactive peptides, polyunsaturated fatty acids (Agrawal, 2005; Aguilar-Toala et al., 2018; Tsuda, 2018).

Clinically proven consequences of consuming probiotic bacteria, include the following: anti-carcinogenic and anti-mutagenic activities, combating infection by *Heliobacter pylori*, treatment of the inflammatory bowel disease, prevention and treatment of gastrointestinal disorders, increase in activity of the immunological system, antimicrobial activity, reduction of lactose intolerance, and reduction in blood cholesterol levels (Agrawal, 2005; Shah, 2007). Recently, there have been reports on the potential benefit

of probiotics for human skin (Krutmann, 2009) and against colds and flu (Leyer, Li, Mubasher, Reifer, & Ouwehand, 2009).

2.4 Previous research on tea-yoghurt systems

2.4.1 Interaction of polyphenol-dairy protein system

In recent years there has been an increased interest in determining the reactions of milk proteins with some functional groups of other compounds in food. For example, cross-linking of milk proteins is regarded as important, because of its potential effects on the textural properties of milk products. The cross-linking effect of green tea flavonoids on milk proteins can be used for manufacturing novel milk products with the desired textural properties (Yuksel, Avci, & Erdem, 2010). Polyphenols are well known to bind to proteins in solution and to form complexes whose properties depend on the structure, concentration and ratio of both the polyphenol and the protein (Jongberg, Lund, Skibsted, & Davies, 2014). In spite of the numerous studies relating the nature of the polyphenol-protein interaction in solution, the understanding of this reaction is still partial because of the great diversity of the structures of polyphenolic compounds and proteins (Acuie-Beghin, Sausse, Meudec, Cheynier, & Douillard, 2008; Ren et al., 2018; Yang, Wang, Wang, Xia, & Wu, 2019).

Tea polyphenols were weakly bound to both α-casein and β-casein in solution based on the spectroscopic results and docking studies of Hasni et al. (2011a). The binding order increases together with the number of OH groups with C ≈ EC > EGC > EGCG. β-Casein

formed stronger complexes with tea polyphenols than α-casein, due to the hydrophobic nature of β-casein. Polyphenol-casein interaction is more hydrophobic than hydrophilic (Acuie-Beghin et al., 2008; Yazdi, Corredig, & Dalgleish, 2014). Binding between polyphenols and casein altered casein secondary structure with a major decrease in α-helix and β-sheet accompanied by an increase in random coil and turn structures which led to more protein unfolding. The casein structural changes may be a major factor in the effect of milk on the antioxidant activity of tea polyphenols (Hasni et al., 2011b). Similarly, Kanakis et al. (2011a) found that tea polyphenols could weakly bind to β-lactoglobulin (β-LG) in solution. Both hydrophobic and hydrophilic interactions were observed in the polyphenol- β-LG complexation and the binding altered protein secondary structure with an increase in β-sheet and α-helix, led to protein structural stabilisation. Therefore, the β-LG structural changes could be a major factor in the effect of milk on the antioxidant capacity of tea polyphenols. Work by Ye et al. (2013) reached a similar conclusion in which they stated that Casein micelles are more likely to bind highly polymerized polyphenols while whey proteins are prone to bind and transport smaller molecules. A gallate group in catechins and the *cis*-form enhance the catechin–casein interaction but a pyrogallol group weakens the interaction. The interactions between β-casein and polyphenols transformed the secondary structures of β-casein by interrupting the native irregular structures of random coil and the large loop and the increase of α-helix, intra β-sheet and turn structures (Condict, Kaur, Hung, Ashton, & Kasapis, 2019).

Sabouri, Geng & Corredig, (2015) demonstrated that EGCG molecules associated with caseins when adsorbed at an oil-water interface, and such interfaces could load high

ratios of EGCG to protein. Caseinate-stabilized emulsion could therefore be used as a platform for the delivery of tea polyphenols. The stability, size, and charge of the emulsion particles were not affected by the presence of EGCG. In addition, there were no differences in the amount of sodium caseinate adsorbed at the sodium caseinate–EGCG interface compare to sodium caseinate interface, although the complex formed affected the viscoelastic properties of the protein layer formed at the interface. This finding could be significant in the development of dairy based beverages containing tea polyphenols.

2.4.2 Interaction of polyphenol-dairy lipid system

Literature has also focused on the interactions between polyphenol and dairy lipid. For instance, Gulseren & Corredig (2013) investigated the ability to encapsulate tea polyphenols and physical properties of liposomal dispersions (10%) which were prepared using high-pressure homogenization. The results suggest that milk phospholipids could be employed to prepare tea-polyphenol-bearing liposomes and that the tea catechins may be incorporated in the milk phospholipid bilayer more efficiently than in the case of a soy phospholipid bilayer. They indicated that it might be possible to optimize the polyphenol release (rate and duration of release) and liposomal stability (changes in size and surface charge) through simple physical manipulation in order to generate high performance polyphenol delivery vehicles that retain functionality in food products and demonstrate maximum biological functionality.

2.4.3 Interaction of polyphenol-protein-fat globule system

Zhang et al. (2012) studied the effects of the interactions between milk components and jujube juice using produced skimmed milk, milk fat, and whole milk, respectively. The bioavailability of phenolics and the plasma antioxidant capacity were studied using a rat model for 8 h after the consumption of jujube juice with and without milk components in rats. The addition of skimmed milk to jujube juice resulted in significant changes in the plasma kinetics profile of phenolics, rather than affecting the overall absorption. Milk fat did not interact with jujube juice phenolics. However, when jujube juice was ingested with whole milk, a significant reduction of the bioavailability of phenolic components and an increase in plasma antioxidant capacity was observed.

A consistent increase in the median diameters of the emulsions indicated the formation of complexes of proteins, fat, and phenolic compounds during digestion. An increase in the plasma antioxidant capacity was observed after jujube juice consumption compared to the initial time and a good correlation between antioxidant capacity and phenolic levels was found. The research of Zhang et al. (2012) indicated that the plasma antioxidant capacity was associated with the content of polyphenols. In addition, milk ultrafiltration showed no significant effect on neither the bioavailability nor the antioxidant activity of jujube juice phenolic in rats based on the results of different milk preparations. Therefore, the effect of the constituents of milk in these preparations other than proteins and fats can be neglected.

It has been found that polyphenols have a significant affinity for proteins and peptides that contain a high proportion of proline residues in their sequences (Rashidinejad, Birch, & Everett, 2016). Due to the high amount of proline present in milk proteins, it could be expected that additional interactions between polyphenols and peptides may present during the early digestion and which could be a hindrance to the polyphenol's absorption through the intestinal brush border.

2.4.4 The complex of polyphenol-dairy components in digestion experiments

The addition of the tea extracts to dairy matrices promoted formation of polyphenol-protein complex which significantly improved polyphenol stability in a simulated gastrointestinal environment and enhanced the antioxidant activity (Bandyopadhyay, Ghosh, & Ghosh, 2012). The health effects of green tea polyphenols could be harnessed by ensuring that their biological properties are preserved. For instance, they could prevent the oxidation of vitamins and other nutrients to preserve higher quality nutrient intake. The structure of the dairy matrix was an important factor influencing protein and lipid digestion as well as bioaccessibility of polyphenols from the green tea extract. Liquid and semi-liquid matrices such as milk and yoghurt were more easily digested and therefore showed greater polyphenol bioaccessibility after digestion (Lamothe, Azimy, Bazinet, Couillard, & Britten, 2014).

2.4.5 The influences of interactions between polyphenol and dairy components on textural property in yoghurt

A study of Wang, Kristo, and LaPointe (2019) reported the possible influences of interaction between phenolic and dairy components on physical properties in an apple pomace-yoghurt system. Their results indicated the addition of apple pomace in low volume (≤1% w/w) increased the firmness, cohesiveness and consistency of the yoghurt ($p < 0.05$) which could be explained by the reinforcement of gel at low temperature. The study also found that the increase of firmness was mainly attributed to the increase of total solids contributed by apple pomace, as well as the interactions between apple particles and the dairy protein matrix, resulting in a denser and more rigid gel structure which is supported by the results of O'Shea, Arendt, and Gallagher (2012) and Puvanenthiran, Stevovitch-Rykner, McCann, and Day (2014). The studies above suggested that low volume polyphenols could improve the texture of yoghurt as a new additive.

2.4.6 The application of acidified dairy gel as a simulate yoghurt sysytem

Glucono-δ-lactone (GDL) is a legal additive in food industry as a coagulant and pH control agent. Compared with other food acids, GDL provides a gradual, progressive, and continuous decrease of pH to equilibrium due to its slow hydrolysis to gluconic acid. Therefore, it is used as a slow release acidifier. During its hydrolysis, the initial sweet taste becomes slightly acidic, making the final flavour of aqueous solution less tart than that of other acidifiers (Chen, Chen, & Lin, 2004; Mession et al., 2017). Due to the slow

acidification by GDL which is similar to the fermentation progress of yoghurt, a GDL-induced acidified dairy gel system to mimic yoghurt matrix could be created.

2.4.7 Secondary stucture of tea-ADG system

2.4.7.1 Fourier transform infrared spectroscopy (FT-IR) spectrum

Normally, the differences in four regions (water region (3500 to 3000 cm^{-1}), amide I (1700 to 1600 cm^{-1}), amide II (1600 to 1500 cm^{-1}) and amide III (1400 to 1200 cm^{-1})) are used for the determination of the secondary structure of proteins (Guerrero, Kerry, & de la Caba, 2014).

The amide I vibration, absorption near 1650 cm^{-1}, arises mainly from the C=O stretching vibration with minor contributions from the out of phase casein (CN) stretching vibration, the C-CN (calcium casein) deformation and NH in plane bend. The latter is responsible for the sensitivity of the amide I band to N-deuteration of the backbone. In proteins, the extent to which the several internal coordinates contribute to the amide I normal mode depends on the backbone structure. The amide I vibration is hardly affected by the nature of the side chain. It depends however on the secondary structure of the backbone and is therefore the amide vibration that is most commonly used for secondary structure analysis (Goormaghtigh, Gasper, Benard, Goldsztein, & Raussens, 2009).

The amide II mode is the out of phase combination of the N-H in plane bend and the C-N stretching vibration with smaller contributions from the C=O in plane bend and the C-C and N-C stretching vibrations. As for the amide I vibration, the amide II vibration of

proteins is hardly affected by side chain vibrations but the correlation between protein secondary structure and frequency is less straightforward than for the amide I vibration. Nevertheless, it provides valuable structural information and secondary structure prediction can be done with the amide II band alone.

The amide III mode of NMA (*N*-methyl acetamide) is the in-phase combination of the N-H bending and the C-N stretching vibration with small contributions from the C=O in plane bending and the C-C stretching vibration. In polypeptides, the composition of this mode is more complex, since it depends on side chain structure and since N-H bending contributes to several modes in the 1400 to 1200-cm^{-1} region. In spite of side chain contributions to the amide III mode, this mode can be used for secondary structure prediction upon N-deuteration.

2.4.7.2 Circular dichriosm (CD) spectrum

CD spectrum is widely used for determination of secondary structure of protein. Circular dichroism is the difference in the absorption of left-handed circularly polarized light (L-CPL) and right-handed circularly polarized light (R-CPL) and occurs when a molecule contains one or more chiral chromophores (light-absorbing groups) (Kelly, Jess, & Price, 2005).

Measurements carried out in the visible and ultra-violet region of the electro-magnetic spectrum monitor electronic transitions, and, if the molecule under study contains chiral chromophores then one CPL state will be absorbed to a greater extent than the other and the CD signal over the corresponding wavelengths will be non-zero. A circular dichroism

signal can be positive or negative, depending on whether L-CPL is absorbed to a greater extent than R-CPL (CD signal positive) or to a lesser extent (CD signal negative) (Berova, Di Bari, & Pescitelli, 2007).

2.4.7.3 Dynamic light scattering (DLS)

Dynamic light scattering (DLS) is commonly used for determining the characteristics of colloidal suspensions. The geometrical structure and state of motion of small particles can be measured base on scattering light. As DLS measures the apparent hydrodynamic radii of particles, it is well adapted to measure not only the basic sizes of particles but can also be used to determine changes in their sizes as a result of processing. Milk is an ideal subject for DLS, due to the minuteness of protein particles (the casein micelles), as they are unable to be observed by light microscopy but large enough to scatter light strongly (Alexander & Dalgleish, 2006). Therefore, the DLS measurement is useful for determining the characteristics of tea-ADG system.

The secondary structure and particle size of protein molecules are important in terms of microstructure and stability of the food matrix. FT-IR spectroscopy is one of the most versatile analytical tools used across various disciplines including the secondary structure determination of protein. The CD spectroscopy are well known non-destructive technique for structural characterization of proteins and polypeptides while the DLS is one of the most popular light scattering techniques because it allows the measurement of particle sizing down to 1 nm diameter. To obtain a comprehensive understanding of the ADG, FT-IR, CD and DLS were used in our study.

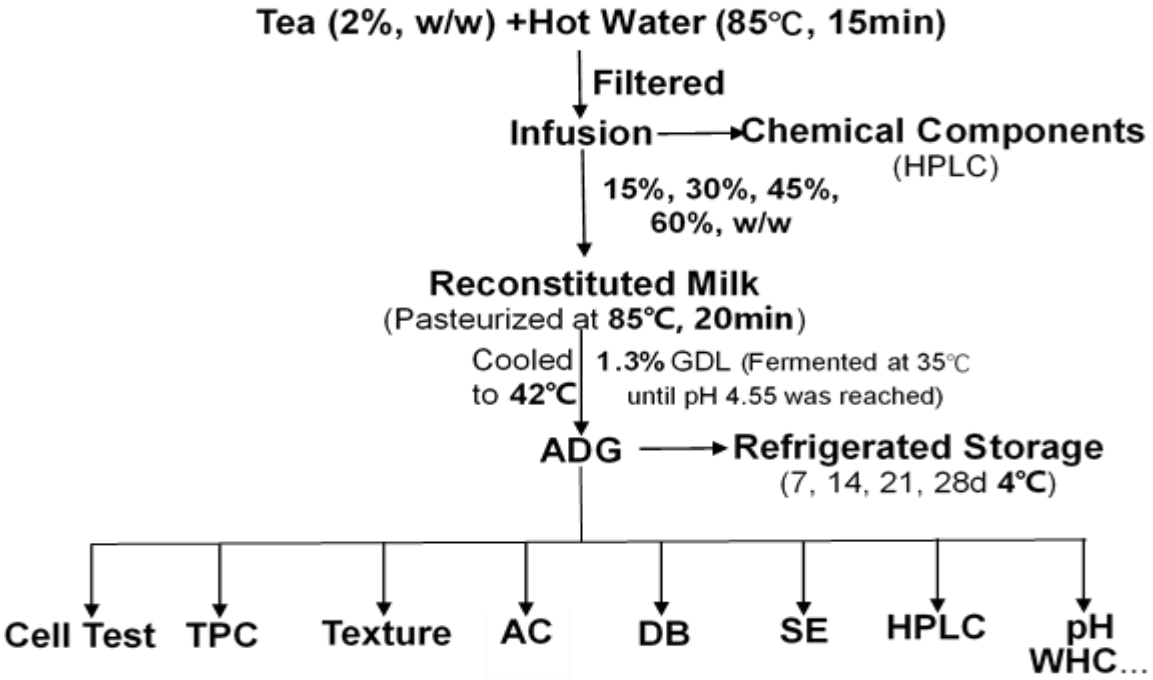
Chapter 3

Materials and methods

3.1 Materials

Skimmed milk powder (SMP, fat 1.2%, protein 33.0%, Pams, New Zealand), bagged black tea (premium Ceylon tea, Dilmah, Peliyagoda, Sri Lanka) and loose green tea leaves (Tea Ren Tea Co., Ltd., Taiwan, China) were purchased locally. Loose oolong tea (Dancong tea) leaves were provided by Chaoshan Kongfu Tea Co., Ltd. (Guangdong, China). All tea samples were packed in vacuumed aluminum foil mylar bags and stored in cool and dry condition. DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2,2-Diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) and gallic acid were purchased from Sigma, USA. Trolox (6-hydroxy-2,5,7,8-tetramethylchloromane-2-carboxylic acid) was purchased from Acros Organic (NS, USA). Phosphate buffered saline was purchased from Oxioid Ltd., Hampshire, England). Methanol was purchased from ECP-Laboratory Reagent (Auckland, New Zealand). D-Hank's solution was purchased form Hao Yang Biological Manufacture Co., Ltd (Tianjin, China). William's Medium E (1×) was purchased form Gibco, Life Technologies Ltd. (NY, USA). Catechin, caffeic acid, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin gallate were purchased from Aladdin Co. (Shanghai, China). Acetonitrile were of HPLC grade. Acetic acid was of analytical-reagent grade. Ultrapure water was generated by a Milli-Q water purification system. All other reagents were of analytical grade.

3.2 Technical route



Abbreviations:

GDL: Glucono delta-lactone
Cell test: Cellular antioxidant activity test and anti-proliferation test
HPLC: High performance liquid chromatography
TPC: Total phenolic content
AC: Antioxidant capacity (DPPH, FRAP, ABTS)
SE: Sensory rvaluation
WHC: Water holding capacity

3.3 Methods

3.3.1 Preparation of tea infusion

Bagged black tea, and loose tea leaves for oolong tea and green tea, were used to make tea infusions. One tea bag (containing 2 g of tea), or 2g loose tea leaves, per 100 mL RO (reverse osmosis) water was extracted using of 85 °C (RO) water for 15 min in a water bath. After infusion, the bags and tea leaves were removed and filtered by filter paper

(Whatman, grade No. 541) and the solutions were cooled to 40 °C in ice water for further experiments (Yuksel et al., 2010).

3.3.2 Heat treatment and acidification

The glucono-δ-lactone (GDL) gels were made according to Vega and Grover (2011). Reconstituted skim milk, as the principle model substrate, was made from SMP and tea infusion. All skimmed milks contained 3.3% protein, 0.12% fat and 9.1% total solids. The reconstituted skim milks containing 0%, 15%, 30%, 45% and 60% (w/w) of tea infusion were made before acidification. Different concentrations of reconstituted milk were used to ensure the final tea-milk had same level of total solids content (10% w/w). The proportion of tea infusion in the mixture was altered to give the appropriate content of tea extracts in tea-milk mixture. The reconstituted milk was heated in a water bath at 85 °C for 20 min. After rapidly cooling to 40 °C in an ice water bath and mix with tea infusions, GDL (1.3% w/w) was added and dissolved. The reconstituted milks containing different levels of tea extracts were incubated at 35 °C until pH 4.55 was reached. The formed set gels were cooled to 4 ± 1 °C for further analysis and were homogenized to make stirred gels. The samples were subjected to Texture Profile Analysis (TPA) directly after production and other experiments were carried out after 1, 7, 14, 21 and 28 days of cold storage at 4 °C.

3.3.3 pH and Ca^{2+} content measurement

The pH of ADG was determined using a calibrated digital pH meter (SevenEasy pH, Mettler-toledo GmbH, Schwerzenbach, Switzerland).

The free Ca^{2+} content in ADG system was determined by a portable Calcium Ion Selective Electrode (B-751, LAQUAtwin Calcium Ca++ Ion Meter, Horiba, Japan). The Horiba B-751 LAQUAtwin Calcium Ion Meter is a compact meter for a quick measurement of calcium ion using a flat sensor with ion selective membrane which delivers a reliable and direct measurement of a drop of the sample from 0.3mL. The instrument was calibrated daily with 150 & 2000ppm calcium standards included in the kit (Kosasih, Bhandari, Prakash, Bansal, & Gaiani, 2016).

3.3.4 Texture characteristics

Textural attributes of set gel. The TPA of set ADG was carried out using Texture Analyser TA-XT2 (Stable Micro Systems, UK) according to Vercet, Oria, Marquina, Crelier, and López-Buesa (2002) with slight modifications. The samples were analysed at 15 °C. The texture analyses were performed, using 5 Kg load cell, by two sequential compression events separated by a 5 s stop interval (10 mm penetration at 1 mm/s, P/25 aluminium cylinder probe and trigger force was 10 g). Hardness and adhesiveness values were calculated from the obtained profiles using the software provided by Stable Microsystems. Hardness (g) is first peak force and adhesiveness (g·s) is curve area above negative peak. Firmness (g) and cohesiveness (g) are related with, respectively, maximum

and minimum force obtained during extrusion of the ADG sample through the annular gap between the probe and jar wall (Najgebauer-Lejko, Zmudzinski, Ptaszek, & Socha, 2014).

Textural attributes of stirred gel. The textural properties of stirred gel was characterized by back-extrusion method described by Ciron, Gee, Kelly, and Auty (2010b). A 5 Kg load cell was used and the samples were tested in cylinder pot (50 mm internal dia.) at 15 °C, using an extrusion disc (Φ = 35mm) operating at a fixed speed of 1.0 mm/s to a depth of 30 mm. Firmness and cohesiveness values were calculated from the obtained profiles using the software provided by Stable Microsystems.

3.3.5 phase stability

The water holding capacity (WHC) and syneresis rate of the ADG were measured according to the method of Ciron et al. (2010b) with some modifications. Gel samples (1.0 g) in 1.5-mL Axygen® microtubes were centrifuged at 15,000 × g) for 15 min at 25 °C using a Heraeus® Multifuge X3R centrifuge (Heraeus Co., Hanau, Germany); WHC was expressed as % (supernatant/sample, w/w).

The determination of the extent of syneresis (EOS) was carried out by placing stirred ADG samples (1.0 g) in 1.5 mL tubes and then centrifuged at 101 × g for 60 min at 5 °C; EOS was expressed as % (pellet/sample, w/w). Quadruplicate measurements were made for the determination of WHC and EOS of the stirred ADGs.

3.3.6 Antioxidant capacity evaluation

Sample extraction. The sample (1 g) was placed in a 50 mL plastic pot with 20 mL 50% methanol solution and stirred overnight (speed 3, RT 15 Power 15-position analogue hotplate stirrer, IKA, Staufen Im Breisgau, Germany) at ambient temperature. The supernatant of samples was collected after centrifugation (2500 × g for 20 min). Extracts were stored at -20 °C until required.

Total phenolic content (TPC). Folin–Ciocalteu method is widely used in routine analysis (Turkmen, Sari, & Velioglu, 2006) The TPC of the sample extracts was determined spectrophotometrically in triplicate using Folin-Ciocalteu (F-C) reagent according to the method described by Deng et al. (2013) with slight modifications. A 500 µL Sample was added to a test tube followed by 2.5 mL of 0.2 M Folin-Ciocalteu reagent and 2.0 mL of sodium carbonate (7.5 g/100 mL). The sample was mixed thoroughly and stored in the dark for 2 h before the absorbance was measured at 760 nm using VWR V-1200 Spectrophotometer (VWR International Co., Pennsylvania, USA). TPC was expressed as mg gallic acid equivalents (GAE) per 100 g of fresh material.

DPPH assay. The DPPH radical scavenging activity was assayed by the method used by Al-Al-Dabbas et al. (2007). DPPH was dissolved in methanol at a concentration of 0.1mM. To 500 µL of sample extract, 1 mL 0.1mM DPPH solution and 1.5 mL of methanol were added and mixed by vortex. The absorbance was measured at 517 nm (VWR International Co., Pennsylvania, USA) after the solution was kept at room temperature for 30 min in the dark, methanol was used as the control. The DPPH radical scavenging activity was

expressed as mg Trolox equivalents (TE) per 100 g of fresh material.

Ferric reducing/antioxidant power (FRAP) assay. FRAP was assessed according to Khanizadeh, Tsao, Rekika, Yang, and DeEll (2007) with slight modifications. A fresh working solution of FRAP reagent was prepared each time by mixing acetate buffer (300 µM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCL, and 20 mM $FeCl_3 \bullet 7H_2O$ at 10:1:1 (v/v/v). A 250 µL aliquot of an iron (II) sulphate ($FeSO_4 \bullet 7H_2O$) standard or sample extract was added to 2.5 mL of the FRAP reagent and the absorbance at 593 nm recorded immediately after the addition of the sample and again after 2 h incubation at 37 °C (Le, 2012). The results were expressed as µmol Fe^{2+}/g sample.

ABTS radical scavenging capacity. The ABTS radical scavenging assay was based on the method of Elfalleh et al. (2009). $ABTS^{\bullet+}$ was prepared by mixing colourless ABTS stock solution (7mM in water) with 2.45 nM potassium persulfate and then maintaining the reaction for 16 hours in the dark at room temperature. On the day of analysis, the $ABTS^{\bullet+}$ solution was diluted with PBS (phosphate buffer saline) (pH 7.4) to an absorbance of 0.70 (± 0.02) at 734 nm and 3 mL transferred to a cuvette. After the addition of 300 µL Trolox or sample extract, the mixture was well mixed, allow to stand 6 min and absorbance read at 734nm. The results were expressed as Trolox equivalents (TE).

3.3.7 Microstructure of ADG

Scanning electron microscope (SEM). The bulk microstructure characteristic method was developed according to Kalab (1979). Approximately 3 mm^3 cubes of chilled samples were

coated in a thin layer of low melting point agarose (3%) before being placed into primary fixative (3% glutaraldehyde 0.1M sodium cacodylate buffer, pH 7.2) for at least 8 hours at ambient temperature. The samples were then washed three times (10-15 minutes each) in sodium cacodylate buffer (0.1M, pH 7.2) followed by post fixation in 1% osmium tetroxide (in sodium cacodylate buffer) for 1 hour at room temperature and another three buffer washes. Finally, the samples were dehydrated in a graded ethanol series (25%, 50%, 75%, 95% and 100%) for 12.5 ± 2.5 min each before a final 100% ethanol wash for 1 h.

Samples were critical point dried using liquid CO_2 as the C_P (pressure coefficient) fluid and 100% ethanol as the intermediary (Polaron E3000 series II critical point drying apparatus). Samples were torn to expose their structure, mounted on to aluminium stubs, sputter coated with approximately 200nm of gold (Baltec SCD 050 sputter coater) and viewed in the FEI Quanta 200 Environmental SEM at an accelerating voltage of 20kV (Domagala, 2009) . Analyses of ADG microstructure were conducted at Massey University, Palmerston North, New Zealand.

Confocal laser scanning microscopy (CLSM) methods. The planar microstructure of the protein arrangement in ADG was investigated using CLSM. The method was adapted from (Ciron et al., 2010b) with modifications. A small amount of sample was placed in a cavity slide and 40 µL each of 0.2 g/L Nile red (in methanol) and fast green (in water) were added before being covered with a coverslip.

Imaging was carried out using the Leica DM6000B SP5 confocal laser scanning microscope system with LAS AF software (version 2.7.3.9723; Leica Microsystems CMS GmbH).

Images were acquired with an HCX PL APO CS 10x (N.A. 0.40), HCX PL FLUOTAR 40x (N.A. 0.75) and HCX PL APO CS 100x oil (N.A. 1.40). Nile red and fast green were sequentially imaged through excitation at 488nm (argon laser) and 633nm (HeNe 633 laser) (respectively) and emission collection at 498-569nm and 643-787nm (respectively).

3.3.8 X-ray diffraction (XRD)

Dried samples were prepared by grinding the lyophilized samples and then screening the samples with an 80-mesh sieve (particle size < 0.178mm). The XRD studies were carried out using a Bruker AXS D8 Advance powder X-ray diffractometer with Cu-Kα radiation (D8 Advance, Bruker, Karlsruhe, Germany) set at a tube load of 40 kV and 40mA. An anti-scatter slit of 1° divergence and receiving slits were used. 12 Diffractograms were taken between 5 and 70° (2θ) at a rate of 12° 2θ min^{-1} and with a step size of 0.05° (2θ). Diffractograms of ADG powders were obtained (Chen et al., 2013; Zhao et al., 2015).

3.3.9 Fourier transform-infrared spectroscopy (FT-IR)

Pellets for analysis were formed by pressing 2mg lyophilized ADG powder and 200 mg of KBr together (Rozenberg, Lansky, Shoham, & Shoham, 2019).

Samples were analysed for the effects of the treatments on the secondary structure of proteins using a VERTEX 70 Fourier transform infrared (FT-IR) spectrometer (Bruker Co., German) in the range of 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} and averaging 16 scans for each spectrum as described by Markoska, Huppertz, Grewal, and Vasiljevic (2019). Spectrograph software (F. Menges "Spectrograph - optical spectroscopy

software", Version 1.2.9, 2018, http://www.effemm2.de/spectragryph/) was used for calculation of spectrum. They were normalized for protein concentration as previously described by Militello et al. (2004).

3.3.10 Particle size

The size distribution of ADG samples were measured using a LA-960S laser scattering particle size distribution analyser (Horiba Co., Japan). Samples were diluted 5 times with distilled water before measurements. The refractive index for samples was set at 1.34 (Markoska et al., 2019).

3.3.11 High performance liquid chromatography (HPLC)

The tea extracts, and ADGs, were centrifuged at 10000 × g for 20min and then filtered through a 0.45 mm Millipore filter. A 1260 Infinity HPLC System equipped with G1311B, G1329B, G1318A, and G1315D (Agilent Co. Ltd., California, USA) was used. An Eclipse Plus C18 column (4.6mm internal diameter × 100 mm long; 3.5 µm particle size, Agilent Co. Ltd., California, USA) was used and maintained at a temperature of 25 ± 0.5 °C. A flow rate of 1.2 mL/min was used during the separation; the injected volume was 10 µL. The mobile phase consists of a combination of solvent A [deionized water with 2% (volume/volume) acetic acid] and solvent B (acetonitrile). The elution profile for catechin separation was as follows: 0 minutes, 100% A; 20 minutes, 70% A, 30% B. Absorbance at 280 nm was used for the real-time monitoring of peak intensities (Lin, Lo, Chen, & Chen, 2016).

3.3.12 Circular dichroism (CD)

Far-UV CD spectra were recorded at 25 °C on a MOS-450 CD spectropolarimeter (BIOLOGIC Co., France) by a 0.1-cm path-length cuvette using the supernatant of samples and a step of 0.1 nm, a scan rate of 20 nm s^{-1}, 4 s of delay and a scan average of three. The supernatant of samples was obtained by centrifugation at 10000 × g for 20 min. The acquired spectra were corrected for distilled water (Hudson, Ecroyd, Dehle, Musgrave, & Carver, 2009).

3.3.13 Cytotoxicity and anti-proliferation

3.3.14.1 Cell culture

HepG2 cell lines were obtained from Stem Cell Bank of Chinese Academy of Sciences in Beijing (No. SCSP-510). HepG2 cell were cultured in minimum Eagle's medium from Hyclone (GE Healthcare Life Sciences, Marlborough, MA, US), supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine, Earle's balanced salts and were retained in an incubator at 37 °C containing 5% CO_2.

3.3.14.2 Cytotoxicity on HepG2 cell

The supernatants of ADG samples were harvested by centrifugation (8,000 × g, 20 min at ambient temperature) and then filtered using a 0.45 μm filter and stored at 4 °C before use.

Cytotoxicity was measured using the method of Wolfe and Liu (2007) with modifications. HepG2 cells were seeded at 4 × 10^4/well on a 96-well plate in 100 μL of growth medium

and incubated for 24 h at 37 °C with 5% CO_2. The medium was removed, and the cells were washed with PBS. Treatments of ADG supernatants or antioxidant compounds in 100 µL of the treatment medium (WME (Williams' Medium E) supplemented with 2 mM L-glutamine and 10 mM Hepes) were applied to the cells, and the plates were incubated at 37 °C, 5% CO_2 for 24 h. The treatment medium was removed, and the cells were washed with PBS. A volume of 50 µL/well methylene blue staining solution (98% Hanks Balanced Salt Solution (HBSS), 0.67% glutaraldehyde, 0.6% methylene blue) was applied to each well, and the plate was incubated at 37 °C for 1 h. The dye was removed, and the plate was immersed in fresh deionized water three times, or until the water was clear. The water was tipped out of the wells, and the plate was allowed to air-dry briefly before 100 µL of elution solution (49% PBS, 50% ethanol, 1% acetic acid) was added to each well. The microplate was placed on a bench-top shaker for 20 min to allow uniform elution. The absorbance was read at 570 nm with blank subtraction using the MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA). Concentrations of ADGs that decreased the absorbance by >10% when compared to the control were considered cytotoxic.

3.3.14.3 Anti-proliferation

HepG2 cells were seeded at a density of 2.5×10^4 cells/well on a 96-well microplate for anti-proliferation activity analysis, and 4×10^4 cells/well for cytotoxicity analysis. After incubation, the cells were stained with methylene blue solution (Sigma-Aldrich, St. Louis, MO) and 0.6% methylene blue (BBL, Cockeysville, MD) with HBSS at 37 °C for 1 h. Cells were then washed with water and dried. The elution solution was used to elute the methylene blue stain, and then the plate was agitated at room temperature for 15 min

(Wang et al., 2017). The absorbance was measured at 570 nm by an MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA).

3.3.14 Cell antioxidant activity (CAA)

3.3.15.1 Preparation of solutions

A 0.5 mmol L^{-1} DCFH-DA (dichloro-dihydro-fluorescein diacetate) in ethanol and a 12 mmol L^{-1} AAPH in treatment medium were prepared, aliquoted and stored at 4 °C, respectively. Then, they were diluted with treatment medium separately to prepare working DCFH-DA (25 µmol L^{-1}) and AAPH (600 µmol L^{-1}) solutions. Quercetin was dissolved in ethanol to configure the concentration at 1 mg mL^{-1}, before further dilution to different concentrations from 2 to 20 mM L^{-1} in working DCFH-DA solution. ADG solutions were dissolved and diluted to different concentrations from 2 to 20 mg mL^{-1} in working media solution and there was no toxic effect on HepG2 cells at those concentrations, as assessed by cytotoxicity (Shen et al., 2019).

3.3.15.2 CAA assay

The CAA assay was conducted as described by Wolfe and Liu (2007). Hep G2 cells were used as cell model for analysis; quercetin was used as standard to calculate the CAA value. With and without PBS wash treatments were used in this assay. The steps are as followed: HepG2 cells were plated in a 96-well black plate with a density of 6×10^4 cells/well and incubated at 37 °C, 5% CO_2 for 24 h. After 24 h, 100 µL of appropriate concentration of extracts or controls were dissolved in treatment medium containing DCFH-DA (50 mmol/L) and incubated for 1 h. For PBS wash protocol, cells were washed with PBS once;

for no PBS wash protocol, cells were not washed before adding ABAP (2,2’ -azobis (2-amidinopropane) dihydrochloride). Finally, 600 mmol/L ABAP was applied to the cells in 100 mL of HBSS and the 96-well microplate was placed into FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 37 °C. Emission at 535 nm was measured after excitation at 485 nm every 5 min for 12 cycles.

Figure 3.1 The fundamental mechanism of the cellular antioxidant activity (CAA) assay. Adapted from Wolfe and Liu (2007)

Quantification of CAA. After blank subtraction from the fluorescence readings, the area under the curve of fluorescence versus time was integrated to calculate the CAA value at each concentration

of pure phytochemical compound or extract as follows:

$$\text{CAA unit} = 100 - (\int SA / \int CA) \times 100$$

where $\int SA$ is the integrated area under the sample fluorescence versus time curve and $\int CA$ is the integrated area from the control curve. The median effective dose (EC50) was determined for the pure phytochemical compounds and fruit extracts from the median effect plot of log (fa/fu) versus log (dose), where fa is the fraction affected and fu is the fraction unaffected by the treatment. To quantify intra-experimental variation, the EC50 values were stated as mean ± SD for triplicate sets of data obtained from the same experiment. Inter-experimental variation was obtained for some representative pure phytochemical compounds and fruit extracts by averaging the fluorescence values from triplicate wells in each trial to obtain one EC50 value per experiment and calculating the mean ± SD for at least four trials. In each experiment, quercetin was used as a standard, and cellular antioxidant activities for pure phytochemical compounds were expressed as micromoles of quercetin equivalents (QE) per 100 μmol of compound, whereas for fruit extracts they were expressed as micromoles of QE per 100 g of fruit. To compare the antioxidant quality of different fruits, CAA was also calculated as micromoles of QE per 100 μmol of total phenolics.

After the CAA unit was calculated from the integrated area under the fluorescence versus time curve, and the results were expressed as micromoles of quercetin equivalents (QE) per 100 g in fresh weight (μmol QE/100 g FW).

3.3.15 Statistical analysis

All measurements were performed in triplicate. Analysis of variance, principle component analysis (PCA) and "t" tests were carried out by Minitab 17.0 and the significance level was set at $P \leq 0.05$.

Chapter 4

Physicochemical and textural properties of tea enriched ADG

Currently, awareness of the link between diet and nutrition has been growing, thus there has been an increased demand for healthier products with a consequent rise in interest in functional and nutritional product by the food industry. Tea extracts have been extensively used not only as feed additives (Fang et al., 2019; Kolling et al., 2018; Winkler et al., 2015) directly employed on livestock to improve the quality and quantity of animal products, but also as food additives in modern food industry to either as preservation (Fernandez, Aguero, & Jagus, 2018; Huvaere et al., 2011; Yuan, Zhang, Tang, & Sun, 2016) to prolong the shelf life of food products or to improve the nutritional value of food products (Glibowski, Karwowska, Latoch, Nosowska, & Udeh, 2019; Lysoniewska, Kalisz, & Mitek, 2011).

The effects of tea extracts on physicochemical and textural properties of food matrices have been reported by Glibowski et al. (2019); Najgebauer-Lejko (2019) and Chatterjee, Das, Das, and Das (2018) but how the tea extracts influence the stability of ADG during cold storage are not fully understood.

The acidified dairy gels were made with the inclusion of green, oolong and black tea as of the method in Chapter 3.3.2. Figure 4.1 to 4.3 illustrate the gels which were made, and it is obvious that the colour of the gel was altered depending upon the type of tea infusion used.

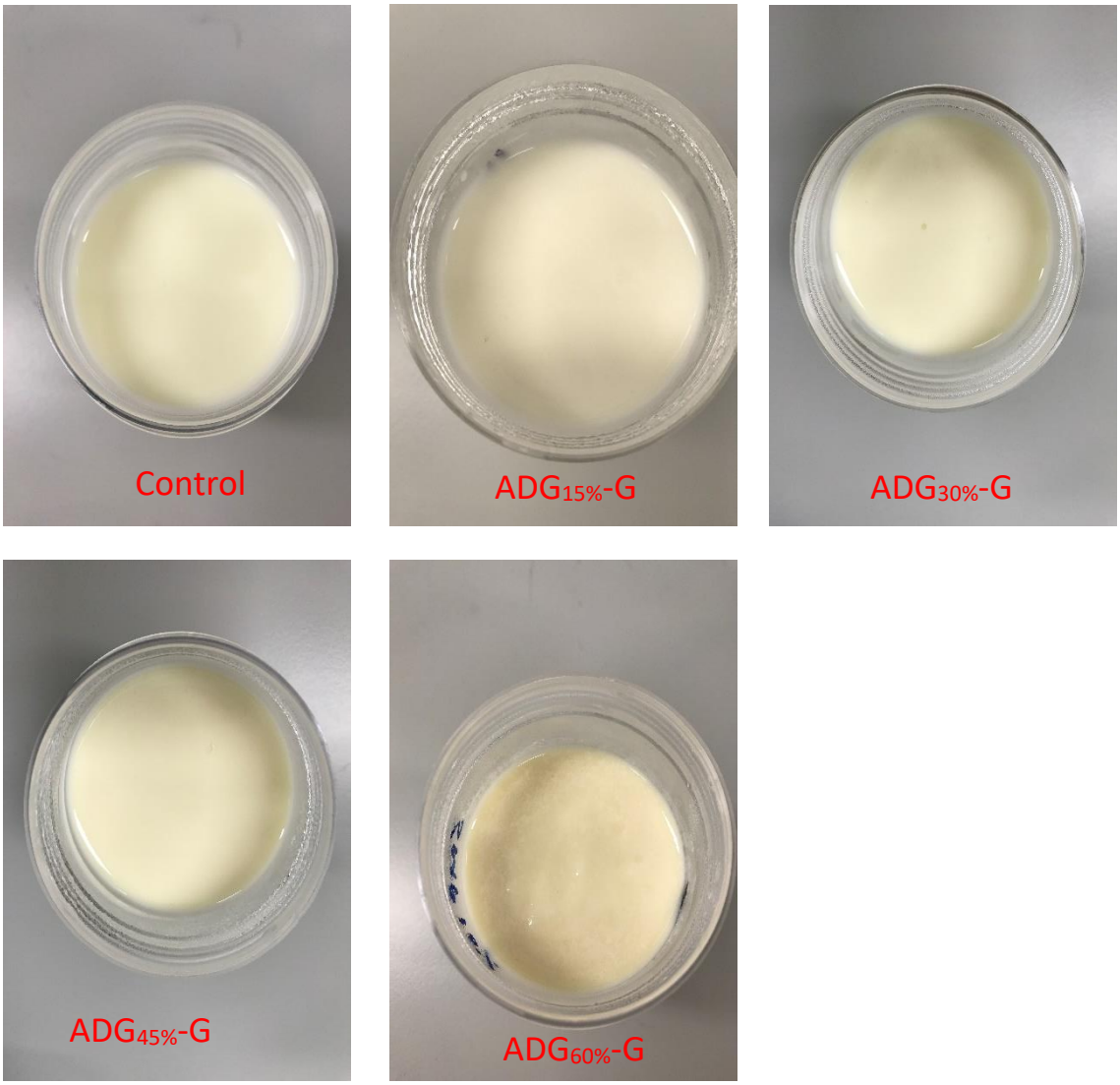


Figure 4.1 Photograph of green tea enriched acidified dairy gels.

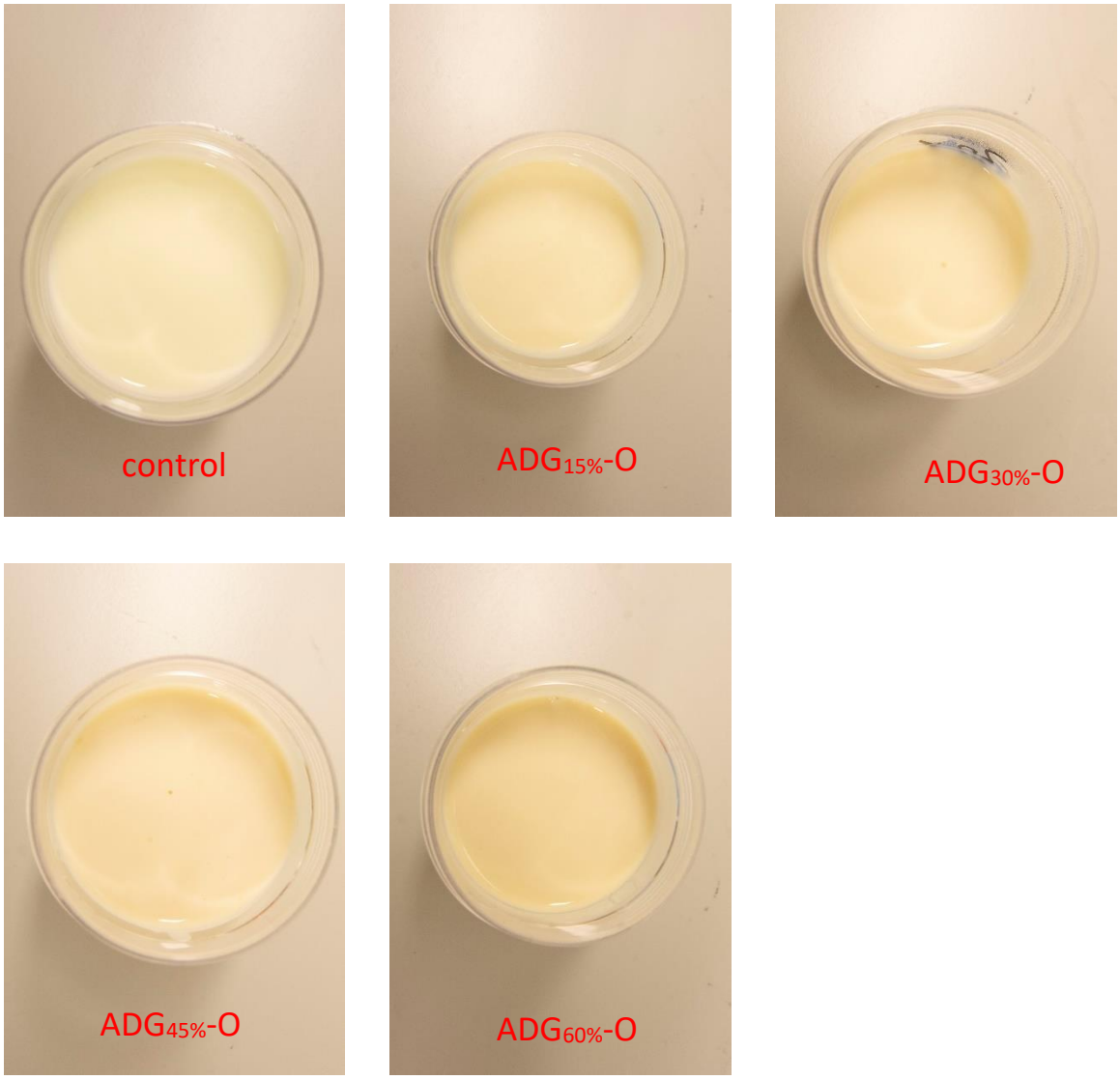


Figure 4.2 Photograph of oolong tea enriched acidified dairy gels.

Figure 4.3 Photograph of black tea enriched acidified dairy gels.

4.1 Results of physicochemical and textural properties of green tea enriched ADG

4.1.1 Physicochemical characteristics

The physicochemical characteristics of green tea enriched ADGs (ADG-G) and control (plain ADG) are shown in **Table 4.1**.

Table 4.1 Physicochemical characteristics between different green tea formulations during a 28-day cold storage (4 °C)

Physicochemical properties	Storage time (Days)	Formulation				
		ADG	$ADG_{15\%}$-G	$ADG_{30\%}$-G	$ADG_{45\%}$-G	$ADG_{60\%}$-G
pH value	1	4.55 ± 0.01^{Aa}	4.55 ± 0.01^{Aa}	4.55 ± 0.01^{Aa}	4.55 ± 0.02^{Aa}	4.56 ± 0.01^{Aa}
	7	4.40 ± 0.01^{Ba}	4.41 ± 0.01^{Ba}	4.37 ± 0.02^{Bc}	4.39 ± 0.01^{Bb}	4.42 ± 0.02^{Ba}
	14	4.35 ± 0.01^{Cb}	4.39 ± 0.02^{Ca}	4.36 ± 0.01^{Bb}	4.35 ± 0.02^{Cb}	4.39 ± 0.01^{Ca}
	21	4.33 ± 0.01^{Dc}	4.35 ± 0.01^{Da}	4.35 ± 0.01^{Cb}	4.32 ± 0.01^{Dc}	4.36 ± 0.01^{Da}
	28	4.30 ± 0.01^{Eb}	4.31 ± 0.01^{Eb}	4.30 ± 0.01^{Db}	4.29 ± 0.01^{Eb}	4.33 ± 0.02^{Ea}
Ca^{2+} concentration	1	463.33 ± 5.77^{Aa}	383.33 ± 5.77^{Ab}	356.67 ± 5.77^{Ac}	323.33 ± 5.77^{Ad}	376.67 ± 5.77^{Ab}
	7	413.33 ± 5.77^{Ba}	350.00 ± 10.00^{Bb}	326.67 ± 5.77^{Ab}	290.00 ± 10.00^{Bc}	283.33 ± 577^{Bc}
	14	403.33 ± 11.55^{Ba}	300.00 ± 10.00^{Cb}	243.33 ± 5.77^{Cd}	240.00 ± 10.00^{Dd}	273.33 ± 5.77^{Bc}
	21	376.67 ± 5.77^{Ca}	323.33 ± 15.28^{Db}	286.67 ± 15.28^{Bc}	263.33 ± 15.28^{Cc}	250.00 ± 10.00^{Cd}
	28	353.33 ± 11.55^{Da}	303.33 ± 5.77^{Db}	293.33 ± 20.82^{Bb}	270.00 ± 10.00^{Cc}	240.00 ± 10.00^{Cd}
EOS/%	1	36.61 ± 1.41^{Ab}	31.94 ± 0.48^{Aa}	30.27 ± 1.47^{Ca}	30.49 ± 1.39^{Ca}	30.45 ± 1.50^{Ba}
	7	32.75 ± 0.95^{Bb}	30.35 ± 1.01^{Ac}	34.56 ± 0.54^{Ba}	29.97 ± 1.16^{Cd}	31.40 ± 0.89^{Bb}
	14	33.20 ± 0.58^{Bb}	33.10 ± 1.18^{Ab}	37.10 ± 1.01^{Aa}	36.69 ± 0.91^{Aa}	31.41 ± 2.20^{Bb}
	21	27.69 ± 1.30^{Db}	32.05 ± 2.11^{Ab}	38.07 ± 0.98^{Aa}	38.15 ± 1.25^{Aa}	35.15 ± 2.05^{Ac}
	28	30.78 ± 1.51^{Cc}	32.47 ± 1.07^{Ab}	32.26 ± 1.68^{Bb}	34.26 ± 1.60^{Ba}	32.00 ± 1.13^{Bb}
WHC/%	1	15.13 ± 0.68^{Ba}	14.69 ± 0.39^{Ba}	13.19 ± 0.68^{Db}	13.76 ± 0.65^{Db}	13.67 ± 0.35^{Cb}
	7	17.50 ± 0.28^{Aa}	15.39 ± 0.25^{Ab}	13.26 ± 0.45^{Dc}	12.92 ± 0.91^{Dc}	14.61 ± 0.48^{Bb}
	14	16.01 ± 0.83^{Ba}	13.42 ± 0.79^{Cb}	13.97 ± 0.51^{Cb}	13.96 ± 0.22^{Cb}	16.36 ± 0.48^{Aa}
	21	15.66 ± 1.05^{Ba}	14.63 ± 0.60^{Bb}	14.66 ± 0.47^{Bb}	14.93 ± 0.41^{Bb}	14.65 ± 0.61^{Bb}
	28	18.04 ± 0.73^{Aa}	16.00 ± 0.77^{Ab}	15.93 ± 0.24^{Ab}	16.56 ± 0.86^{Ab}	17.15 ± 0.97^{Aa}

$^{A-E}$ Means ± SD within a column with different superscripts differ ($P \leq 0.05$).

$^{a-e}$ Means ± SD within a row with different superscripts differ ($P \leq 0.05$).

Formulation: ADG: acidified dairy gel; $ADG_{15\%}$-G: acidified dairy gel containing 15% (w/w) green tea infusion; $ADG_{30\%}$-G: acidified dairy gel containing 30% (w/w) green tea infusion; $ADG_{45\%}$-G: acidified dairy gel containing 45% (w/w) green tea infusion; $ADG_{60\%}$-G: acidified dairy gel containing 60% (w/w) green tea infusion;

EOS: Extend of syneresis; WHC: Water holding capacity.

The pH value showed a decreasing trend among all samples during cold storage, but no significant difference was observed between the different formulations. The minor differences of pH value between different formulations through could storage could be related to the buffering capacity of proteins or colloidal calcium phosphate (CCP) (Ozcan,

Horne, & Lucey, 2011; Salaün, Mietton, & Gaucheron, 2005).

The Ca^{2+} concentration showed a general decreasing trend with some fluctuations among all samples during the cold storage while the plain ADG exhibited the highest values in Ca^{2+} concentration from day 1 to day 28. The decrease of Ca^{2+} content in plain ADG showed the binding ability of calcium ions with CCP or directly bound to caseins (McIntyre, O' Sullivan, & O' Riordan, 2016). Although the calcium ion could directly bind with CCP or casein, the lower calcium content of ADGs-G indicated the calcium ions were affected by tea polyphenols. Ai et al. (2019a) added tea polyphenols and $Ca(OH)_2$ into egg white to explore the interaction between polyphenol and egg protein under a Ca^{2+}-enriched environment and the results suggested that the addition of polyphenol improved the interaction of nonspecific cross-linking, but weakened the other forces and increases surface hydrophobicity via calcium bridges which could lead to a decrease of free calcium and also supported our results. Yamada (2007) and his colleagues found that the calcium ion increased the amount of tea stain greatly due to the combination of phenolic compounds and calcium ions which demonstrated that calcium ions could form calcium-bridged polyphenols or matrix to lower the solubility of tea ingredients because its bridging effects using infrared spectroscopy (IR) and matrix-assisted laser desorption ionization technique with a time-of-flight mass spectrometer (MALDI-TOF-MS). A study of Carnovale and his colleagues (2016) also illustrated that the Ca^{2+} can increase the particle size in milk gel when polyphenols are present because of the increasing occurrence of EGCG-Ca^{2+}-EGCG bridging which could be an explanation of our results in a molecular point of view.

Weak gels flow more easily than stronger gels, which result in higher risk of shrinkage and subsequent expulsion of whey protein (Ciron, Gee, Kelly, & Auty, 2010a), so a good-quality ADG should retain water (Pimentel, Garcia, & Prudencio, 2012). All green tea enriched ADGs obtained lower results of EOS (lower is better) compared to plain ADG on day 1. However, the plain ADG exhibited the lowest value for EOS on day 28 ($P \leq 0.05$). When considering the results for WHC (higher is better), plain ADG illustrated higher values from day 1 to day 28 compared with green tea enriched samples. The results of EOS and WHC indicated the addition of green tea infusion weakened the dairy gel system. Whey separation generally occurs when the gel network is damaged or if the gel undergoes substantial structural rearrangement. Further, whey syneresis is due to inability to hold water in the protein matrix of the yoghurt gel (Narayana & Gupta, 2013). Lower whey syneresis rate suggested that the protein structure was less spongy and less dense after the addition of green tea infusion, and hence, gel structure might not be able to hold all the water, leaving some water as whey syneresis.

Interestingly, all samples with green tea infusion showed similar level of stability in terms of EOS and better on WHC through cold storage ($P < 0.05$). There was also a water-recovering in control group through cold storage both on EOS and WHC ($P < 0.05$). Reports have shown that exopolysaccharides (EPSs), a natural polymer, can act as an agent which is capable of thickening, stabilising, emulsifying, gelling as well as water-binding in the food system and EPSs can be produced by certain strains, such as *Lactobacillus paracasei*, therefore a better result in EOS was observed through cold storage (Pimentel et al., 2012). Moreover, a decreasing trend of EOS appeared in the control samples through cold

storage indicated an improvement of the water retention capacity of the system although there was no microbe used in the methodology, which suggested the interactions between polyphenols and proteins in the gradually acidified environment may form polymers with a similar effect as EPSs.

The results of EOS indicated that the tea components decreased the formation of polymers between protein by interacting with protein or deactivated the formation. The explanation for the contrary between the results of WHC and EOS could be that during preparation of samples, the high centrifuge speed in WHC procedure damaged the structure of the system. The covalent conjugation between tea polyphenols and dairy proteins suggests that amino acid side chain residues bound with polyphenols. Yuksel et al. (2010) used the anionic fluorescent probe of the aromatic sulphonic class (1-anilinonapthalene-8-sulfonate, ANS) to determine the different results of WHC between low and high level of tea infusions enriched protein samples. It could be due to the amount of amino acid side chain residue sites were far more than polyphenols in the reaction system at low level of tea infusions. With the increase of tea infusions, the polyphenol binding equivalent of covalent conjugates increased continuously. In other words, the small number of polyphenols could not saturate the residue site of amino acid side chains therefore one polyphenol molecular links one protein molecular with another. When the number of polyphenols saturated the residue site of amino acid side chains, one protein molecular links with several polyphenol molecules and less capable to link with protein molecules (Yang et al., 2019).

4.1.2 Textural Characteristics

The texture parameters of ADG-G determined by means of TPA and back-extrusion test (BET) on the set gel and BET on the stirred gel are presented in **Table 4.2**. Firmness is regarded as a critical textural characteristic of ADGs, along with other parameters, such as hardness and cohesiveness (Domagala, Sady, Grega, & Bonczar, 2006; Najgebauer-Lejko, 2019).

The addition of green tea extracts did not significantly affect the hardness in set ADG samples ($P < 0.05$). Although an increase was observed in the $ADG_{15\%}$-G in the adhesiveness compared to control higher content of tea infusion showed negative effects.

Table 4.2 Textural characteristics between different green tea formulations during a 28-day cold storage (4 °C)

Texture properties		Storage time (Days)	Formulation				
			ADG	$ADG_{15\%}$-G	$ADG_{30\%}$-G	$ADG_{45\%}$-G	$ADG_{60\%}$-G
Set ADG	Hardness/g	0	72.97 ± 5.66^{a}	66.57 ± 3.01^{a}	64.93 ± 6.76^{a}	65.20 ± 4.70^{a}	67.03 ± 7.20^{a}
	Adhesiveness/g·s		-16.01 ± 2.37^{a}	-17.17 ± 3.64^{a}	-10.88 ± 1.30^{b}	-13.99 ± 1.98^{b}	-13.36 ± 2.98^{b}
Firmness/g		1	21.10 ± 1.32^{Aa}	19.47 ± 1.46^{Ba}	19.57 ± 2.68^{Aa}	18.80 ± 1.91^{Aa}	21.73 ± 1.68^{Aa}
		7	22.43 ± 1.65^{Aa}	23.53 ± 2.27^{Aa}	20.27 ± 2.02^{Aa}	20.47 ± 0.75^{Aa}	21.97 ± 2.00^{Aa}
		14	22.87 ± 1.50^{Aa}	20.73 ± 0.75^{Aa}	18.97 ± 1.87^{Ab}	19.03 ± 1.97^{Ab}	21.67 ± 3.02^{Aa}
		21	20.83 ± 1.27^{Aa}	23.43 ± 3.00^{Aa}	19.17 ± 1.10^{Ab}	19.27 ± 1.50^{Ab}	19.87 ± 2.65^{Aa}
		28	19.17 ± 1.12^{Ba}	21.57 ± 1.56^{Aa}	17.70 ± 2.23^{Aa}	18.80 ± 1.57^{Aa}	21.67 ± 3.10^{Aa}
Cohesiveness/g		1	-13.50 ± 1.15^{Ba}	-12.27 ± 0.91^{Aa}	-12.53 ± 2.08^{Aa}	-11.77 ± 1.82^{Aa}	-12.83 ± 0.84^{Aa}
		7	-14.73 ± 1.21^{Ba}	-15.20 ± 1.22^{Ba}	-13.47 ± 1.67^{Aa}	-12.67 ± 0.81^{Aa}	-14.03 ± 1.15^{Aa}
		14	-14.23 ± 0.59^{Ba}	-13.60 ± 0.79^{Ba}	-12.27 ± 2.12^{Aa}	-11.90 ± 1.68^{Aa}	-13.70 ± 1.35^{Aa}
		21	-13.83 ± 0.93^{Ba}	-15.03 ± 1.79^{Bb}	-11.93 ± 0.87^{Aa}	-12.73 ± 0.65^{Aa}	-11.90 ± 1.38^{Aa}
		28	-11.60 ± 1.61^{Aa}	-12.90 ± 0.53^{Aa}	-11.00 ± 1.71^{Aa}	-12.27 ± 1.29^{Aa}	-12.7 ± 1.51^{Aa}

[A-E] Means ± SD within a column with different superscripts differ ($P \leq 0.05$).

[a-e] Means ± SD within a row with different superscripts differ ($P \leq 0.05$).

Formulation: ADG: acidified dairy gel; $ADG_{15\%}$-G: acidified dairy gel containing 15% (w/w) green tea infusion; $ADG_{30\%}$-G: acidified dairy gel containing 30% (w/w) green tea infusion; $ADG_{45\%}$-G: acidified dairy gel containing 45% (w/w) green tea infusion; $ADG_{60\%}$-G: acidified dairy gel containing 60% (w/w) green tea infusion;

EOS: Extend of syneresis; WHC: Water holding capacity

ADGs-G showed no significant difference in either firmness or cohesiveness on day 1 and day 28 ($P < 0.05$) except for the ADG15%-G which showed higher firmness on day 28 and better cohesiveness results between day 7 to day 14. This not only indicates that the ADG system was overall stable during the 28day cold storage, but also suggests that the addition of low level of green tea extracts improved the performance of ADG in terms of texture.

Donmez, Mogol, and Gokmen (2017) and Najgebauer-Lejko (2019) also found the effect of tea addition on the dairy gel mechanism. Donmez et al. (2017) noticed modified consistency and rate of syneresis in yoghurts supplemented with green tea powder and green coffee powder compared to a control sample. Najgebauer-Lejko (2019) reported the cohesiveness and index of viscosity of pu-erh tea enriched yoghurt were concentration-dependent in pu-erh tea treatments compared to control samples. The effect depended on the type of polyphenolic substances and their concentration. The retaining the hardness and adhesiveness between plain ADG, $ADG_{15\%}$-G suggested that low level of green tea infusion (15%) has the potential to be a texture modifier in gel food matrix.

4.2 Physicochemical and Textural Properties of Oolong Tea Enriched ADG

4.2.1 Physicochemical Characteristics

The physicochemical characteristics of oolong tea enriched ADGs and control (plain ADG) are shown in **Table 4.3**.

Table 4.3 Physicochemical characteristics between different oolong tea formulations during a 28-day cold storage (4 °C)

[A-E] Means ± SD within a column with different superscripts differ ($P \leq 0.05$).
[a-e] Means ± SD within a row with different superscripts differ ($P \leq 0.05$).
Formulation: ADG: acidified dairy gel; $ADG_{15\%}$-O: acidified dairy gel containing 15% (w/w) oolong tea infusion; $ADG_{30\%}$-O: acidified dairy gel containing 30% (w/w) oolong tea infusion; $ADG_{45\%}$-O: acidified dairy gel containing 45% (w/w) oolong tea infusion; $ADG_{60\%}$-O: acidified dairy gel containing 60% (w/w) oolong tea infusion;
EOS: Extend of syneresis; WHC: Water holding capacity.

Physicochemical properties	Storage time (Days)	Formulation				
		ADG	$ADG_{15\%}$-O	$ADG_{30\%}$-O	$ADG_{45\%}$-O	$ADG_{60\%}$-O
pH value	1	4.54 ± 0.02^{Aa}	4.54 ± 0.02^{Aa}	4.53 ± 0.02^{Aa}	4.53 ± 0.02^{Aa}	4.53 ± 0.01^{Aa}
	7	4.47 ± 0.02^{Ba}	4.39 ± 0.02^{Bc}	4.39 ± 0.01^{Bc}	4.41 ± 0.02^{Bc}	4.44 ± 0.02^{Bb}
	14	4.42 ± 0.02^{Ca}	4.35 ± 0.01^{Cc}	4.35 ± 0.01^{Cc}	4.34 ± 0.02^{Cc}	4.37 ± 0.01^{Cb}
	21	4.38 ± 0.01^{Da}	4.32 ± 0.01^{Dc}	4.33 ± 0.01^{Cb}	4.32 ± 0.01^{Cc}	4.32 ± 0.01^{Dc}
	28	4.35 ± 0.01^{Ea}	4.28 ± 0.01^{Ed}	4.29 ± 0.01^{Dc}	4.32 ± 0.01^{Cb}	4.30 ± 0.01^{Dc}
Ca^{2+} concentration	1	500.00 ± 20.00^{Aa}	356.67 ± 11.55^{Ab}	346.67 ± 5.77^{Ab}	296.67 ± 15.28^{Ac}	273.33 ± 15.28^{Ac}
	7	453.33 ± 15.28^{Ba}	316.67 ± 5.77^{Cb}	283.33 ± 5.77^{Cc}	253.33 ± 11.55^{Bd}	236.67 ± 15.28^{Bd}
	14	423.33 ± 15.28^{Ca}	333.33 ± 5.77^{Bb}	303.33 ± 5.77^{Bc}	263.33 ± 15.28^{Bd}	243.33 ± 5.77^{Be}
	21	376.67 ± 11.55^{Da}	360.00 ± 10.00^{Ab}	306.67 ± 5.77^{Bc}	250.00 ± 10.00^{Bd}	236.67 ± 5.77^{Bd}
	28	356.67 ± 5.77^{Da}	336.67 ± 5.77^{Bb}	283.33 ± 15.28^{Cc}	246.67 ± 5.77^{Bd}	226.67 ± 5.77^{Be}
EOS/%	1	35.96 ± 0.68^{Ab}	28.82 ± 0.72^{Cb}	27.22 ± 0.97^{Cc}	30.32 ± 0.30^{Ca}	27.72 ± 1.10^{Dc}
	7	32.52 ± 1.14^{Bd}	27.93 ± 0.37^{Cc}	31.98 ± 0.61^{Ba}	30.23 ± 0.78^{Cb}	31.98 ± 0.82^{Ca}
	14	29.09 ± 0.42^{Dd}	32.06 ± 0.58^{Bb}	32.07 ± 0.85^{Bb}	28.12 ± 0.72^{Dc}	34.90 ± 1.00^{Ba}
	21	31.21 ± 0.33^{Cc}	32.43 ± 1.55^{Bb}	36.46 ± 0.75^{Aa}	34.01 ± 0.43^{Ba}	31.64 ± 1.30^{Cb}
	28	30.93 ± 0.56^{Cc}	34.76 ± 1.20^{Ab}	36.44 ± 0.90^{Ab}	36.27 ± 1.09^{Ab}	39.35 ± 1.51^{Aa}
WHC/%	1	17.57 ± 0.58^{Aa}	15.54 ± 0.22^{Bb}	15.87 ± 0.41^{Bb}	15.48 ± 0.36^{Bb}	14.75 ± 0.27^{Ac}
	7	16.99 ± 0.88^{Aa}	16.90 ± 0.41^{Ca}	17.25 ± 0.49^{Aa}	16.99 ± 0.88^{Aa}	12.12 ± 0.84^{Db}
	14	17.97 ± 0.45^{Aa}	15.62 ± 0.37^{Cc}	15.18 ± 0.70^{Bc}	17.14 ± 0.51^{Ab}	13.41 ± 0.41^{Cd}
	21	16.59 ± 0.26^{Ba}	16.16 ± 1.17^{Aa}	15.33 ± 0.97^{Bb}	14.39 ± 0.29^{Cc}	15.81 ± 0.40^{Aa}
	28	16.08 ± 0.52^{Ca}	14.77 ± 0.26^{Cb}	15.62 ± 0.30^{Ba}	15.66 ± 0.31^{Ba}	13.79 ± 0.44^{Bc}

Glibowski et al. (2019) reported that storage time caused minor changes in the acidity value of dairy gels and that the pH value showed no significant differences between samples with different concentrations of tea infusion added, with the control being similar to the results presented in this study. The plain ADG got highest values in Ca^{2+} concentration from day 1 to day 28 while the Ca^{2+} concentration showed a general

decreasing trend among all samples during the cold storage. For oolong tea enriched ADG (ADG-O), the decreases of Ca^{2+} concentration appeared in all ADG-O samples after adding oolong tea infusion, and the Ca^{2+} concentration was opposite to the content of tea infusion. The pH value of plain ADG was higher than ADG-O samples.

The decrease of Ca^{2+} concentration in plain ADG suggested that the calcium ions could bind with dairy protein naturally to form calcium-caseinate while the more intense decrease of Ca^{2+} concentration in ADG-O samples indicated the appearance of tea polyphenols promoted the interaction of calcium ions and dairy protein.

Ai et al. (2019a) added tea polyphenols and $Ca(OH)_2$ into egg white to explore the interaction between polyphenol and egg protein under a Ca^{2+}-enriched environment. Their results suggested that the addition of polyphenol improves the interaction of nonspecific cross-linking but weakens other forces and increases surface hydrophobicity via calcium bridges.

The water holding capacity and syneresis of a gel matrix are related to its stability, functional properties, texture and the method of preservation of the food (Kruif, Anema, Zhu, Havea, & Coker, 2015). ADGs-O samples gained lower results of EOS compared to plain ADG (lower is better) on day 1 but became higher on day 28 ($P \leq 0.05$) and ADG-O samples showed an increasing trend while the plain ADG showed a decreasing trend. As for WHC, plain ADG acquired higher results (higher is better) compared to ADG-O from day 1 to day 28 ($P \leq 0.05$). The results of EOS and WHC indicated that the addition of oolong tea infusion will weaken the structure and damage the stability of ADG. Cartasev

and Rudic (2017) reported that exopolysaccharides (EPSs), a microbe-generated polymer, can act as an agent which can improve the textural properties and water-binding capacity in the food system, therefore a better result in EOS was observed through cold storage. Interestingly, a decreasing trend of EOS appeared in the control samples through cold storage indicated an improvement of the water retention capacity of the system although no microbe was used which suggested the presence of polyphenols in the gradually acidified dairy system may form polymers with similar effects as EPSs. The covalent conjugation between tea polyphenols and dairy proteins was due to amino acid side chain residues bonding with polyphenols. Kanakis and colleagues (2011b) reported that the protein would bond with tea polyphenol as the order of EGCG > ECG > EC > C. With the increase of tea infusions, the polyphenol binding equivalent of covalent conjugates increased continuously, and the binding polyphenol shifted from EGCG to C. In other words, the small number of polyphenols could not occupy all the residue site of amino acid side chains therefore one polyphenol molecular links one protein molecular with another. When the number of polyphenols saturated the residue site of amino acid side chains, one protein molecular links with several polyphenol molecules and less capable to link with protein molecules (Yang et al., 2019).

4.2.2 Texture characteristics

The textural characteristics of ADGs are shown in **Table 4.4**. The firmness is a critical textural characteristic of ADGs along with other parameters, such as hardness and cohesiveness (Domagala et al., 2006).

Table 4.4 Texture characteristics between different oolong tea formulations during a 28-day cold storage (4 °C)

Texture properties		Storage time (Days)	Formulation				
			ADG	$ADG_{15\%}$-O	$ADG_{30\%}$-O	$ADG_{45\%}$-O	$ADG_{60\%}$-O
Set ADG	Hardness/g	0	72.07 ± 5.42^{a}	60.53 ± 1.87^{c}	64.10 ± 6.60^{b}	65.00 ± 2.71^{b}	67.70 ± 8.03^{b}
	Adhesiveness/g·s		-26.65 ± 1.44^{c}	-16.99 ± 1.90^{a}	-17.47 ± 2.54^{a}	-20.59 ± 3.10^{a}	-23.14 ± 4.76^{b}
Firmness/g		1	21.93 ± 0.55^{Aa}	19.77 ± 1.04^{Ab}	19.60 ± 1.00^{Ab}	20.23 ± 1.29^{Ab}	20.000 ± 1.40^{Aa}
		7	21.97 ± 1.30^{Aa}	19.63 ± 1.46^{Aa}	20.67 ± 1.29^{Aa}	22.50 ± 1.77^{Aa}	22.10 ± 2.34^{Aa}
		14	24.90 ± 4.56^{Aa}	21.27 ± 1.31^{Aa}	21.40 ± 3.58^{Aa}	22.17 ± 1.96^{Aa}	21.63 ± 0.61^{Aa}
		21	22.80 ± 2.14^{Aa}	21.10 ± 1.47^{Aa}	20.80 ± 1.32^{Aa}	21.27 ± 3.02^{Aa}	19.57 ± 1.29^{Aa}
		28	21.63 ± 2.32^{Aa}	18.83 ± 0.78^{Ba}	21.60 ± 1.11^{Aa}	19.43 ± 1.11^{Aa}	21.60 ± 2.41^{Aa}
Cohesiveness/g		1	-14.47 ± 0.67^{Ab}	-12.70 ± 0.80^{Aa}	-12.77 ± 0.93^{Aa}	-13.27 ± 1.29^{Aa}	-12.20 ± 1.57^{Aa}
		7	-14.27 ± 1.42^{Aa}	-12.67 ± 1.25^{Aa}	-13.23 ± 1.27^{Aa}	-14.50 ± 1.78^{Aa}	-13.97 ± 1.31^{Aa}
		14	-16.43 ± 3.23^{Aa}	-13.77 ± 1.78^{Aa}	-13.93 ± 2.78^{Aa}	-15.00 ± 2.02^{Aa}	-14.70 ± 0.99^{Aa}
		21	-15.07 ± 2.34^{Aa}	-15.70 ± 2.86^{Ba}	-13.57 ± 1.43^{Aa}	-12.50 ± 1.61^{Aa}	-12.20 ± 1.40^{Aa}
		28	-14.27 ± 1.97^{Aa}	-12.00 ± 0.76^{Aa}	-13.83 ± 1.29^{Aa}	-12.97 ± 1.91^{Aa}	-14.27 ± 2.02^{Aa}

[A-E] Means ± SD within a column with different superscripts differ ($P \leq 0.05$).

[a-e] Means ± SD within a row with different superscripts differ ($P \leq 0.05$).

Formulation: ADG: acidified dairy gel; $ADG_{15\%}$-O: acidified dairy gel containing 15% (w/w) oolong tea infusion; $ADG_{30\%}$-O: acidified dairy gel containing 30% (w/w) oolong tea infusion; $ADG_{45\%}$-O: acidified dairy gel containing 45% (w/w) oolong tea infusion; $ADG_{60\%}$-O: acidified dairy gel containing 60% (w/w) oolong tea infusion;

ADG enriched with oolong tea infusion gained worse results regardless of concentration in terms of hardness and adhesiveness compared with the control in the shape of set ADG ($P < 0.05$). These results also affected the textural properties of stirred samples, ADGs-O samples showed worse performance in both firmness and cohesiveness on day 1 compared to the control sample, however by day 28 their firmness and cohesiveness values were similar to those of the control sample ($P < 0.05$). As for the set ADG, the plain ADG also achieved better results than ADG-O samples. The textural results were similar to the results of EOS and WHC, and this suggested that the addition of oolong tea infusion changed the formation and weakened the structure of the ADG system.

4.3 Physicochemical and textural properties of black tea enriched ADG

4.3.1 Physicochemical characteristics

The physicochemical characteristics of black tea enriched ADGs (ADG-B) are shown in **Table 4.5**.

Table 4.5 Physicochemical characteristics between different black tea formulations during a 28-day cold storage (4 °C)

Physicochemical properties	Storage time (Days)	Formulation				
		ADG	$ADG_{15\%}$ -B	$ADG_{30\%}$-B	$ADG_{45\%}$-B	$ADG_{60\%}$-B
pH value	1	4.55 ± 0.01^{Aa}	4.53 ± 0.01^{Ab}	4.54 ± 0.01^{Ab}	4.54 ± 0.01^{Ab}	4.53 ± 0.01^{Ab}
	7	4.35 ± 0.01^{Bc}	4.37 ± 0.01^{Bb}	4.36 ± 0.01^{Bc}	4.34 ± 0.01^{Cd}	4.40 ± 0.01^{Ba}
	14	4.29 ± 0.01^{Db}	4.28 ± 0.01^{Dc}	4.36 ± 0.01^{Ba}	4.36 ± 0.01^{Ba}	4.28 ± 0.01^{Dc}
	21	4.30 ± 0.01^{Dc}	4.33 ± 0.01^{Ca}	4.32 ± 0.01^{Cb}	4.28 ± 0.02^{Dd}	4.29 ± 0.01^{Dd}
	28	4.32 ± 0.01^{Ca}	4.28 ± 0.02^{Dc}	4.25 ± 0.01^{Dc}	4.29 ± 0.01^{Dc}	4.31 ± 0.01^{Cb}
Ca^{2+} concentration	1	450.00 ± 0.00^{Ba}	443.33 ± 5.77^{Aa}	446.67 ± 15.28^{Aa}	353.33 ± 5.77^{Ab}	273.33 ± 15.28^{Bc}
	7	500.00 ± 10.00^{Aa}	356.67 ± 11.55^{Bb}	316.67 ± 5.77^{Cc}	253.33 ± 5.77^{Cd}	326.67 ± 15.28^{Ab}
	14	356.67 ± 5.77^{Ca}	323.33 ± 5.77^{Cb}	326.67 ± 5.77^{Bb}	253.33 ± 5.77^{Cc}	263.33 ± 5.77^{Cc}
	21	370.00 ± 10.00^{Ca}	$326.6\ 7 \pm 15.28^{Bb}$	293.33 ± 5.77^{Db}	266.67 ± 5.77^{Bc}	246.67 ± 11.55^{Cc}
	28	330.00 ± 10.00^{Da}	336.67 ± 5.77^{Ba}	316.67 ± 5.77^{Cb}	246.67 ± 5.77^{Cd}	290.00 ± 10.00^{Bc}
EOS/%	1	39.95 ± 0.89^{Aa}	24.05 ± 0.78^{Ad}	29.48 ± 0.73^{Bc}	35.09 ± 1.15^{Bb}	29.01 ± 1.10^{Ac}
	7	37.74 ± 1.21^{Ba}	22.99 ± 0.52^{Ad}	31.16 ± 0.90^{Ab}	39.02 ± 1.22^{Aa}	27.50 ± 0.80^{Bc}
	14	21.28 ± 0.80^{Dc}	23.06 ± 1.23^{Ab}	24.83 ± 0.20^{Db}	20.34 ± 0.59^{Ec}	28.42 ± 0.52^{Aa}
	21	17.21 ± 0.39^{Ee}	22.10 ± 0.92^{Bd}	24.68 ± 0.68^{Dc}	28.92 ± 0.60^{Da}	26.36 ± 0.78^{Bb}
	28	23.37 ± 0.33^{Cd}	23.30 ± 0.96^{Ad}	28.25 ± 0.33^{Cb}	30.20 ± 0.27^{Ca}	26.87 ± 0.14^{Bc}
WHC/%	1	17.13 ± 0.81^{Eb}	19.56 ± 1.04^{Ba}	21.10 ± 0.63^{Ba}	15.63 ± 0.12^{Dc}	15.30 ± 0.48^{Cc}
	7	24.33 ± 0.69^{Ba}	22.69 ± 0.23^{Ab}	23.50 ± 0.80^{Aa}	20.49 ± 0.79^{Ac}	21.01 ± 0.65^{Ac}
	14	27.31 ± 0.72^{Aa}	21.93 ± 0.65^{Ab}	20.69 ± 0.34^{Cc}	16.34 ± 0.15^{Cd}	15.22 ± 0.63^{Ce}
	21	18.82 ± 0.25^{Db}	16.84 ± 1.16^{Cc}	23.89 ± 0.58^{Aa}	18.07 ± 0.51^{Bc}	18.54 ± 0.94^{Bc}
	28	20.15 ± 0.55^{Cb}	22.20 ± 0.32^{Aa}	19.42 ± 0.37^{Cb}	19.82 ± 0.32^{Ab}	19.37 ± 0.84^{Bb}

$^{A-E}$ Means ± SD within a column with different superscripts differ ($P \leq 0.05$).
$^{a-e}$ Means ± SD within a row with different superscripts differ ($P \leq 0.05$).
Formulation: ADG: acidified dairy gel; $ADG_{15\%}$-B: acidified dairy gel containing 15% (w/w) black tea infusion; $ADG_{30\%}$-B: acidified dairy gel containing 30% (w/w) black tea infusion; $ADG_{45\%}$-B: acidified dairy gel containing 45% (w/w) black tea infusion; $ADG_{60\%}$-B: acidified dairy gel containing 60% (w/w) black tea infusion;
EOS: Extend of syneresis; WHC: Water holding capacity.

Among the different formulations, the plain ADG achieved the highest values in pH value, Ca^{2+} concentration and EOS. Decreases in pH value and Ca^{2+} concentration of ADGs along with an increasing EOS were observed through cold storage. The decreases of Ca^{2+} concentration was only observed in $ADG_{45\%}$-B and $ADG_{60\%}$-B on day 1 which indicated the

sequence of interaction between protein and polyphenol is prior to polyphenol and Ca^{2+} is probably because the interaction between calcium ion and polyphenols or oxalate in tea, confirming the observation of Charrier, Savage, and Vanhanen (2002) who mentioned that the oxalate in teas would bind with calcium ion. Additionally, Yamada (2007) and his colleagues used infrared spectroscopy (IR) and matrix-assisted laser desorption ionization technique with a time-of-flight mass spectrometer (MALDI-TOF-MS), and found that the calcium ion increased the amount of tea stain greatly due to the combination of phenolic compounds and calcium ions which demonstrated that calcium ions could form calcium-bridged polyphenols or matrix to lower the solubility of tea ingredients because its bridging effects. A study of Carnovale et al. (2016) signified that the Ca^{2+} can increase the particle size in milk gel when polyphenols are present because of the increasing occurrence of EGCG-Ca^{2+}-EGCG bridging which could be an explanation of our results in a molecular point of view.

The minor differences of pH value between different formulations could be related to the buffering capacity of proteins or CCP (Salaün et al., 2005).

The physical properties of the dairy gel system, including the separation of the whey (syneresis) and water retention (water holding capacity), play important roles in the product quality and consumer acceptance (da Silva, de Abreu, & Assumpcao, 2012). All ADGs gained lower results of EOS (lower is better) on day 1 but only the $ADG_{15\%}$-B sample remained low level of EOS through cold storage ($P \leq 0.05$). As for WHC, $ADG_{15\%}$-B and $ADG_{30\%}$-B acquired higher results (higher is better) on day 1 but only $ADG_{15\%}$-B remained

higher results on day 28 ($P \leq 0.05$), thus the low amount of tea infusion may help to maintain the gel structure of ADGs. Interestingly, all samples with black tea infusion showed more stable trends both on EOS and WHC but there was a stronger water-recovering in control group through cold storage. and It is reported that exopolysaccharides (EPSs), produced by certain microbes such as *Lactobacillus mucosae*, resulted in a yoghurt product with improvement of water retaining properties, therefore a decrease in EOS was observed through cold storage (Buldo et al., 2016; London et al., 2015). A decreasing trend of EOS appeared in our experiments through cold storage although no microbe was utilized which indicate the proteins may form a mechanism with similar effect to EPSs in a gradually acidified environment when interact with polyphenols.

4.3.2 Textural characteristics

The textural characteristics of ADGs are shown in **Table 4.6**. The firmness is a critical textural characteristic of ADGs along with other parameters, such as hardness and cohesiveness (Domagala et al., 2006).

Table 4.6 Textural characteristics between different black tea formulations during a 28-day cold storage (4 °C)

Texture properties		Storage time (Days)	Formulation				
			ADG	$ADG_{15\%}$-B	$ADG_{30\%}$-B	$ADG_{45\%}$-B	$ADG_{60\%}$-B
Set ADG	Hardness/g	0	78.63 ± 9.22^{a}	71.70 ± 1.51^{a}	37.70 ± 0.89^{c}	30.87 ± 2.85^{c}	46.43 ± 1.62^{b}
	Adhesiveness/g·s		-26.65 ± 10.01^{a}	-27.56 ± 1.67^{a}	-7.65 ± 2.20^{b}	-3.37 ± 1.32^{b}	-2.52 ± 1.11^{b}
Firmness/g		1	14.77 ± 0.46^{Ba}	14.73 ± 0.35^{Ba}	14.57 ± 0.31^{Ba}	14.07 ± 0.42^{Ba}	14.70 ± 1.04^{Aa}
		7	20.10 ± 0.90^{Aa}	20.43 ± 1.25^{Aa}	17.13 ± 0.72^{Ab}	16.40 ± 0.72^{Ab}	15.17 ± 1.08^{Ab}
		14	21.13 ± 0.67^{Aa}	20.07 ± 0.86^{Aa}	18.60 ± 0.87^{Ab}	16.37 ± 0.83^{Bb}	15.33 ± 1.60^{Ab}
		21	21.93 ± 0.55^{Aa}	19.83 ± 1.15^{Aa}	17.70 ± 0.27^{Ab}	16.30 ± 0.87^{Bb}	16.73 ± 0.15^{Ab}
		28	20.53 ± 0.32^{Aa}	18.80 ± 0.82^{Aa}	18.20 ± 0.87^{Aa}	19.03 ± 1.61^{Aa}	16.57 ± 0.85^{Ab}
Cohesiveness/g		1	-9.50 ± 0.27^{Ca}	-9.07 ± 0.06^{Bb}	-8.67 ± 0.40^{Bb}	-8.53 ± 0.23^{Bb}	-9.40 ± 0.61^{Bb}
		7	-12.43 ± 0.50^{Ba}	-12.63 ± 1.00^{Aa}	-10.93 ± 0.35^{Ab}	-10.53 ± 0.67^{Ac}	-9.37 ± 0.31^{Bc}
		14	-13.27 ± 0.42^{Ba}	-12.83 ± 1.24^{Aa}	-12.47 ± 1.25^{Aa}	-10.27 ± 0.40^{Ab}	-8.83 ± 0.71^{Bb}
		21	-14.13 ± 0.51^{Aa}	-12.80 ± 0.27^{Aa}	-12.33 ± 1.10^{Ab}	-10.07 ± 0.59^{Bb}	-10.13 ± 0.06^{Bb}
		28	-13.73 ± 0.12^{Aa}	-12.57 ± 0.15^{Ab}	-11.80 ± 1.21^{Ab}	-10.43 ± 0.67^{Ac}	-10.43 ± 0.40^{Bc}

[A-E] Means ± SD within a column with different superscripts differ ($P \leq 0.05$).

[a-e] Means ±SD within a row with different superscripts differ ($P \leq 0.05$).

Formulation: ADG: acidified dairy gel; $ADG_{15\%}$-B: acidified dairy gel containing 15% (w/w) black tea infusion; $ADG_{30\%}$-B: acidified dairy gel containing 30% (w/w) black tea infusion; $ADG_{45\%}$-B: acidified dairy gel containing 45% (w/w) black tea infusion; $ADG_{60\%}$-B: acidified dairy gel containing 60% (w/w) black tea infusion;

There was little significant difference in firmness and cohesiveness between different formulations owing to homogenization. All ADG-B samples and the control showed an increasing trend in firmness and cohesiveness through cold storage which is similar to the results reported by Bruzzone, Ares, and Gimenez (2013) and Vieira et al. (2019). As for set ADG, retaining the hardness and adhesiveness between plain ADG and $ADG_{15\%}$-B suggested that the addition of 15% tea infusion did not affect the extent to which the gel could undergo the deformation before its disruption nor the force to remove the ADG adhered to the spoon or mouth during eating the ADG (Kumar & Mishra, 2003).

4.4 Conclusion

Although the main characteristics are similar among three types of tea enriched ADG, there still are some differences.

The Ca^{2+} concentration of $ADG_{15\%}$-B and $ADG_{30\%}$-B remained the same level as that of plain ADG on day 1 but showed instant decrease on ADG-G and ADG-O samples. The results of EOS and WHC of ADG-B samples also achieved better results than ADG-G and ADG-O generally.

The main reason for the results could be due to the different processes of tea making. This in turn makes black tea theaflavins-abound, whereas in green tea and oolong tea are catechin-abound. In the meanwhile, protein would interact with tea polyphenols as the order of theaflavins > EGCG > ECG > C > EC > EGC (Bourassa, Côté, Hutchandani, Samson, & Tajmir-Riahi, 2013). Combine the Ca^{2+} concentration of three types of ADG together, we can elicit that the order proteins interact with Ca^{2+} is between proteins interact with theaflavins and EGCG which makes the sequence as theaflavins >Ca^{2+} > EGCG > ECG > C > EC > EGC which is in agreement with Ai et al. (2019b) who explored the effects of tea polyphenol and $Ca(OH)_2$ on the intermolecular forces and mechanical, rheological, and microstructural characteristics of duck egg white gel.

The textural properties did not show significant differences among three types of tea enriched ADGs, this could be due to the homogenization process which happened before

cold storage as Nguyen, Ong, Kentish, and Gras (2015) reported that Homogenisation can change the microstructure, syneresis and rheological properties of buffalo yoghurt.

Thus, tea infusion can be successfully applied as a functional additive to probiotic dairy gel (yoghurt-like matrix) as it can increase the overall stability of the final product. On the other hand, the concentration of tea infusion must be carefully chosen as it negatively influences the textural parameters in higher doses.

Chapter 5

Results of antioxidant properties and HPLC profiles of tea enriched ADG

To determine the antioxidant abilities of the obtained ADGs and tea infusion, we chose three methods which allowed us to measure both the ability to reduce pro-oxidant metal ions (FRAP assay), radical scavenging activity (DPPH assay and ABTS assay) and TPC measurement along with cell line tests. The *in vitro* results are shown in **Figures 5.1 to 5.3**.

5.1 *In vitro* antioxidant capacity of green tea enriched ADG

The TPC, DPPH, FRAP and ABTS results of green tea enriched ADG are shown in **Figure 5.1**.

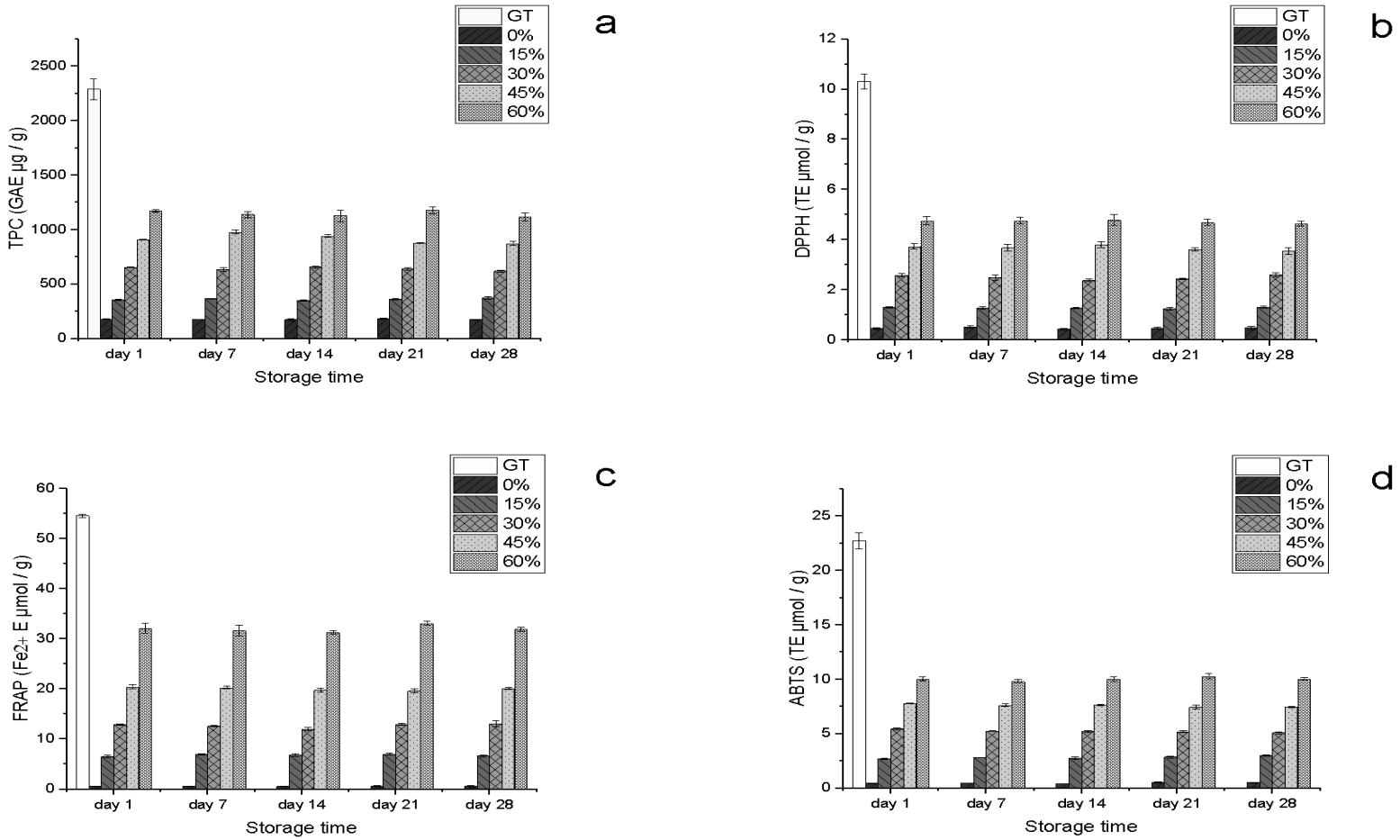


Figure 5.1 Antioxidant capacity parameters (Green tea enriched acidified dairy gel). 0%: plain acidified dairy gel, 15%: acidified dairy gel containing 15% green tea infusion, 30%: acidified dairy gel containing 30% green tea infusion, 45%: acidified dairy gel containing 45% green tea infusion, 60%: acidified dairy gel containing 60% green tea infusion. Figure 5.1a: Total phenolic content (TPC) values of different concentration of tea infusion, expressed as GAE µg/g; Figure 5.1b: DPPH values of different concentration of tea infusion, expressed as TE µmol/g; Figure 5.1c: FRAP values of different concentration of tea infusion, expressed as Fe^{2+} E µmol/g; Figure 5.1d: ABTS values of different concentration of tea infusion, expressed as TE µmol/g;

The strongest antioxidant capacity appeared in green tea infusion, this was observed in the FRAP, DPPH and ABTS radical assays. These values correspond to the TPC results for the samples. The higher the content of tea infusion added in ADGs, the higher were the values of antioxidant capacity and TPC and there were significant differences between different formulation through the whole cold storage (p<0.05). Overall, the addition of green tea infusion significantly enhanced the antioxidant potential of ADG as a 2.5 to 10-fold increase in DPPH, a 13 to 60-fold in FRAP and a 5.5 to 20-fold in ABTS value were observed in comparison to plain ADG. Some antioxidant parameters were not observed to decrease over time, but remained higher level at day 21 compare to day 7 and 14. These results are similar to those reported by Najgebauer-Lejko, Sady, Grega, and Walczycka (2011).

5.2 *In vitro* antioxidant capacity of oolong tea enriched ADG

The TPC, DPPH, FRAP and ABTS results of oolong tea enriched ADG are shown in **Figure 5.2**.

The strongest antioxidant capacity appeared in oolong tea infusion among FRAP, DPPH and ABTS radical assay. These values again corresponded to observed values of TPC in the samples. Again, there was a general observation that the higher content of tea infusion added in ADGs the more antioxidant capacity and TPC was observed, and there were significant differences between different formulation through the cold storage ($p < 0.05$). Overall, the addition of green tea infusion significantly enhanced the antioxidant potential of ADG as a 3 to 7-fold increase in DPPH, an 8 to 30-fold increase in FRAP and a 7 to 18-fold increase in ABTS value were observed in comparison to plain ADG.

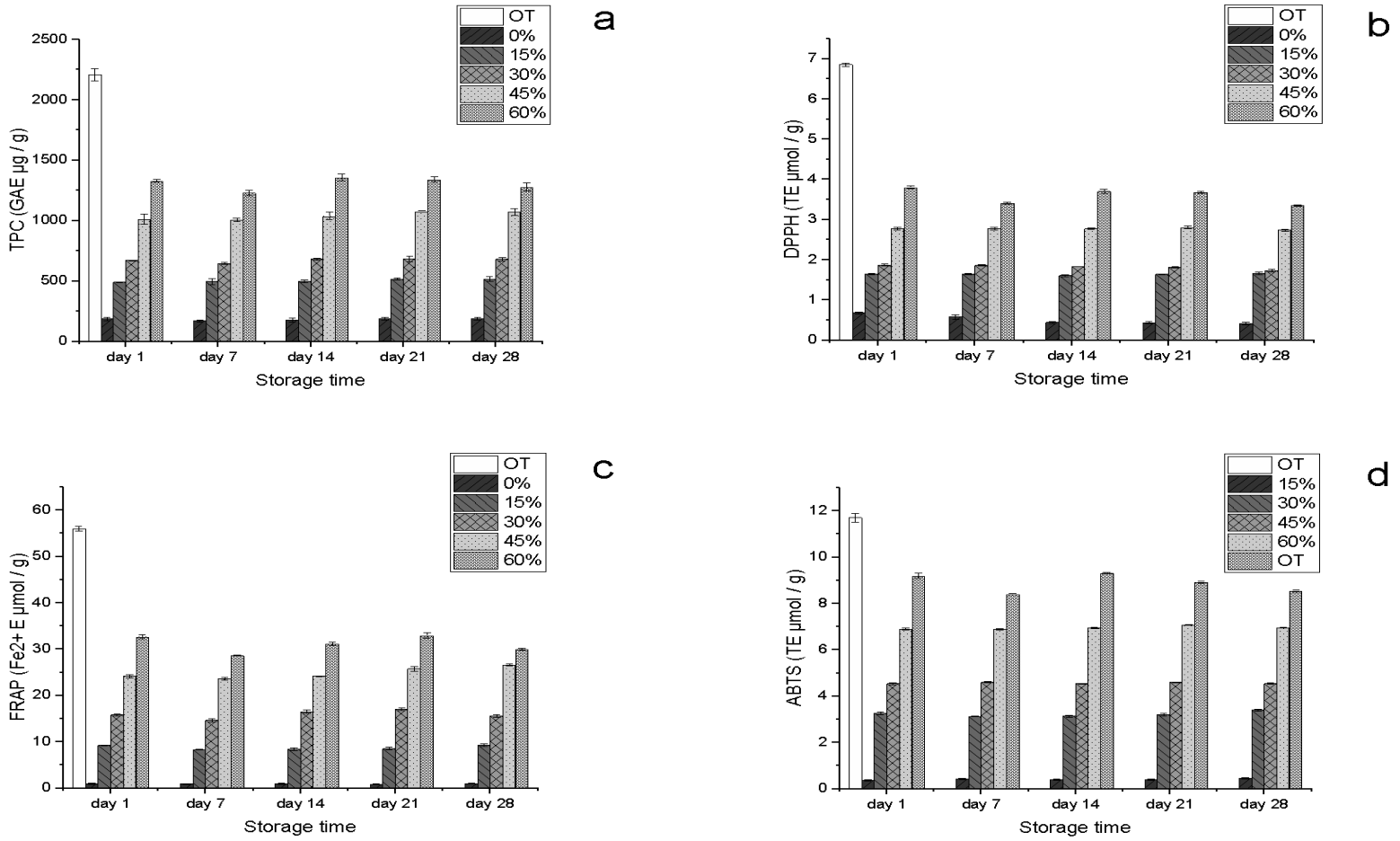


Figure 5.2 Antioxidant capacity parameters (oolong tea enriched acidified dairy gel). 0%: plain acidified dairy gel, 15%: acidified dairy gel containing 15% oolong tea infusion, 30%: acidified dairy gel containing 30% oolong tea infusion, 45%: acidified dairy gel containing 45% oolong tea infusion, 60%: acidified dairy gel containing 60% oolong tea infusion. Figure 5.2a: Total phenolic content (TPC) values of different concentration of tea infusion, expressed as GAE µg/g; Figure 5.2b: DPPH values of different concentration of tea infusion, expressed as TE µmol/g; Figure 5.2c: FRAP values of different concentration of tea infusion, expressed as Fe^{2+} E µmol/g; Figure 5.2d: ABTS values of different concentration of tea infusion, expressed as TE µmol/g;

5.3 *In vitro* antioxidant capacity of black tea enriched ADG

The TPC, DPPH, FRAP and ABTS results of black tea enriched ADG are shown in **Figure 5.3**.

The strongest antioxidant capacity appeared in black tea infusion among FRAP, DPPH and ABTS radical assay which were correspond to the TPC result. The higher content of tea infusion added in ADGs the more antioxidant capacity and TPC would obtain and there were significant differences between different formulation through the cold storage ($P < 0.05$). Overall, the addition of green tea infusion significantly enhanced the antioxidant potential of ADG as a 4 to 12-fold increase in DPPH, an 8 to 40-fold in FRAP and a 6 to 18-fold in ABTS value were observed in comparison to plain ADG.

To summarize the *in vitro* antioxidant experiments, the green tea had the most antioxidant capacity promoting ability followed by the oolong tea and the black tea in terms of FRAP and ABTS results which shared same trend as TPC. The DPPH results showed a different result as: green tea > black tea > oolong tea. The difference could be the different compositions in different types of tea and the mechanism between tea polyphenols and different free radicals.

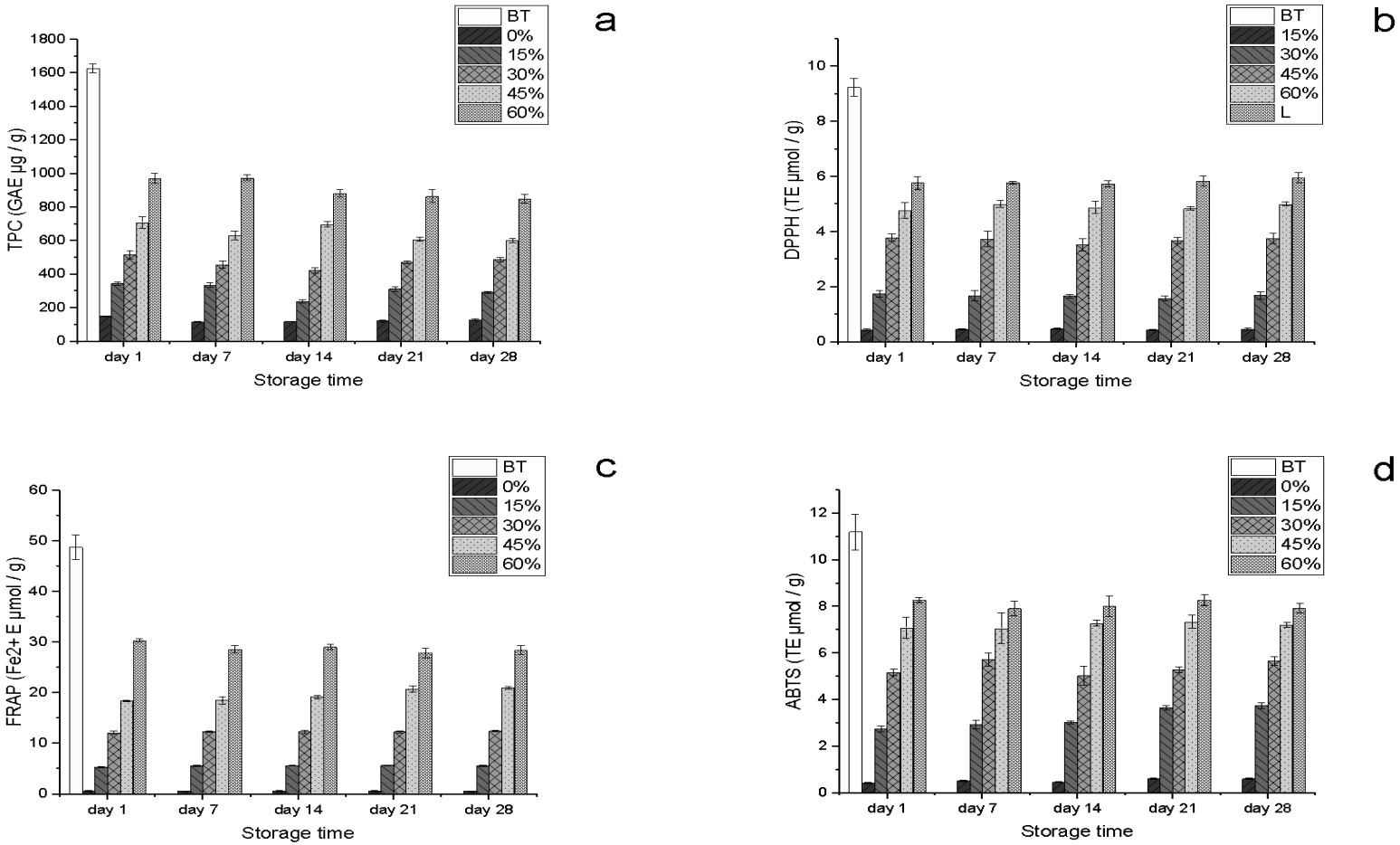


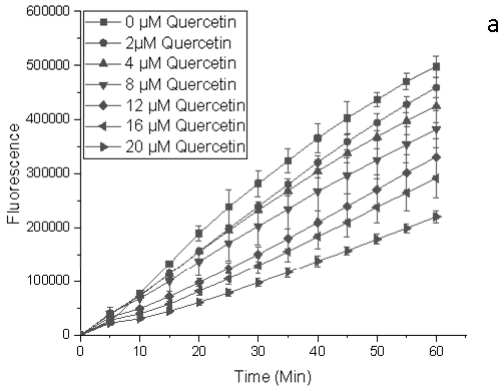
Figure 5.3 Antioxidant capacity parameters (black tea acidified dairy gel). 0%: plain acidified dairy gel, 15%: acidified dairy gel containing 15% black tea infusion, 30%: acidified dairy gel containing 30% black tea infusion, 45%: acidified dairy gel containing 45% black tea infusion, 60%: acidified dairy gel containing 60% black tea infusion. Figure 5.3a: Total phenolic content (TPC) values of different concentration of tea infusion, expressed as GAE µg/g; Figure 5.3b: DPPH values of different concentration of tea infusion, expressed as TE µmol/g; Figure 5.3c: FRAP values of different concentration of tea infusion, expressed as Fe^{2+} E µmol/g; Figure 5.3d: ABTS values of different concentration of tea infusion, expressed as TE µmol/g;

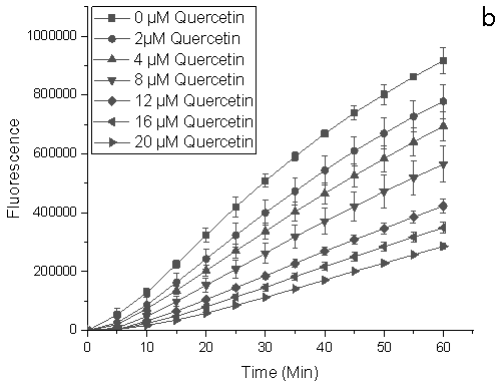
5.4 Cell line tests

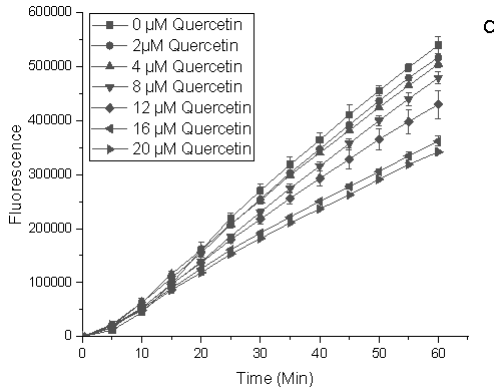
The kinetics of DCFH oxidation in HepG2 cells by peroxyl radicals generated from ABAP is shown in **Figure 5.4**. The increase in fluorescence from DCF (dichlorofluorescein) formation was inhibited by pure phytochemical compounds and ADG extracts in a dose-dependent manner, as demonstrated by the curves generated from cells treated with quercetin (**Figure 5.4a, b**), plain ADG (**Figure 5.4c, d**), ADG60%-G (**Figure 5.4e, f**) ADG60%-O (**Figure 5.4g, h**) and ADG60%-B (**Figure 5.4i, j**). The increase of fluorescein is an indication of the degree of oxidation. If there is an increase in fluorescein then there are more antioxidative activity. Inhibition of oxidation was seen when a PBS wash was conducted between antioxidant and ABAP treatments (**Figure 5.4 a, c, e, g, i**), and when no PBS wash was performed (**Figure 5.4b, d, f, h, i**). Wolfe and Liu (2007) and Liu and Huang (2015) gained similar results when conducted CAA measurements on catechin and black tea extracts, respectively.

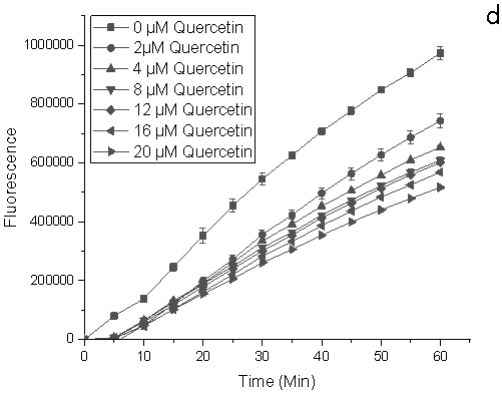
PBS wash

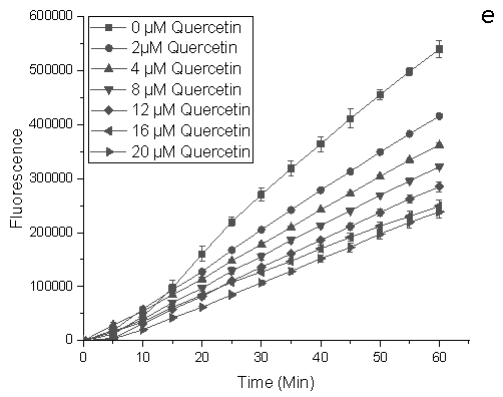
No PBS wash

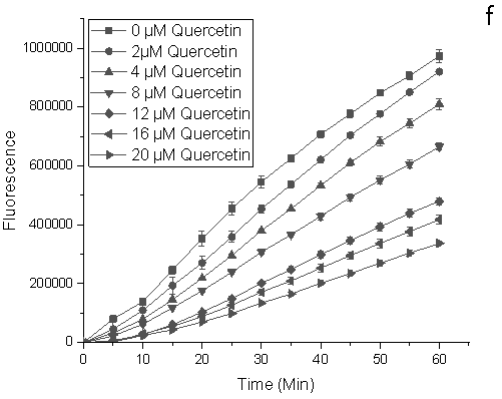












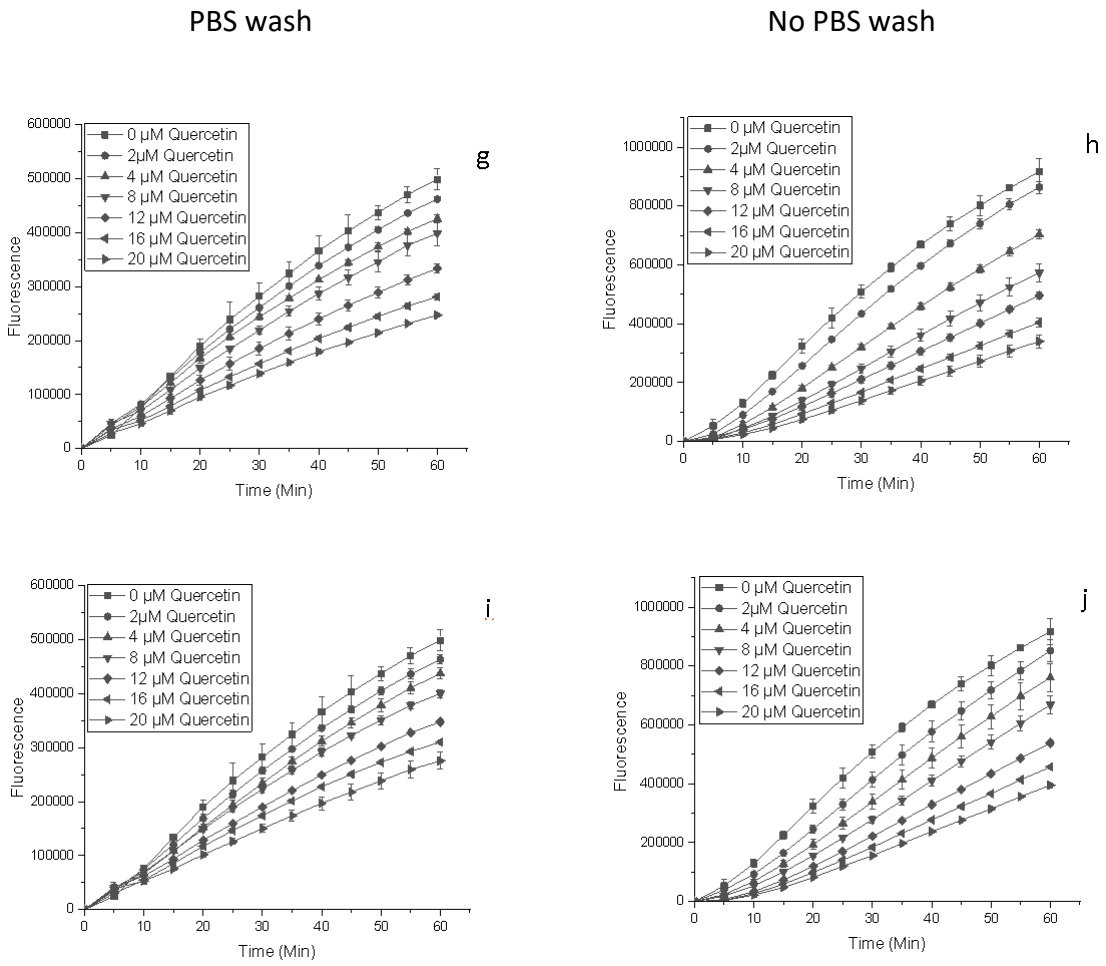


Figure 5.4 Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by quercetin (a, b), Plain ADG (c, d), $ADG_{60\%}$-G (e, f) $ADG_{60\%}$-O (g, h) and $ADG_{60\%}$-B (i, j) over time. the protocol involving a PBS wash between antioxidant and ABAP treatments (a, c, e, g, I, k) and the protocol without PBS wash (b, d, f, h, j, l), to remove antioxidants in the medium not associated with cells. The curves shown in each graph are from a single experiment (mean ± SD, n = 3).

Cellular Antioxidant Activity (CAA). As shown in **Figure 5.4**, the CAA unit of quercetin and ADG samples increased in a concentration-dependent manner following a curvilinear pattern, which showed antioxidant activity in hepG2 cell line. The results indicated that the tea enriched ADGs had the potential to reduce fluorescence levels, to different degrees, inhibiting the oxidation of DCFH to DCF, which effectively reduced the intracellular oxidative state reacting with peroxyl

radicals or ROS/RNS. The results also illustrate that $ADG_{60\%}$-G had the highest antioxidant capacity, followed by $ADG_{60\%}$-O and $ADG_{60\%}$-B. Plain ADG had the CAA unit which indicated variant CAA values are related to the contents of antioxidant components from tea with different geographic origins and processing (Shen et al., 2019).

The results of the CAA assay are presented in **Figure 5.5**. CAA assay was applied to determine the cellular antioxidant capacity of samples by preventing the DCFH oxidation induced by peroxyl radicals in HepG2 cells (human hepatoma cells). In order to conduct the CAA test, sample with highest tea concentration (60% tea infusion) were chosen as these were considered as having the potential to show greatest differences in reactions.

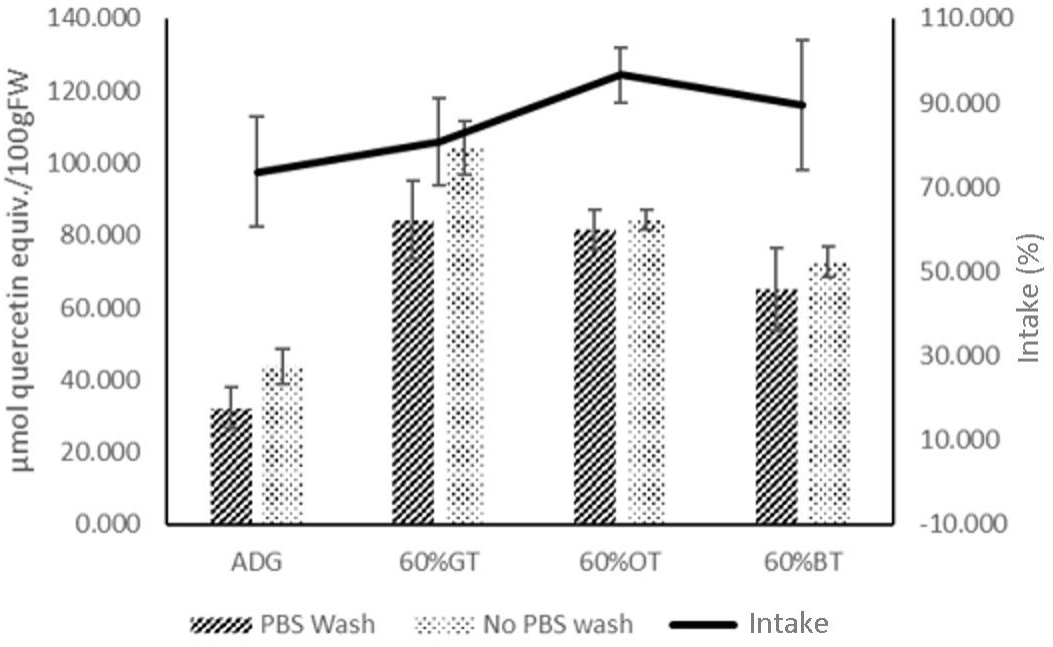


Figure 5.5 Cellular antioxidant activity (CAA) of Plain acidified dairy gel (ADG), acidified dairy gel containing 60% green tea infusion (60%GT), acidified dairy gel containing 60% oolong tea infusion (60%OT), acidified dairy gel containing 60% black tea infusion (60%BT). µmol quercetin equivalent / 100gFW, n=3.

As can be seen in **Figure 5.5**, the addition of tea infusion considerably increased the CAA results of ADGs and $ADG_{60\%}$-G> ADG60%-O> ADG60%-B. The intake rate of $ADG_{60\%}$-O (96.55%) was higher than the $ADG_{60\%}$-G (80.85%) and $ADG_{60\%}$-B (89.45%) which made the $ADG_{60\%}$-O gained similar true intake (PBS wash value) after the samples that were adhesive to the surface of cells were eliminated by a PBS wash compared to $ADG_{60\%}$-G with a lower level of quercetin equivalent.

Cytotoxicity and anti-proliferative activities. The results of cytotoxicity and anti-proliferative activities of ADG samples on the growth of HepG2 human liver cancer cells *in vitro* are summarized in in **Figure 5.6 and 5.7**. The content of samples was considered non-cytotoxic with a cell viability>90%, therefore, we chose 20mg per ml media as the upper limit in this study. All samples showed relatively potent antiproliferative activities on HepG2 cell growth in a dose-dependent manner below the cytotoxic limit and the plain ADG showed highest anti-proliferative activities. The antiproliferative activities of fruits were expressed as the median effective dose (EC_{50}), with a lower EC50 value indicating a higher antiproliferative activity. The plain ADG had the highest antiproliferative activity with the lowest EC_{50} of 9.80 ± 0.48 mg / mL, followed by $ADG_{60\%}$-BT (11.42 ± 0.51 mg / mL), $ADG_{60\%}$-GT (14.37 ± 1.16 mg / mL) and $ADG_{60\%}$-OT (14.45 ±1.07 mg / mL). Sun, Chu, Wu, and Liu (2002) conducted anti-proliferation measurement on common fruits and the antiproliferative activity of cranberry (14.5 ± 0.5 mg / mL) is at similar level as our results. The high anti-proliferative activities of ADGs could be due to their faintly acid.

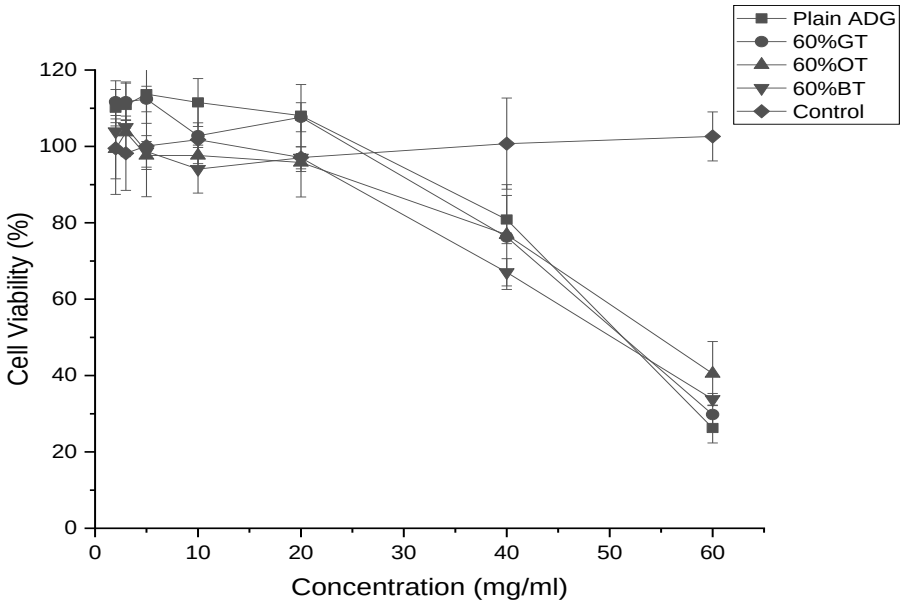


Figure 5.6 Cytotoxicity of Plain acidified dairy gel (ADG), acidified dairy gel containing 60% green tea infusion (60%GT), acidified dairy gel containing 60% oolong tea infusion (60%OT), acidified dairy gel containing 60% black tea infusion (60%BT) on HepG2 cell (mean ± SD, n = 3). The control is HepG2 cell incubated in the medium without ADG.

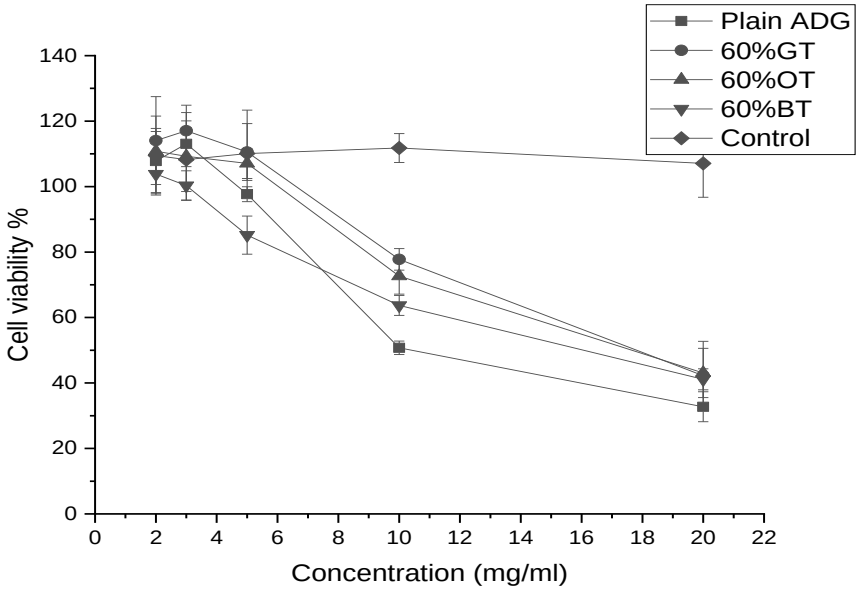


Figure 5.7 Cell viability of plain acidified dairy gel (ADG), acidified dairy gel containing 60% green tea infusion (60%GT), acidified dairy gel containing 60% oolong tea infusion (60%OT), acidified dairy gel containing 60% black tea infusion (60%BT) against HepG2 human liver cancer cells (mean ± SD, n = 3). The control is HepG2 cell incubated in the medium without ADG.

5.5 Results of high performance liquid chromatography (HPLC)

Results of HPLC are shown in **Table 5.1 to 5.2** and **Figure 5.8 to 5.10**. Instrumental precision was measured by repetitive injection of the same homogeneous sample of five concentrations. The recovery measures the closeness between the theoretically added amount and the experimental value with a known amount of catechin derivatives. The recovery of all catechin derivatives at different concentrations was above 80% (**Table 5.1**). The relative standard deviation (R.S.D.) was determined to assess instrumental precision. Intra-assay precision was determined by preparing three independent standard solutions of five different concentrations and measuring the R.S.D. values (**Table 5.2**). The R.S.D. values of both instrumental precision and intra-assay precision of four catechins and caffeic acid were below 3%, indicating the HPLC analytical method for catechin was precise.

Table 5.1 Equations associated with four catechins and caffeic acid (means ± SD, n=3).

Component	Equation[1]	R^2	Recovery Rate
(+)-C	y = 1.15 x + 4.81	>0.99	88.87± 0.353
CA	y = 6.36x + 26.68	>0.99	98.15± 1.639
(-)-EC	y = 1.61x − 53.01	>0.99	80.22 ± 2.420
(-)-EGC	y = 2.74x + 45.21	>0.99	88.98 ± 1.162
(-)-ECG	y = 2.99x + 3.82	>0.99	98.14 ± 0.873

[1]y is the peak area (mAU·s) and x is the concentration of component (μg/ml).

Table 5.2Recoveries of four catechins and caffeic acid (means ± SD, n=3).

Component	Concentration (μg/ml)	Peak area (mAU·s)	R.S.D. (%)
(+)-C	100	117.31 ± 0.629	0.54
	200	235.15 ± 1.600	0.68
	300	351.88 ± 0.804	0.23
	400	464.80 ± 3.937	0.85
	500	576.27 ± 8.994	1.56
CA	100	649.72 ± 4.592	0.71
	200	1300.22 ± 4.563	0.35
	300	1950.12 ± 6.067	0.31
	400	2579.57 ± 14.322	0.56
	500	3188.17 ± 81.024	2.54
(-)-EC	100	130.50 ± 0.932	0.71
	200	260.50 ± 0.287	0.11
	300	410.30 ± 9.201	2.24
	400	570.94 ± 5.333	0.93
	500	781.57 ± 20.003	2.56
(-)-EGC	100	296.42 ± 2.812	0.95
	200	593.49 ± 3.649	0.61
	300	889.17 ± 9.071	1.02
	400	1184.14 ± 4.862	0.41
	500	1370.24 ± 10.475	0.76
(-)-ECG	100	301.09 ± 1.608	0.53
	200	602.95 ± 1.850	0.31
	300	905.13 ± 3.012	0.33
	400	1199.77 ± 9.756	0.81
	500	1498.95 ± 11.225	0.75

The retention time of (+)-C ((+)-catechin), CA (caffeic acid), (-)-EC ((-)-epicatechin), (-)-EGC ((-)-epigallocatechin), (-)-ECG ((-)-epicatechin gallate) was 9.43, 10.31, 11.22, 11.56, 14.63min, respectively. When the tea enriched ADGs are compared with the tea infusion, a massive decline of polyphenols was observed in all ADGs made with green, oolong or black tea infusion which made caffeic acid, (-)-epigallocatechin and (-)-epicatechin gallate (peak 2,4 and 5) undetectable and an increase in protein (peak 0) was observed. When the tea enriched ADGs were compared with the tea infusion sample, it was observed that the (+)-catechin value in tea enriched ADGs were at similar level as the proportion of tea infusion. These results indicate that catechin did not suffer significant damage through the whole progress, even in the high tea-content samples ($ADG_{60\%}$-G, $ADG_{60\%}$-O and $ADG_{60\%}$-O). The results of HPLC indicated that the protein would interact with caffeic acid, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin gallate before it interacts with (+)-catechin. Since the (+)-catechin is the most abundant phenolic compound in all three types of tea, the retention of (+)-catechin can explain the promoted antioxidant capacity of ADGs after adding the tea infusion. The retained catechin increased alongside with the concentration of the addition of tea infusions while the content of retained protein decreased indicated the interaction between protein and polyphenols.

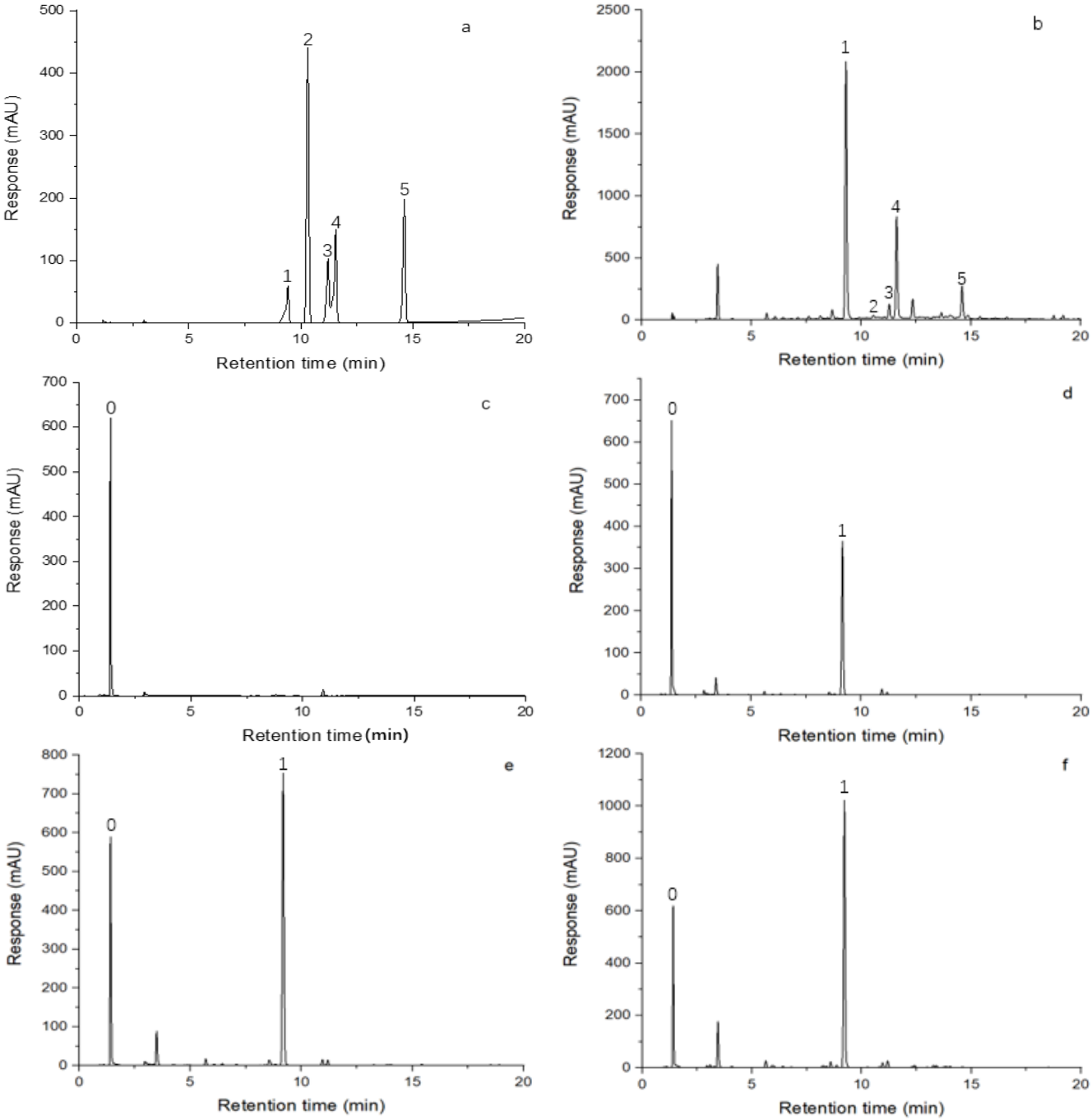


Figure 5.8 HPLC Chromatogram (green tea acidified dairy gel). a to f are standards, green tea infusion, acidified dairy gel (ADG), ADG+15% green tea infusion, ADG+30% green tea infusion, ADG+60% green tea infusion. 0, protein; 1, (+)-catechin; 2, caffeic acid; 3, (-)-epicatechin, 4, (-)-epigallocatechin; 5, (-)-epicatechin gallate.

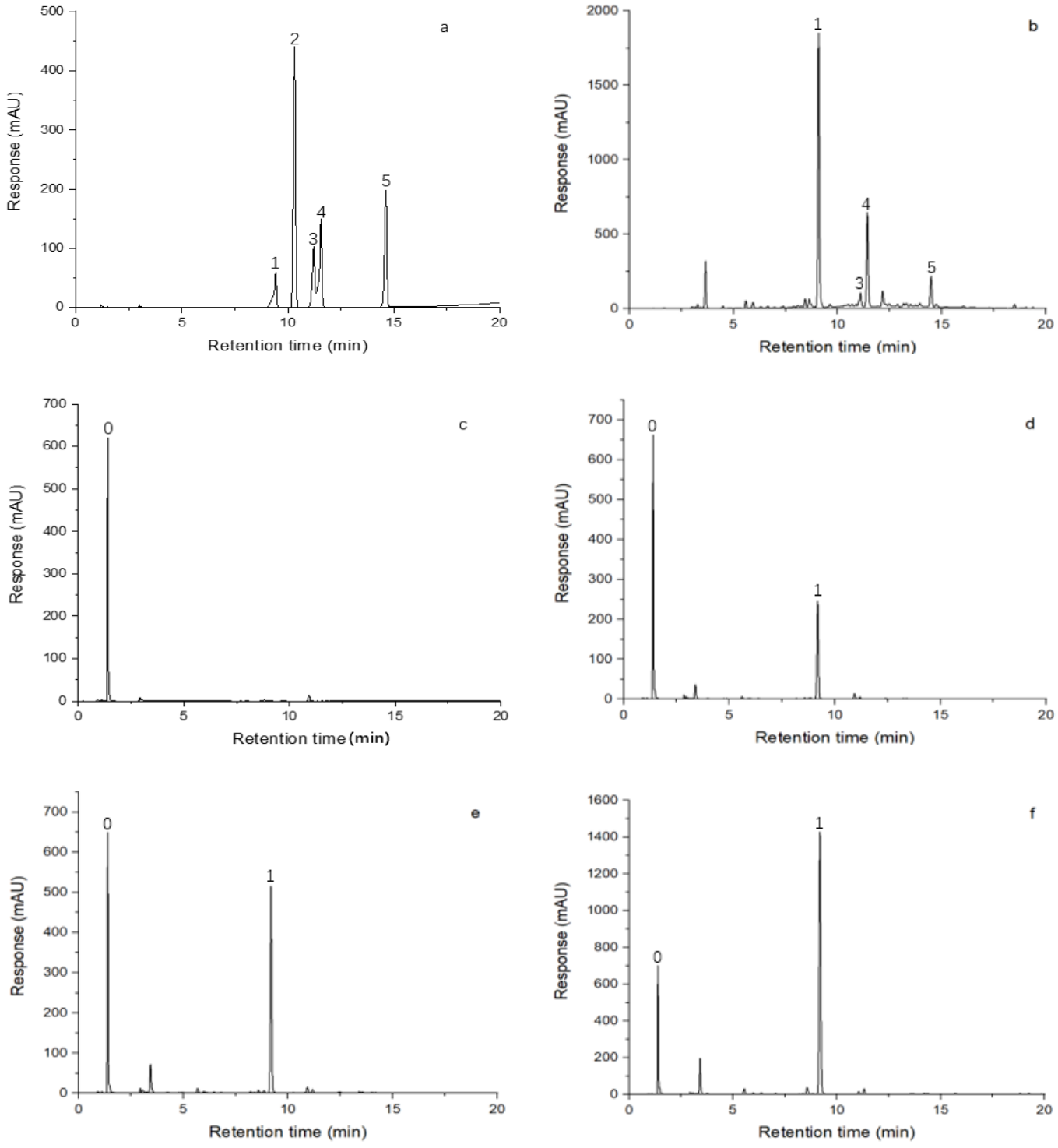


Figure 5.9 HPLC Chromatogram (oolong tea acidified dairy gel). a to f are standards, oolong tea infusion, acidified dairy gel (ADG), ADG+15% black tea infusion, ADG+30% black tea infusion, ADG+60% black tea infusion. 0, protein; 1, (+)-catechin; 2, caffeic acid; 3, (-)-epicatechin, 4, (-)-epigallocatechin; 5, (-)-epicatechin gallate.

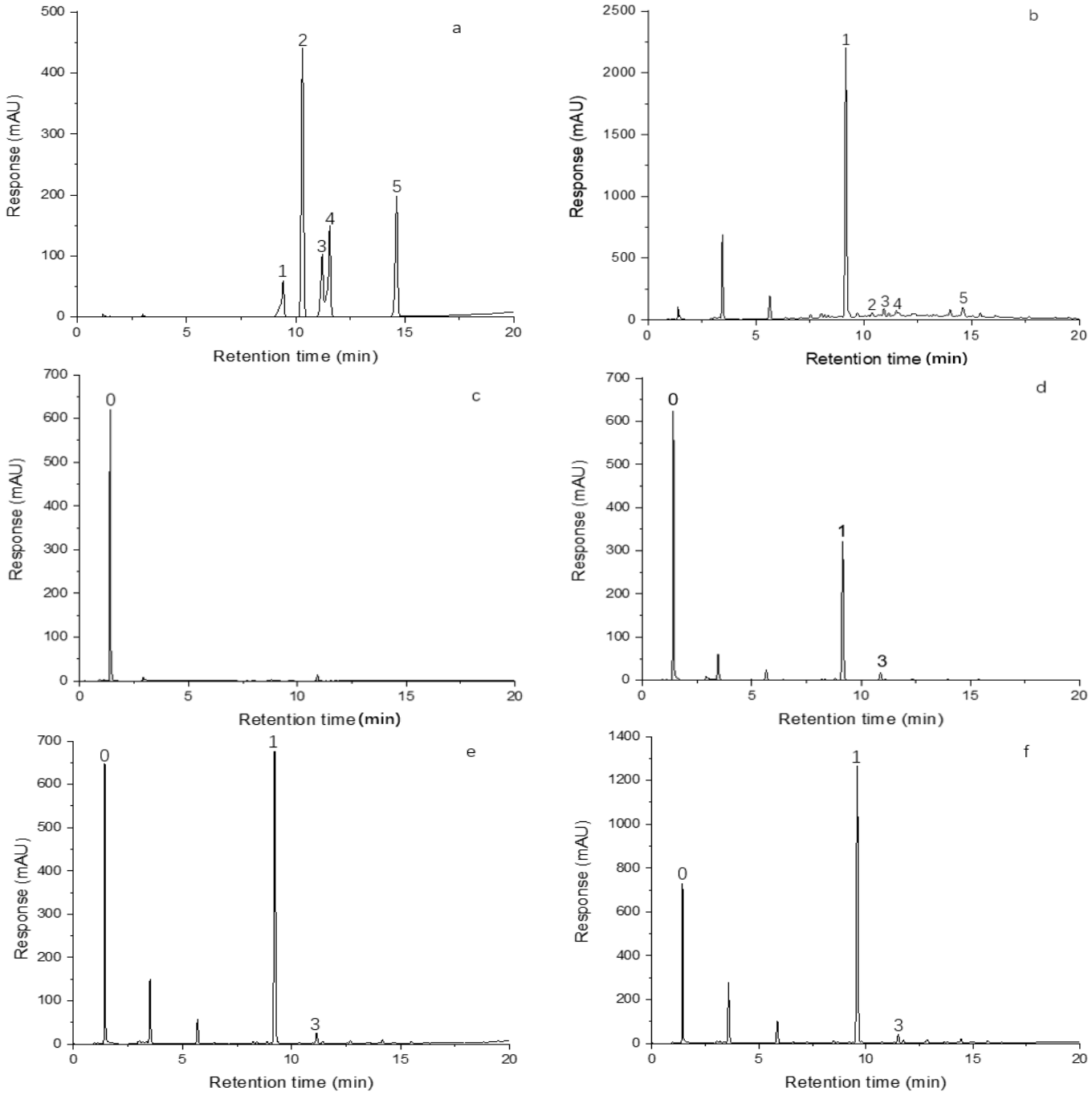


Figure 5.10 HPLC Chromatogram (black tea acidified dairy gel). a to f are standards, black tea infusion, acidified dairy gel (ADG), ADG+15% black tea infusion, ADG+30% black tea infusion, ADG+60% black tea infusion. 0, protein; 1, (+)-catechin; 2, caffeic acid; 3, (-)-epicatechin, 4, (-)-epigallocatechin, 6, (-)-epicatechin gallate.

5.6 Conclusion

Several studies have reported that the antioxidant capacity of green tea is stronger than oolong tea and black tea which is correlated with TPC (Hajiaghaalipour, Sanusi, & Kanthimathi, 2016; Islam, Farooq, & Sehgal, 2018; Jayawardana, Warnasooriya, Thotawattage, Dharmasena, & Liyanage, 2019; Kaur, Farooq, & Sehgal, 2019; Muniandy, Shori, & Baba, 2016). The differences of the results of antioxidant capacity in all parameters (TPC, DPPH, ABTS and FRAP) were minor differences among different storage periods compared to different concentration of tea infusion ($P < 0.05$) which indicated the antioxidant capacity was stable during cold storage and could be adjusted by adding different concentration of tea infusion.

Recently, the development of novel emulsifiers with improved functionality has been given more and more attention based on forming covalent conjugates or physical complexes between different molecular species, such as protein–polysaccharide, protein-polyphenol, or polysaccharide–polyphenol conjugates/complexes or using emulsion among protein and polysaccharide/polyphenol to protect bioactive compounds (Evans, Ratcliffe, & Williams, 2013; Gu et al., 2017; İlyasoğlu, Nadzieja, & Guo, 2019; Li et al., 2019).

The increasing rate in TPC, along with the other antioxidant parameters (DPPH, FRAP, ABTS), were lower than the concentration of tea infusion which suggested the antioxidant capacity of tea infusion was slightly weakened. One possible explanation could be that the polyphenols in the tea infusion were binding with calcium ion or/and protein as reported by Tanizawa, Abe, and Yamada (2007) and Spiro and Chong (1997).

Chapter 6 Results of microstructure of tea enriched ADG

Microstructures of ADGs were observed by SEM and CLSM. Scanning electron micrographs showed the 3D organization of dairy gels. CLSM was used for the determination of the laminar microstructure of dairy gels. The microstructure level of dairy gels corresponded to their textural and rheological properties, the homogeneous casein aggregations, the better gel matrix, resulting in higher hardness or firmness of dairy gel (Pan, Liu, Luo, & Luo, 2019).

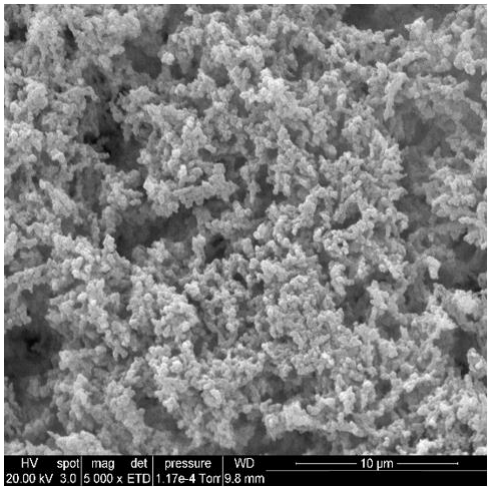
6.1 Microstructure of green tea enriched ADGs

The microstructures of the stirred ADG-G samples are shown in **Figure 8.1** to **Figure 8.4**.

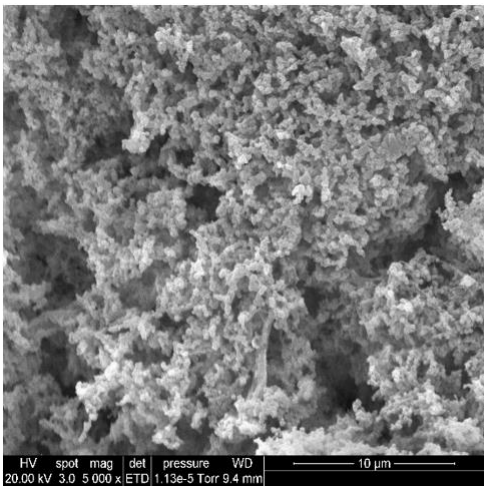
6.1.1 SEM measurement of ADG-Gs

The three-dimensional network of aggregates of casein micelles, the structural elements, were observed in the SEM ADG microstructures illustrated a rod shape or bulk shape consist of small spherules (casein), interspaced by void zones which represents the water and whey serum.

Firstly, we can see an improvement in ADGs which had 45% and 60% green tea infusion after 28 days of cold storage with less bulky area when compared the **Figure 6.1** and **Figure 6.2**. In contrast, the control samples exhibited a continuous branched network, with small void spaces, which lead to a stable structure of the protein network during storage. ADG-Gs made with different concentrations of green tea extract showed different patterns of networks. Similar structures were observed in $ADG_{15\%}$-G and $ADG_{30\%}$-G samples, whereas more compact and aggregated structure was observed in the $ADG_{45\%}$-G (**Figure 6.1d** and **6.2d**) and $ADG_{60\%}$-G (**Figure 6.1e** and **6.2e**).



a



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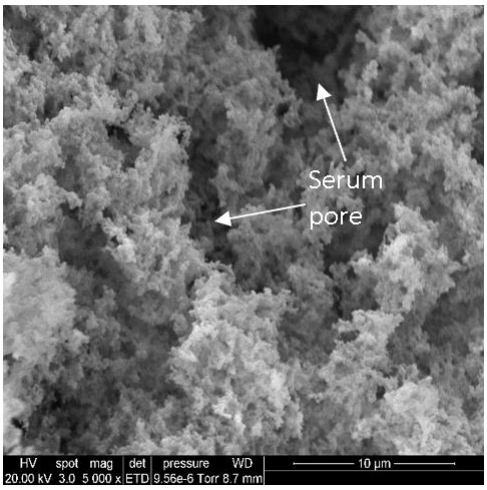

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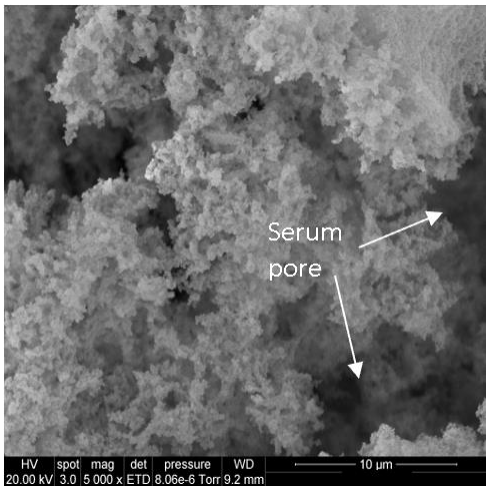

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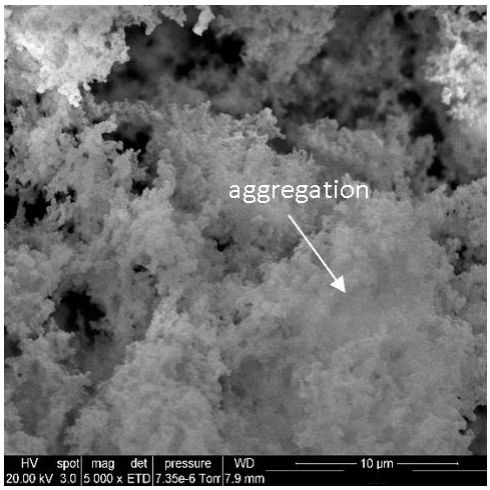
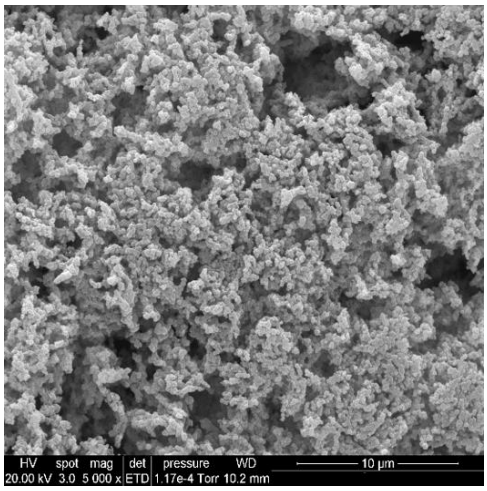


e

Figure 6.1 SEM images of plain ADG (a), ADG with 15% green tea infusion (b), ADG with 30% green tea infusion (c), ADG with 45% green tea infusion (d), ADG with 60% green tea infusion (e) (day 1)



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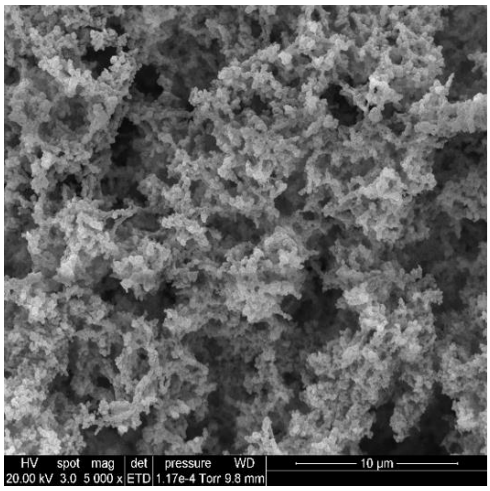

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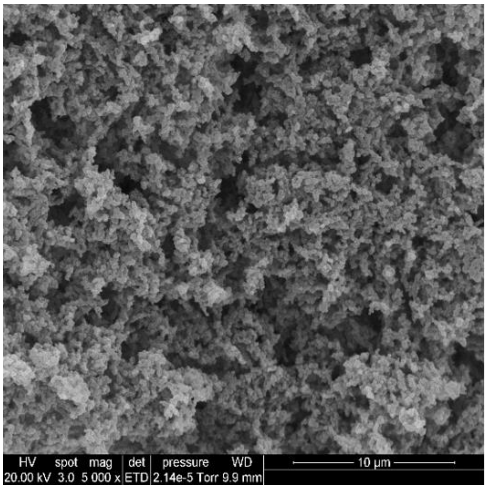

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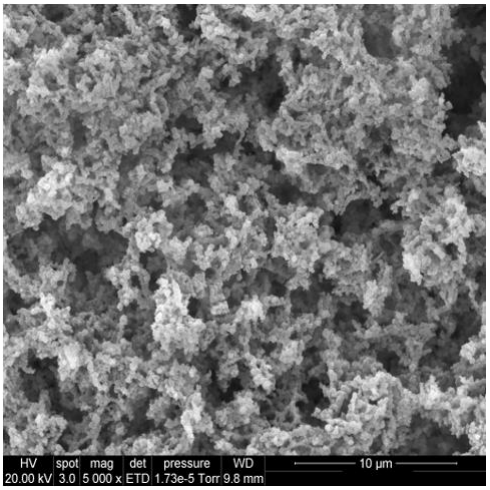

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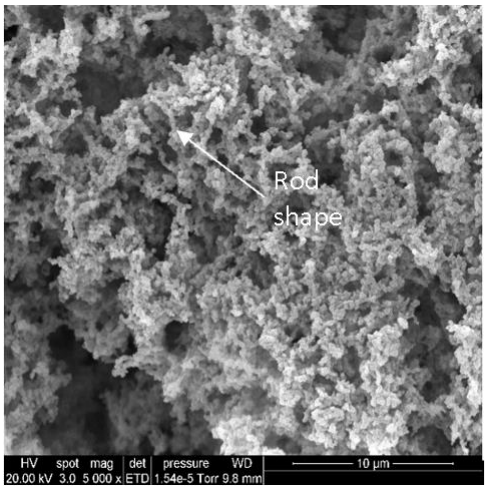


e

Figure 6.2 SEM images of plain ADG (a), ADG with 15% green tea infusion (b), ADG with 30% green tea infusion (c), ADG with 45% green tea infusion (d), ADG with 60% green tea infusion (e) (day 28)

This observation is in agreement with the findings of previous studies, Nguyen, Schwendel, Harland, and Day (2018) and Nguyen, Afsar, and Day (2018), in which the microstructure of low-fat yoghurts from goat, sheep and cow milk as well as bovine milk containing A^1A^1 and A^2A^2 β-casein phenotypes was investigated. Interestingly, more dense structures with smaller pores were observed in $ADG_{45\%}$-G and $ADG_{60\%}$-G samples on day 28 (**Figure 6.2d, e**) compared to day 1 (**Figure 6.1d, e**). This is contrary to the results of Nguyen, Ong, Kentish, and Gras (2014) who found that bigger pores were observed after the storage. The difference could be because they used buffalo milk which has different protein and lipid constitution and that the protein and lipid interactions affected the porosity of the gels.

6.1.2 CLSM measurement of ADG-Gs

CLSM was used for characterizing laminar microstructure of ADG samples and these images are shown in **Figure 6.3** and **6.4**. samples of $ADG_{15\%}$-G (**Figure 6.3b** and **6.4b**) and $ADG_{30\%}$-G (F**igure 6.3c** and **6.4c**) samples had denser protein networks and exhibited less pores than the control (**Figure 6.3a** and **6.4a**) on both day 1 and day 28. The different structure formed depends both on the concentrations of protein and polyphenol and on their ratio. Siebert, Troukhanova, and Lynn (1996) explored polyphenol-protein interaction using catechin and protein. They assumed a model in which each polyphenol molecule is viewed as having a fixed number of binding ends and each protein is viewed as having a fixed number of polyphenol binding sites. So, in a protein solution with low level of polyphenols, the polyphenol could be a bridge to connect two protein molecules to form a denser structure. But in a protein solution with high level of polyphenols, polyphenol molecules will occupy most binding sites on protein molecules and prevent them to

bind with each other therefore a more broken structure with more serum pores was formed. The hypothesis is depicted in **Figure 6.3.**

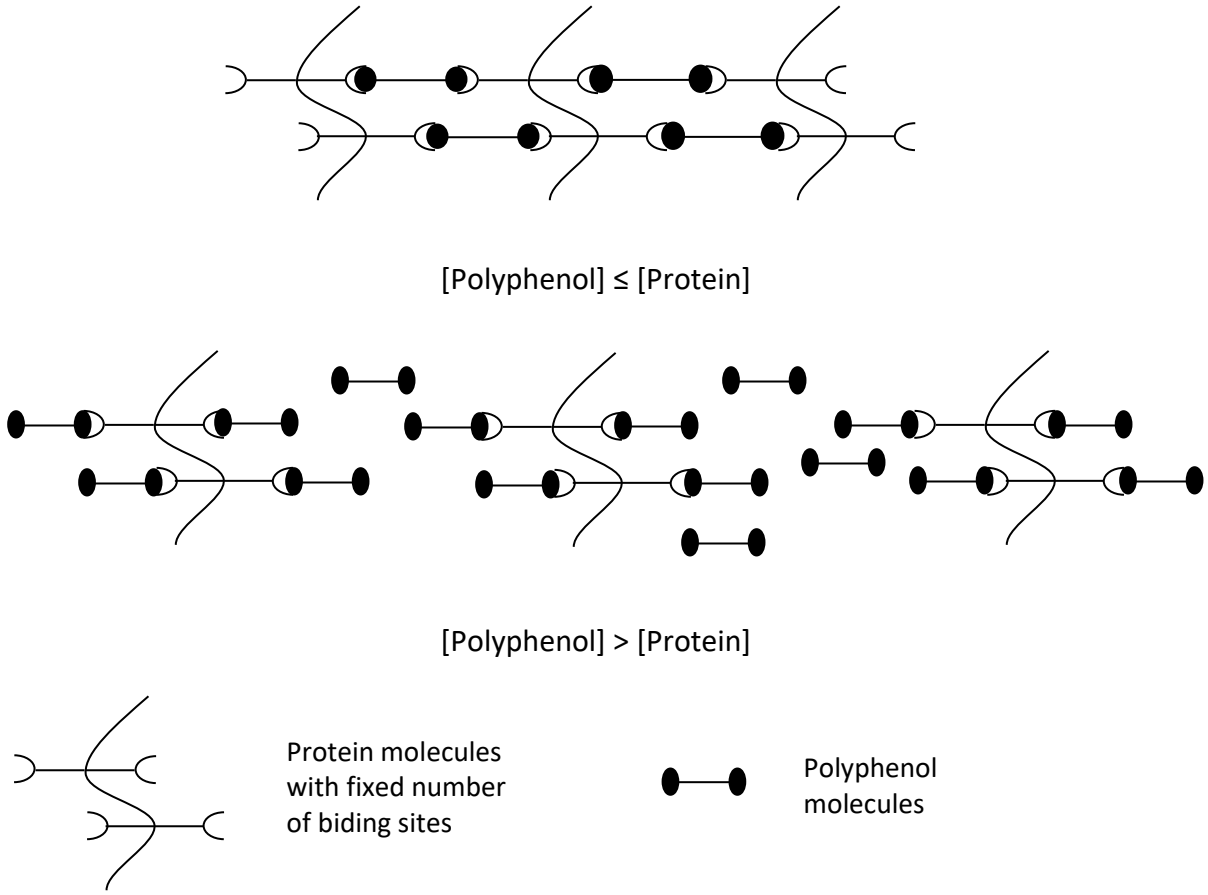
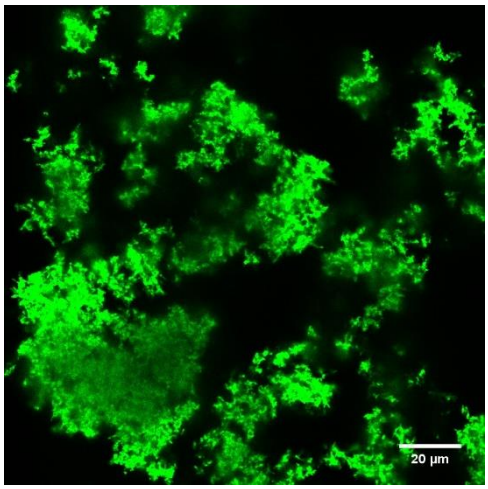


Figure 6.3 Model for protein–polyphenol interaction. Polyphenols are depicted as having two ends that can bind to protein. Proteins are depicted as having a fixed number of binding sites.

More pores appeared in the gels containing 45% and 60% tea infusion which is in accordance to the EOS and WHC results (**Table 4.1**). More significant transformations took place in both $ADG_{45\%}$-G and $ADG_{60\%}$-G which led to a less intense protein network with larger pores. The protein clusters

appeared to be distributed very loosely and the bindings between them were very weak in $ADG_{45\%}$-G (**Figure 6.3d** and **6.4d**) and $ADG_{60\%}$-G (**Figure 6.3e** and **6.4e**) compare to the control (**Figure 6.3a** and **6.4a**), ADG15%-G (**Figure 6.3b** and **6.4b**) and ADG30%-G (**Figure 6.3c** and **6.4c**). Similar structures were reported by Nguyen, Ong, Lefevre, Kentish, and Gras (2014) with buffalo yoghurt. The microstructure improved during the cold storage for all ADGs except for $ADG_{30\%}$-G.



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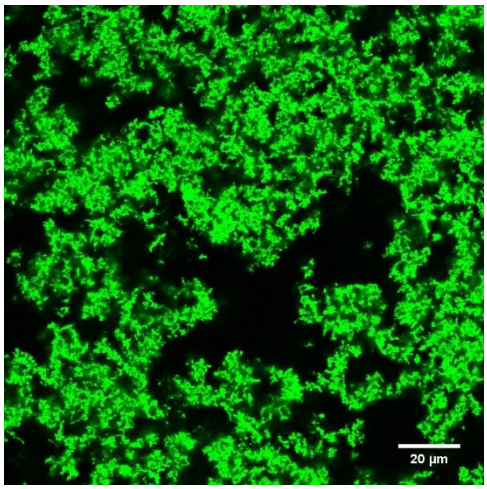

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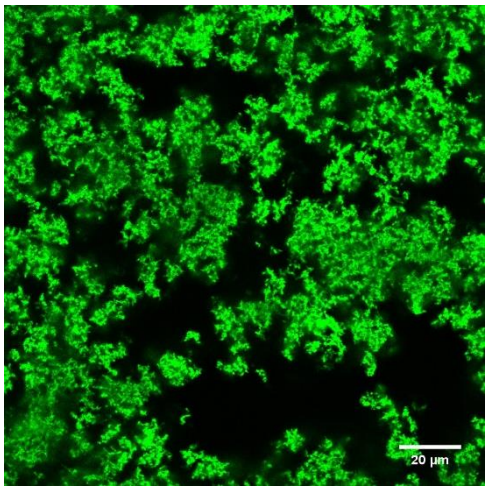

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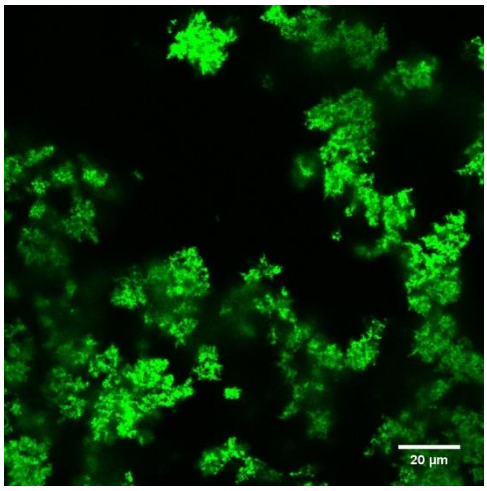

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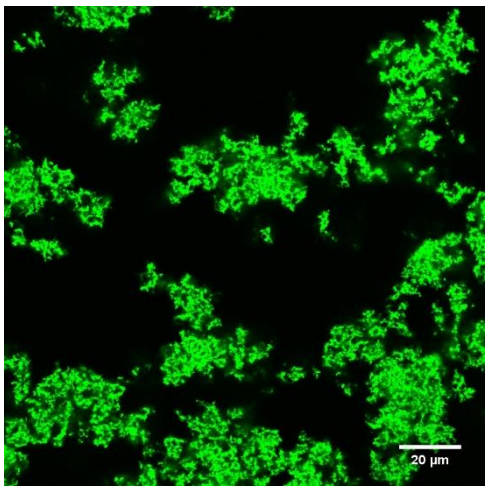
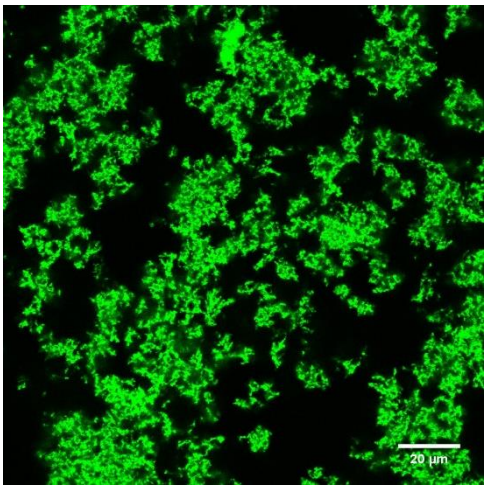


e

Figure 6.4 CLSM images of plain ADG (a), ADG with 15% green tea infusion (b), ADG with 30% green tea infusion (c), ADG with 45% green tea infusion (d), ADG with 60% green tea infusion (e) (day 1). Protein stained by Fast Green FCF appears as green and non-fluorescent areas (dark areas) correspond to the serum pores.



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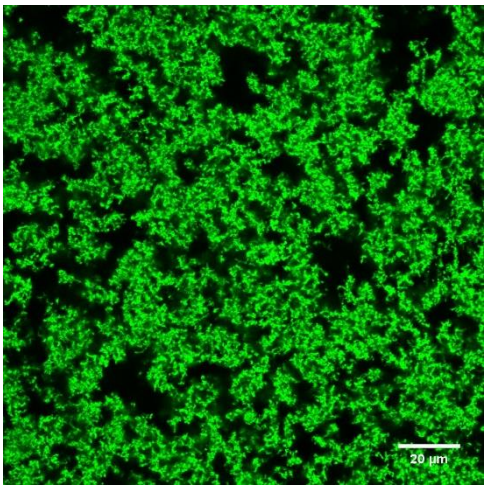

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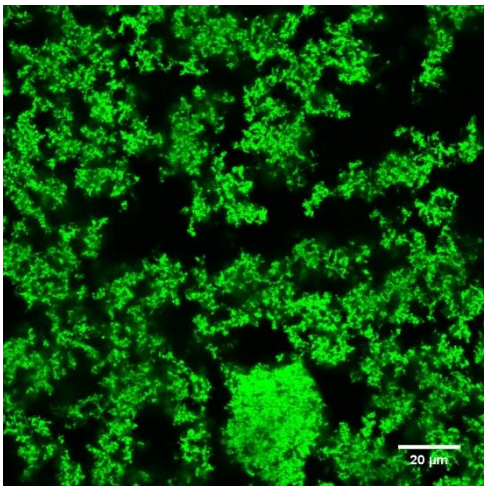

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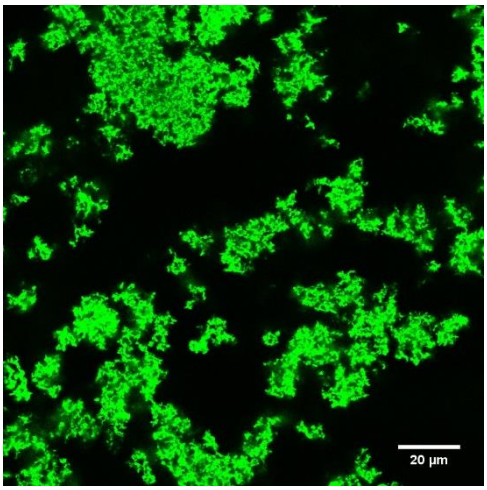

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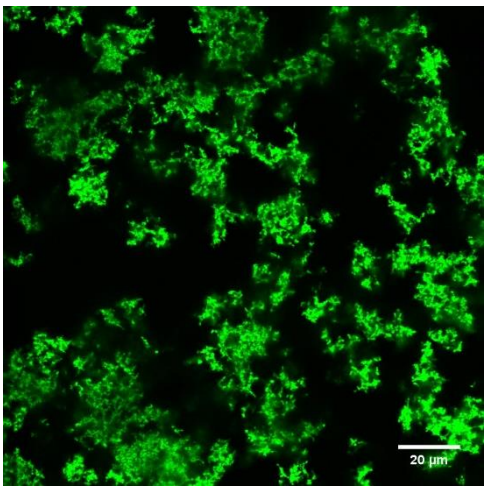


e

Figure 6.5 CLSM images of plain ADG (a), ADG with 15% green tea infusion (b), ADG with 30% green tea infusion (c), ADG with 45% green tea infusion (d), ADG with 60% green tea infusion (e) (day 28). Protein stained by Fast Green FCF appears as green and non-fluorescent areas (dark areas) correspond to the serum pores.

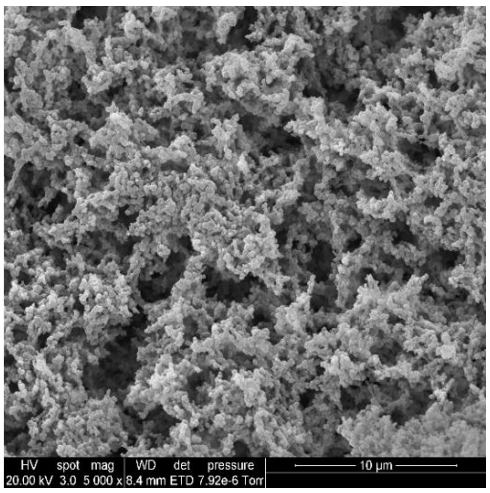
6.2 Microstructure of oolong tea enriched ADGs

The microstructures of the stirred ADG-O samples are shown in **Figure 6.5** to **Figure 6.8**.

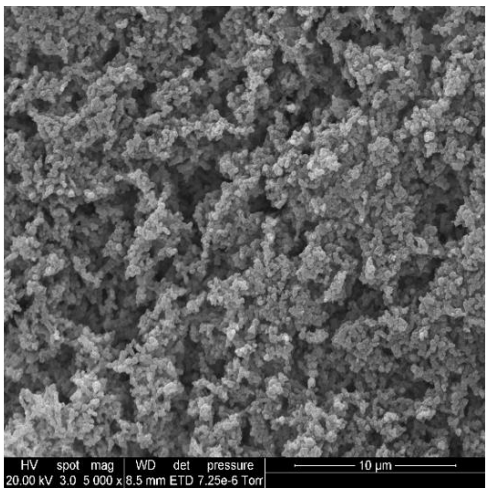
6.2.1 SEM measurement of ADG-Os

The micrographs illustrate the differences in the microstructure of the samples. In general, the microstructure of all samples was similar. The yoghurt protein matrices were composed of casein micelle chains and clusters. The ways in which the casein micelles were linked to each other differed slightly on day 1 (**Figure 6.5**). The protein network of the control (**Figure 6.5a**), $ADG_{15\%}$-O (**Figure 6.5b**) and $ADG_{30\%}$-O (**Figure 6.5c**) were slightly denser than $ADG_{45\%}$-O (**Figure 6.5d**) and $ADG_{60\%}$-O (**Figure 6.5e**) with smaller pores on day 1.

The changes of microstructure of ADG-Os during cold storage were different from ADG-Gs. The control, $ADG_{15\%}$-O and $ADG_{30\%}$-O were less dense after the 28 days of cold storage with more and bigger pores which indicated that ADGs produced with 15% and 30% of oolong tea were more sensitive to the external force and less able to recover to the original structure after deformation. The protein strands of $ADG_{45\%}$-O and $ADG_{60\%}$-O at day 28 of the storage are slightly denser than day 1 (**Figure 6.5d** vs. **6.6d**, **Figure 6.5e** vs. **6.6e**). These observation may be due to transformation of the protein aggregates or the further development of the network resulted by the change of polyphenol-protein ratio which caused by the loss of polyphenols because serum separation during cold storage (Nguyen, Ong, Kentish, et al., 2014).



a



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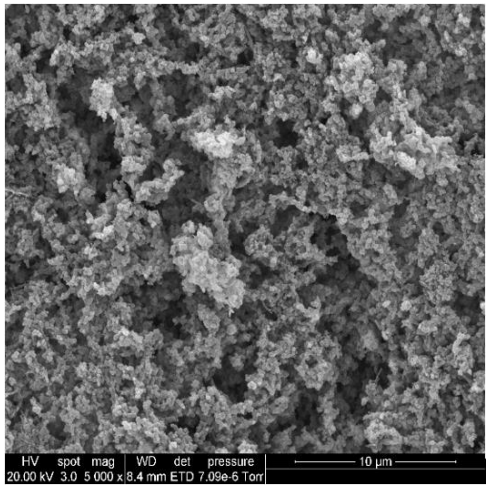

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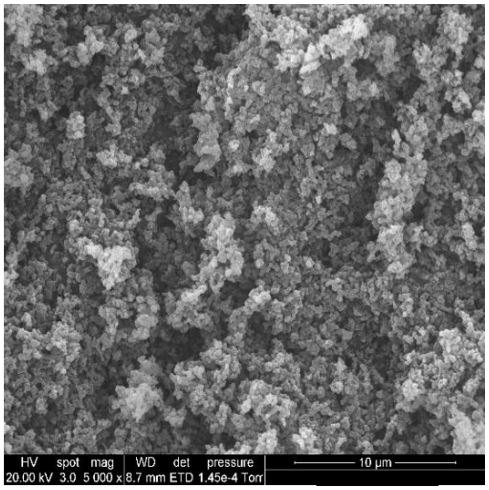

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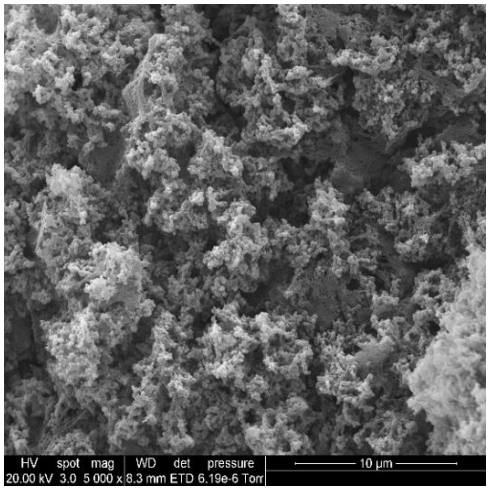
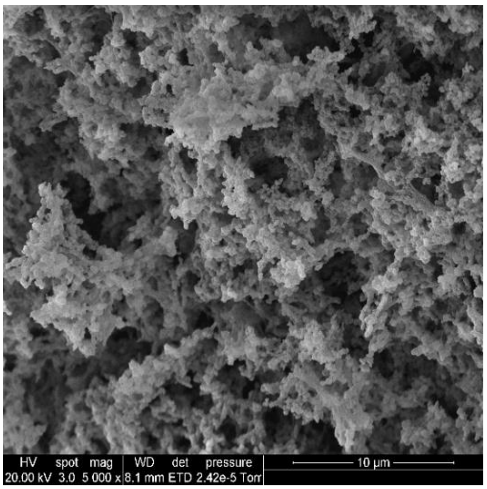


e

Figure 6.6 SEM images of plain ADG (a), ADG with 15% oolong tea infusion (b), ADG with 30% oolong tea infusion (c), ADG with 45% oolong tea infusion (d), ADG with 60% oolong tea infusion (e) (day 1)



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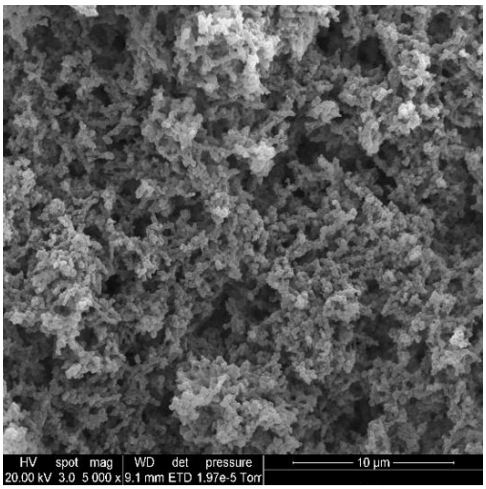

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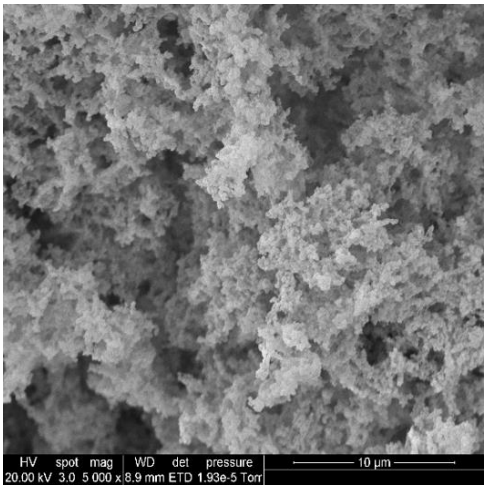

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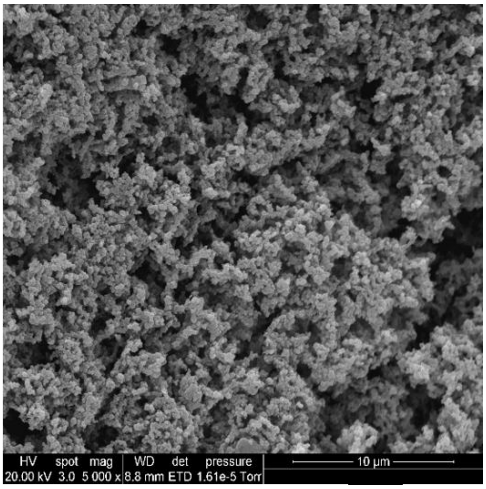

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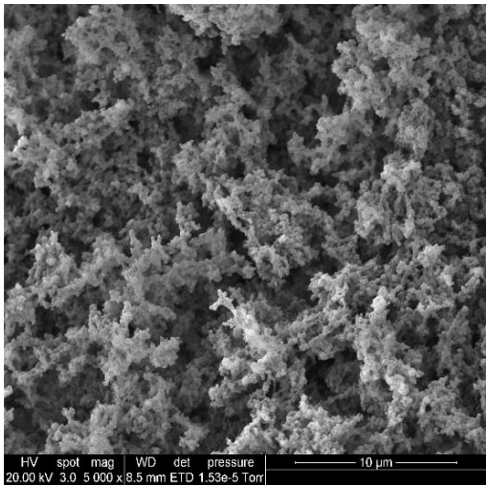
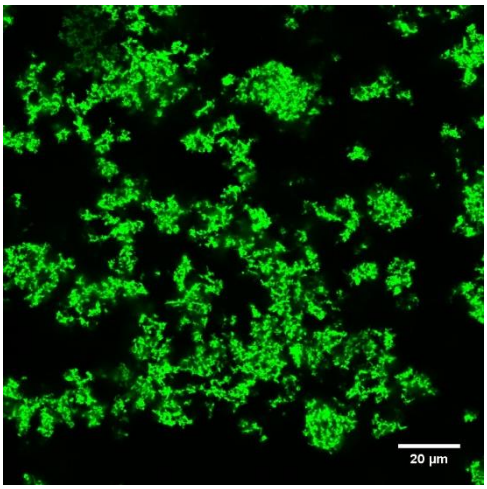


e

Figure 6.7 SEM images of plain ADG (a), ADG with 15% oolong tea infusion (b), ADG with 30% oolong tea infusion (c), ADG with 45% oolong tea infusion (d), ADG with 60% oolong tea infusion (e) (day 28)

6.2.1 CLSM measurement of ADG-Os

Figure 6.7 and **6.8** show the microstructure of ADG made with oolong tea extracts when observed using CLSM. The microstructure ADGs consists of a protein network surrounded by serum pores. The $ADG_{15\%}$-O (**Figure 6.7b** and **6.7b**) and $ADG_{30\%}$-O (**Figure 6.7c** and **6.8c**) had smaller pores and a denser protein network compared with the control yoghurt (**Figure 6.7a** and **6.8a**), which indicated that the low content of oolong tea extract could form a more strong stable structure. The $ADG_{60\%}$-O (**Figure 6.7e** and **6.8e**) exhibited the most porous microstructure and least dense protein network followed by The $ADG_{45\%}$-O (**Figure 6.7d and 6.7d**) that both had bigger pores and a less dense protein network compared with the control yoghurt (**Figure 6.7a** and **6.8a**), which demonstrated that high level of oolong extract generates a weaker yoghurt gel (Nguyen, Afsar, et al., 2018; Nguyen, Schwendel, et al., 2018).



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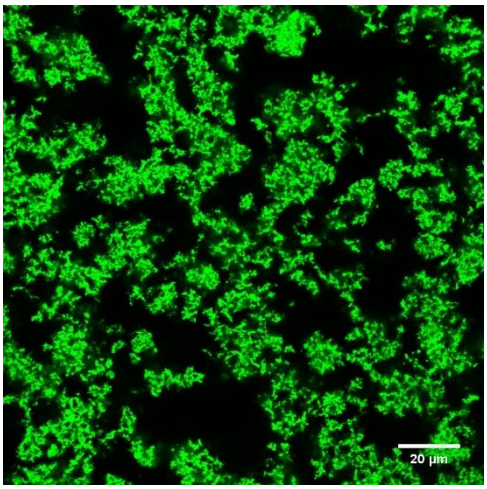

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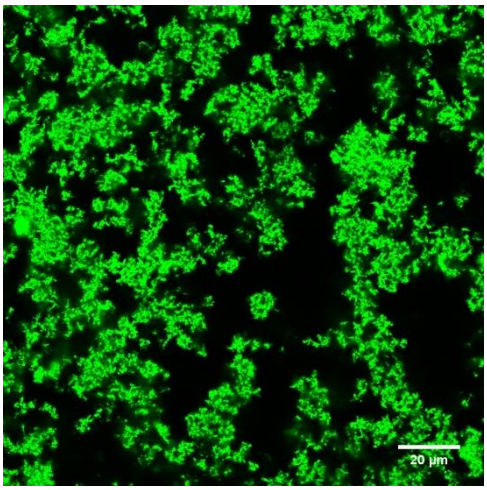

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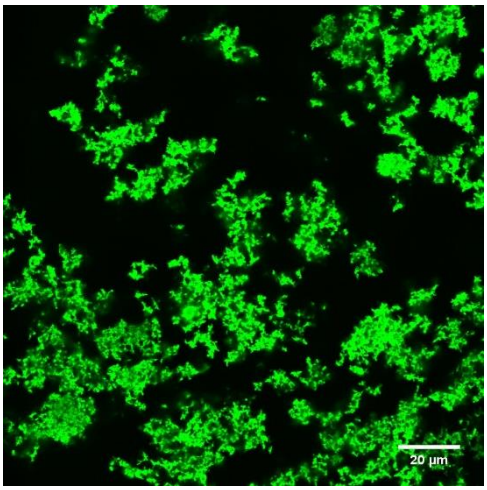

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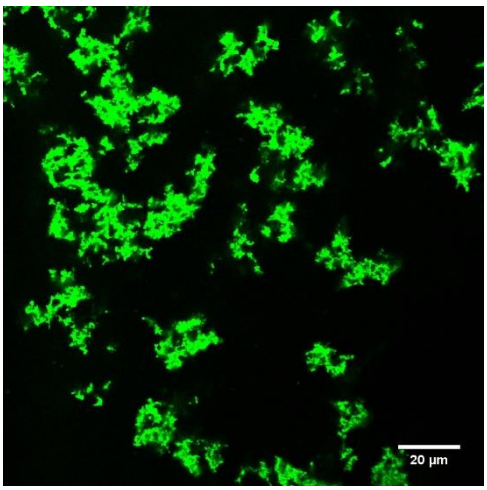
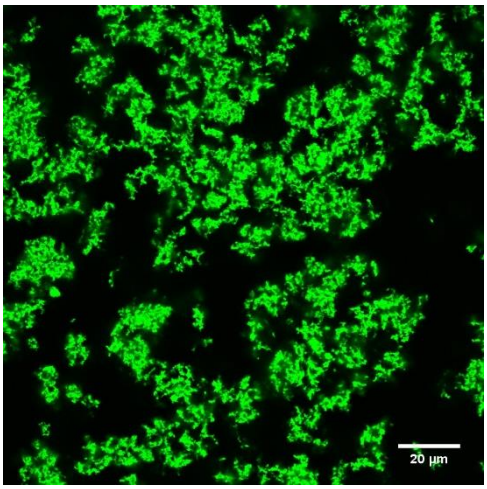
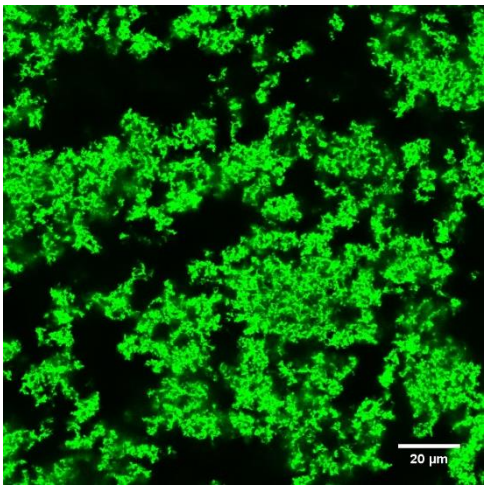


e

Figure 6.8 CLSM images of plain ADG (a), ADG with 15% oolong tea infusion (b), ADG with 30% oolong tea infusion (c), ADG with 45% oolong tea infusion (d), ADG with 60% oolong tea infusion (e) (day 1). Protein stained by Fast Green FCF appears as green and non-fluorescent areas (dark areas) correspond to the serum pores.



a



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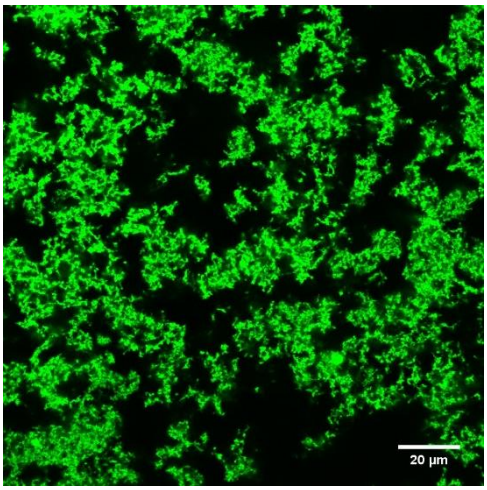

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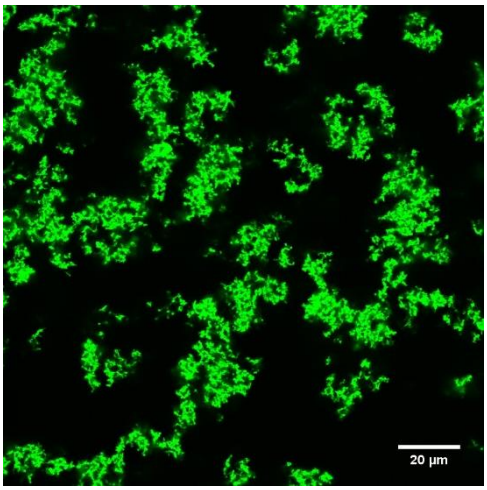

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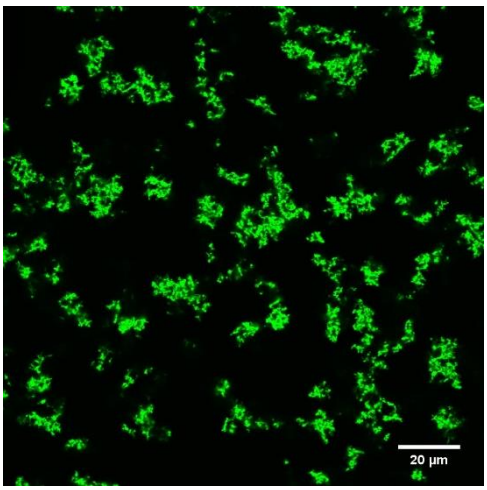


e

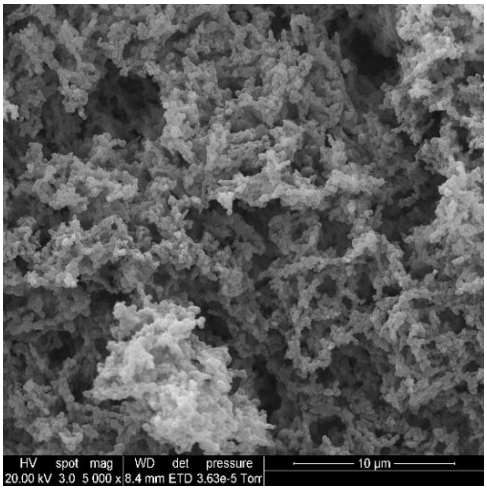
Figure 6.9 CLSM images of plain ADG (a), ADG with 15% oolong tea infusion (b), ADG with 30% oolong tea infusion (c), ADG with 45% oolong tea infusion (d), ADG with 60% oolong tea infusion (e) (day 28). Protein stained by Fast Green FCF appears as green and non-fluorescent areas (dark areas) correspond to the serum pores.

6.3 Microstructure of black tea enriched ADGs

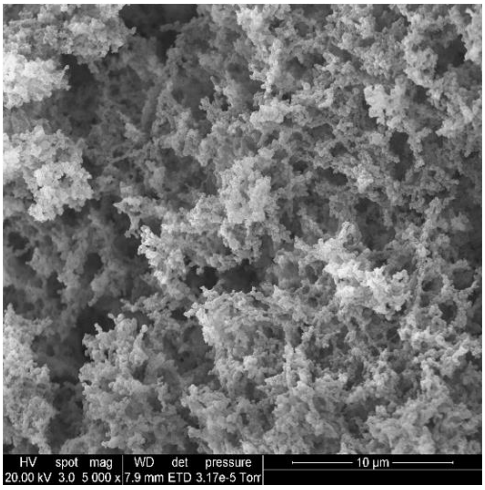
The microstructures of the stirred ADG-B samples are shown in **Figure 6.9** to **Figure 6.12**.

6.3.1 SEM measurement of ADG-Bs

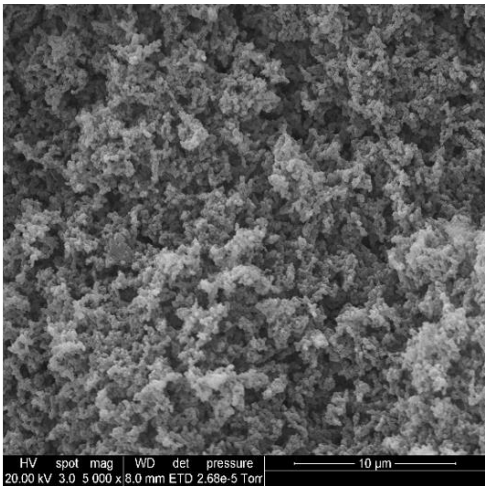
The microstructures of all the ADG-B samples were well-defined and their three-dimensional networks as shown in **Figure 6.9** and **6.10.** Comparing the morphology and the segregated structure of the gel made from the control group (**Figure 6.9a and 6.10a**) and $ADG_{15\%}$-B (**Figure 6.9b and 6.10b**), the z-depth structure was less complex in the $ADG_{15\%}$-B. Also, the later had slightly finer protein arrangement and the pores appeared to be smaller from about 10 – 20 μm to 3 – 10 μm. The $ADG_{30\%}$-B (**Figure 6.9c**) was thicker and denser. It also shows that the sample had fine protein arrangement resulting very small pores in the gel structure (**Figure 6.9c and 6.10c**). Such microstructure explains the better phase stability in the gel samples containing 15% and 30% black tea infusion (EOS and WHC). Generally, all three samples illustrated similar spongy-like interiors with few air cells and highly branched-structure. Yang, Fu, and Li (2012) reported similar results when they added soybean to yoghurt. Fiszman, Lluch, and Salvador (1999) found that the smooth bridge with double network structures of dairy gel seemed to be located at the inside of casein micelles that could maintain the aqueous phase more effectively and reduce EOS which agreed with our results. Some small aggregates started to appear in the images of $ADG_{45\%}$-B (**Figure 6.9d** and **6.10d**) and bigger pores (>10 μm) showed in the image of $ADG_{60\%}$-B (**Figure 6.9e** and **6.10e**) which might be the reasons of instability of $ADG_{45\%}$ and $ADG_{60\%}$.



a



b



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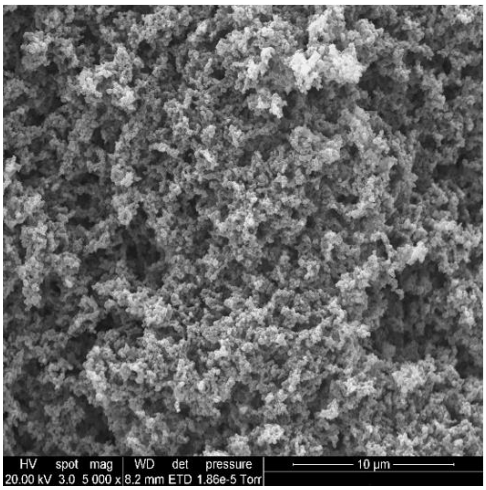
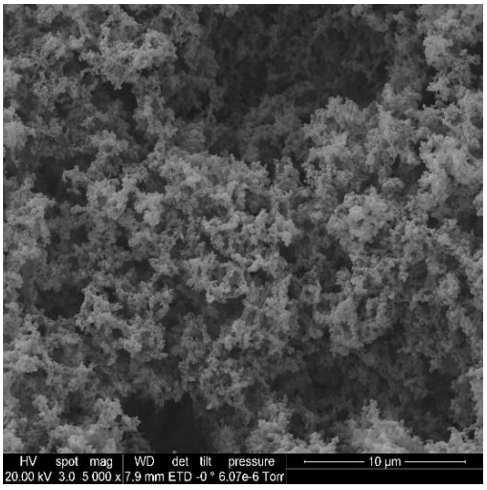
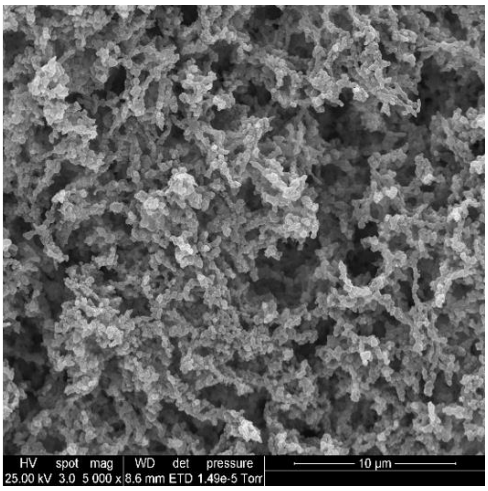


d

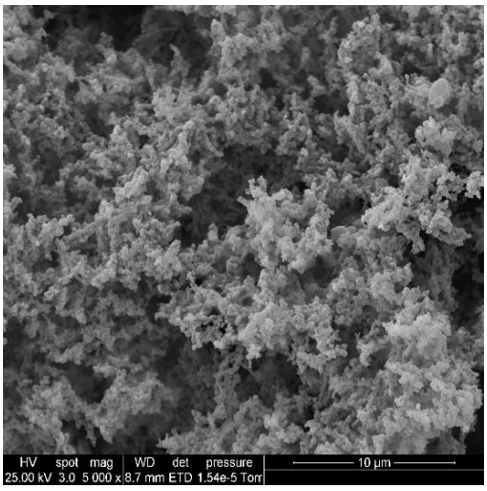


e

Figure 6.10 SEM images of plain ADG (a), ADG with 15% black tea infusion (b), ADG with 30% black tea infusion (c), ADG with 45% black tea infusion (d), ADG with 60% black tea infusion (e) (day 1)



a



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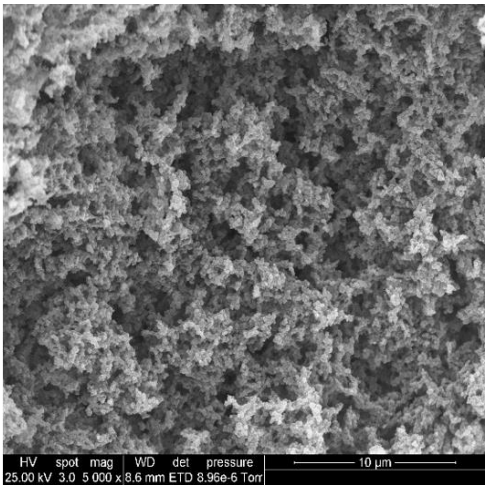

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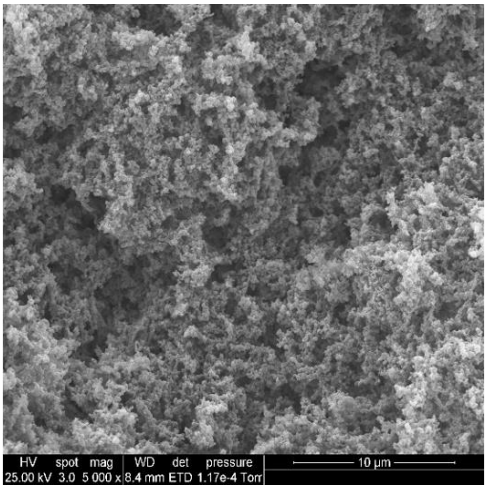


d

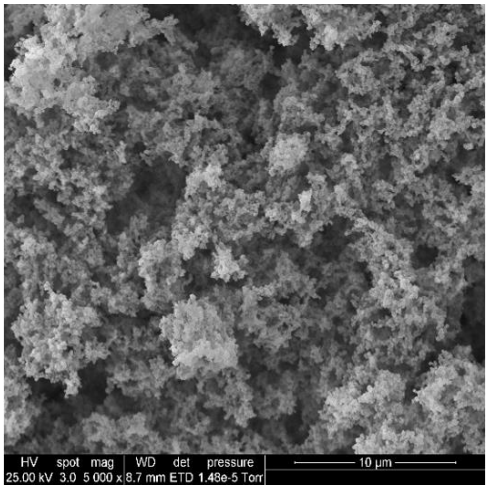
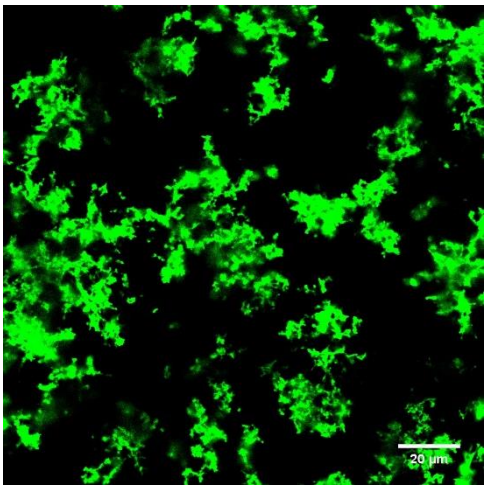


Figure 6.11 SEM images of plain ADG (a), ADG with 15% black tea infusion (b), ADG with 30% black tea infusion (c), ADG with 45% black tea infusion (d), ADG with 60% black tea infusion (e) (day 28)

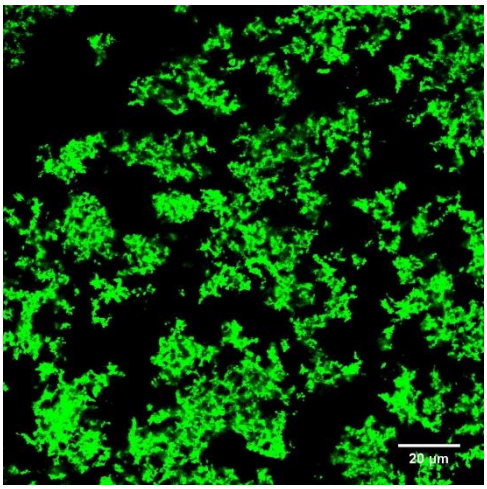
When comparing **Figure 6.9** and **6.10**, more and bigger pores appeared in all samples, especially in samples of $ADG_{45\%}$ which indicated more significant transformations took place when high level of tea infusion was added during storage in later one. $ADG_{60\%}$ remained the same with most and larger pores.

6.3.2 CLSM measurement of ADG-Bs

The microstructures of black tea enriched gels observed by CLSM are shown in **Figure 6.11** and **6.12.** As shown in **Figure 6.11**, tea enriched ADGs had structures of homogeneous connection of aggregated protein clusters with small voids among protein clusters decreased after added the black tea infusion in which $ADG_{45\%}$-G gained the least porous structure. However, after 28 days of cold storage only the control (**Figure 6.12a**), $ADG_{15\%}$-G (**Figure 6.12b**) and $ADG_{30\%}$-G (**Figure 6.12c**) remained evenly distributed structures and protein clusters became larger. On the contrary, the protein clusters in $ADG_{45\%}$-G (**Figure 6.12d**) and $ADG_{60\%}$-G (**Figure 6.12e**) broke down to small pieces after the cold storage.



a



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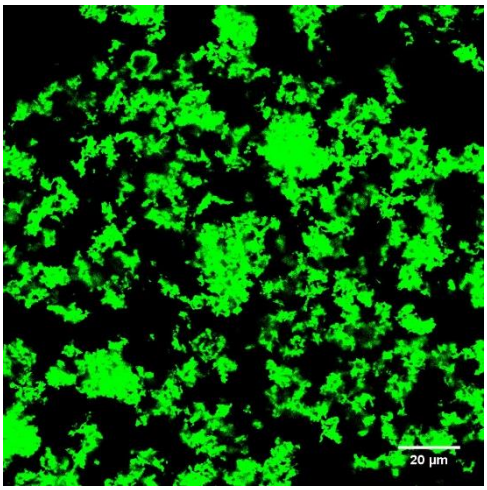

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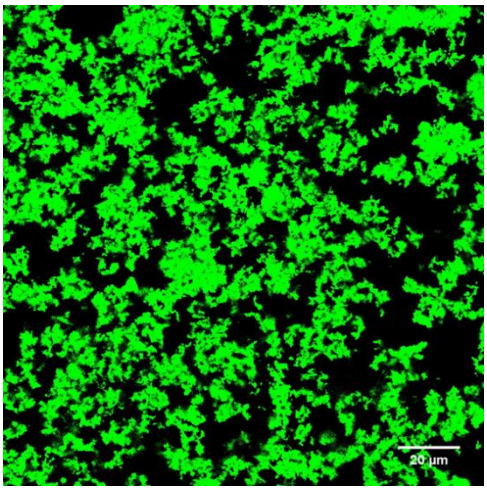

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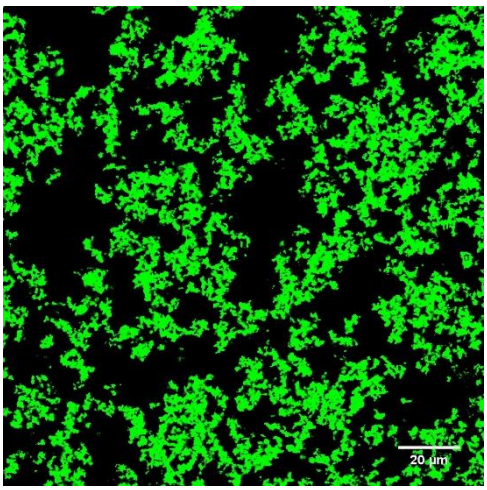
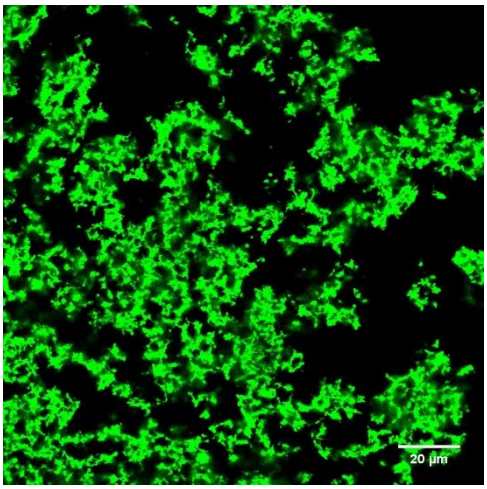
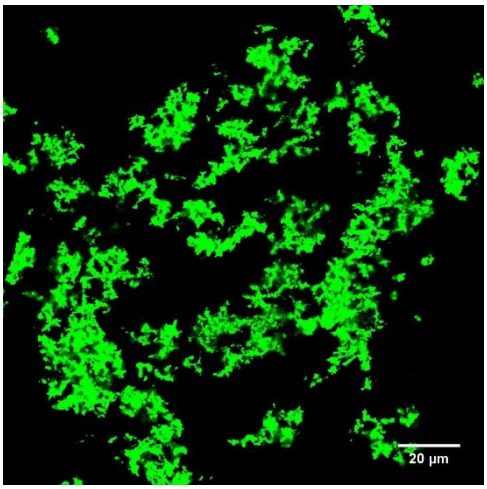


e

Figure 6.12 CLSM images of plain ADG (a), ADG with 15% black tea infusion (b), ADG with 30% black tea infusion (c), ADG with 45% black tea infusion (d), ADG with 60% black tea infusion (e) (day 1). Protein stained by Fast Green FCF appears as green and non-fluorescent areas (dark areas) correspond to the serum pores.



a



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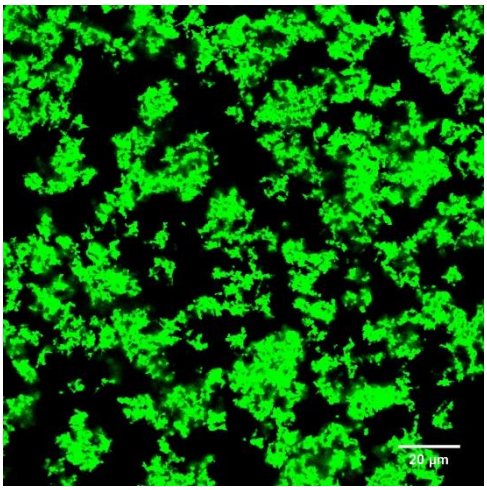

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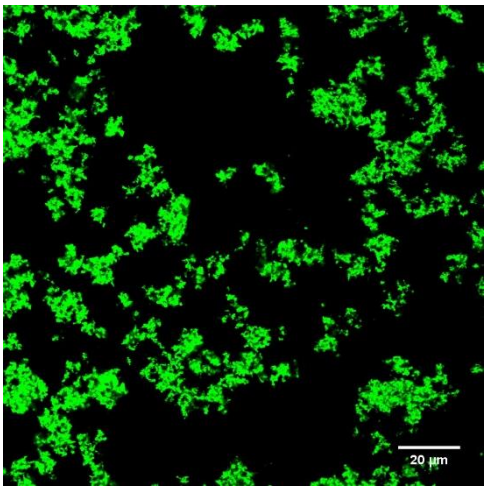

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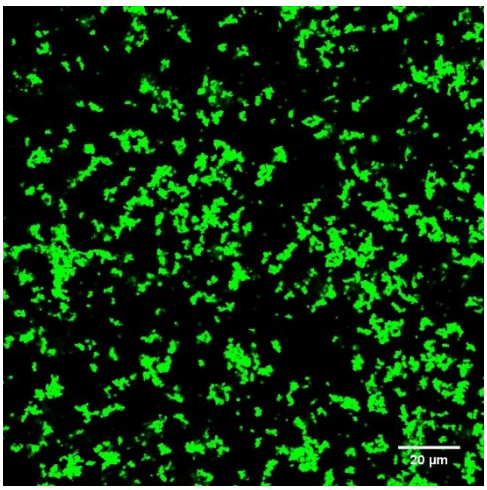


e

Figure 6.13 CLSM images of plain ADG (a), ADG with 15% black tea infusion (b), ADG with 30% black tea infusion (c), ADG with 45% black tea infusion (d), ADG with 60% black tea infusion (e) (day 28). Protein stained by Fast Green FCF appears as green and non-fluorescent areas (dark areas) correspond to the serum pores.

6.4 Conclusion

When comparing tea enriched ADGs and the control, the ADG enriched with tea extracts offered a protein scaffold that strengthened the yoghurt's structure when low level of tea infusion added (15% and 30%). Smaller pores and denser casein micelles were formed in ADG with low level of tea infusion in all types of tea especially for oolong tea. Visualisation of the microstructure revealed that protein-polyphenol complex could behave as active fillers, providing interconnections between protein in the acidified gels with a smaller proportion of localised dense aggregates.

Ai et al. (2019b) added the tea polyphenols (TP) into duck white gel and they found that the degree of non-specific cross-linking and surface hydrophobicity of the proteins after the addition of TP increased significantly, which led to a change the three-dimensional network structure. Also, their supposition on the calcium bridge which causes proteins to aggregate and form cluster-like or granular microstructures could be an explanation of the formation of microstructure when proteins interacted with calcium and polyphenols.

Zhou et al. (2019) used TP to modify the egg white (EW) then utilize EW on surimi. They stated that TP cross-linked with EW proteins, thereby yielding a more compact and denser gel network which agree with our results of low content of tea extracts.

These reports are in agreement with the observations of how the tea polyphenol from green, oolong and black tea interacted with the protein networks in the acidified dairy gels created in this thesis. The next chapter illustrates the chemical bonding of such gels.

Chapter 7

Results of secondary structures and particle size of tea enriched ADG

7.1 Fourier Transform Infrared Spectroscopy (FT-IR) of tea enriched ADG

The FT-IR spectra of ADGs in in the range of 4000-400 cm^{-1} are shown in **Figures 7.1** to **7.3.**

For the ADGs investigated, FT-IR spectra showed similar trends in the water region (3000 to 3500 cm^{-1}). The broad peak in the water region located in 3300-3500 cm^{-1} band is the fingerprint area which represents stretching vibration of O–H groups of hydroxyl group engaged in intramolecular hydrogen bond originating from phenols (3200-3400 cm^{-1}) and N–H group from amino acids (3400-3500 cm^{-1}) (Pagacz-Kostrzewa, Saldyka, Wierzejewska, Khomenko, & Doroschuk, 2016). Kanakis et al. (2011b) reported a common fingerprint band for protein secondary structure: α-helix (1657–1651 cm^{-1}), β-sheet (1634–1608 cm^{-1}), turn (1670–1667 cm^{-1}), and β-antiparallel (1691–1686 cm^{-1}).

The ADG samples which were enriched with three types of tea showed similar trends in spectra (**Figure 7.1** to **7.3).** The O–H bond and N–H bond decreased after tea infusions were added, except ADG with 60% tea infusion. The possible explanation could be the extra O–H bonds in the phenolic molecules exceeded the O–H bonds loss after the proteins interacted with polyphenols in the ADG with 60% tea infusion. The main differences were observed in the amide I, amide II and amide III region.

For the ADG-G samples, the noticeable band shifts were noted in the region of flexural vibrations for the protein amide I band (mark as I) at 1600-1700 cm^{-1}, amide II band (mark as II) at 1500-1600 cm^{-1} and amide III band (mark as III) at ~1200-1400 cm^{-1} as shown in **Figure 7.1** to **7.3**.

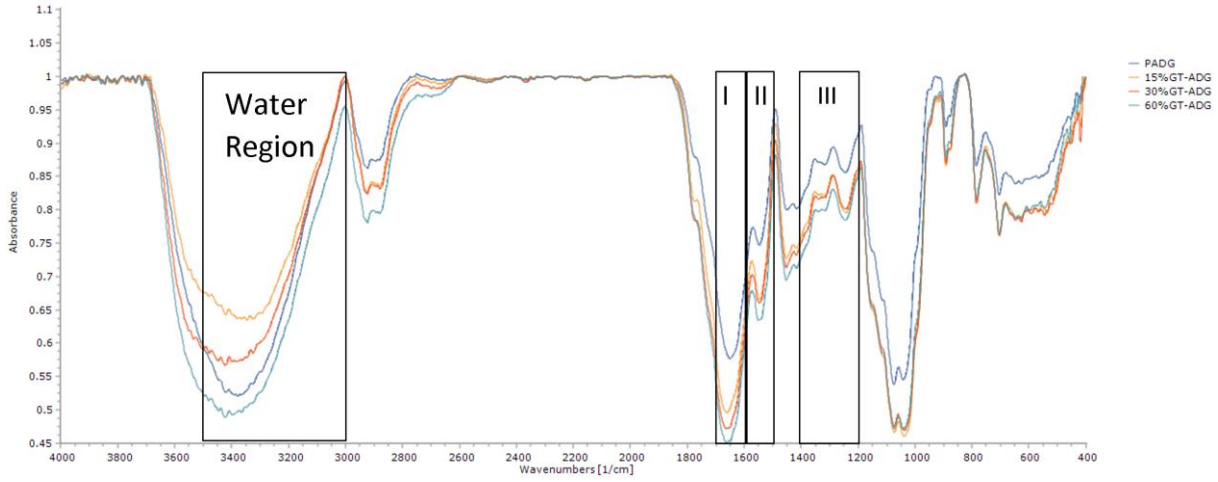


Figure 7.1 FT-IR spectra of green tea enriched acidified dairy gel. PADG: plain acidified dairy gel, 15%GT-ADG: acidified dairy gel containing 15% green tea infusion, 30%GT-ADG: acidified dairy gel containing 30% green tea infusion, 60%GT-ADG: acidified dairy gel containing 60% green tea infusion.

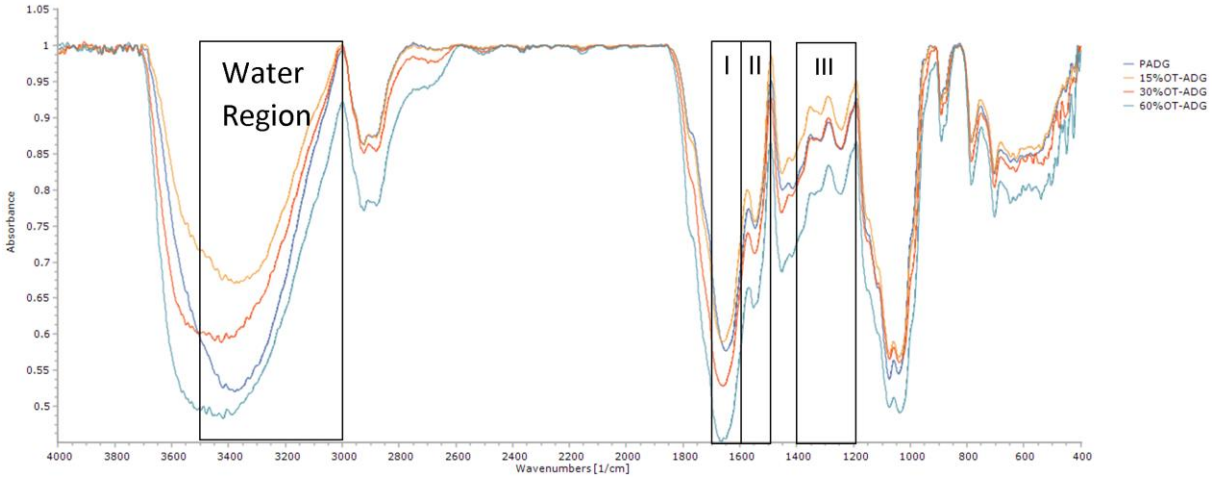


Figure 7.2 FT-IR spectra of oolong tea enriched acidified dairy gel. PADG: plain acidified dairy gel, 15%OT-ADG: acidified dairy gel containing 15% oolong tea infusion, 30%OT-ADG: acidified dairy gel containing 30% oolong tea infusion, 60%OT-ADG: acidified dairy gel containing 60% oolong tea infusion.

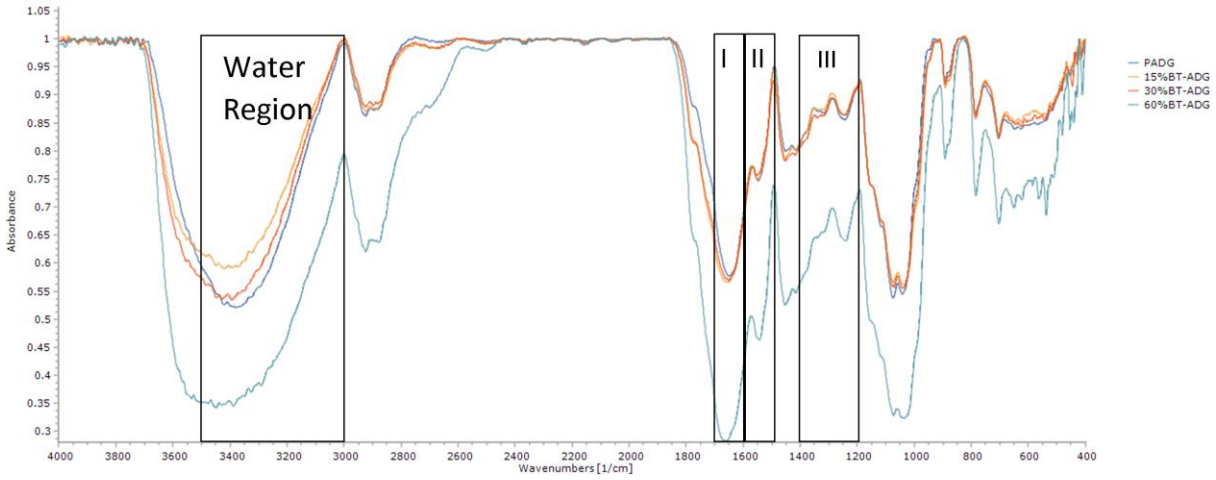


Figure 7.3 FT-IR spectra of black tea enriched acidified dairy gel. PADG: plain acidified dairy gel, 15%BT-ADG: acidified dairy gel containing 15% black tea infusion, 30%BT-ADG: acidified dairy gel containing 30% black tea infusion, 60%BT-ADG: acidified dairy gel containing 60% black tea infusion.

Amide I band consists of 1695 cm^{-1} (aggregated β sheets), 1670 cm^{-1}, 1663 cm^{-1} (β turns), 1653 cm^{-1} (α helix), 1645 cm^{-1} (unordered structures) (Rozenberg et al., 2019). The increasing peak at ~1660 cm^{-1} compared to the control in **Figure 7.1** indicated that the protein structure reformed with more β turns and α helix after addition of green tea infusion. The same trend was observed in $ADG_{30\%}$-O, $ADG_{60\%}$-O (**Figure 7.2**) and $ADG_{60\%}$-B (**Figure 7.3**). However, $ADG_{15\%}$-B and $ADG_{30\%}$-B (**Figure 7.3**) remain the same level at ~1660 cm^{-1} and the ADG15%-O (**Figure 7.2**) decreased at 1660 cm^{-1}. The different patterns between the three types of tea could be the different constitution of polyphenols.

Amide II occurs in the 1600–1500 cm^{-1} region and derives mainly from N–H bending and from the C–N stretching vibration (Akyuz, Akyuz, Celik, & Atak, 2018). The peak in amide III at ~1242 cm^{-1} would be related to O-H bending vibration (Oliveira et al., 2016). The changes in amide II and amide III is the same as amide I. The N–H bending, C–N stretching and O–H bending increased after the addition of tea infusions except for ADG with 15% oolong tea infusion.

Sun, Liang, Yu, Tan, and Cui (2016) reported that infrared absorption peak of N–H had red shift and was caused by a hydrogen bond. In our results, it was suspected that the electrostatic forces between dairy protein and tea polyphenols weakened the formation of hydrogen bond except for 15% oolong tea-ADG sample.

7.2 Circular dichroism (CD) plots of tea enriched ADG

Understanding the characterization of protein-folding intermediates is important and CD spectrum is extensively used for the determination of protein-folding intermediates (Woody, 2004) as UV CD spectra is a direct reflection of protein secondary structure (Wu, Zhang, Kong, & Hua, 2009). When the chromophores of the amides of the polypeptide backbone of proteins are aligned in arrays, their optical transitions are shifted or split into multiple transitions due to "exciton" interactions (Greenfield, 2006).

UV CD spectra were showed in the **Figure 7.4** to **7.6.** Optical rotatory activity of α-helix and β-structure in the 180–260 nm region permitted the use of CD studies for investigation of conformational changes.

Figure 7.4 shows the CD spectra of green tea enriched ADG. The spectra presented a negative peak near 185 nm. The intensity of peaks in CD spectra reflected the magnitude of the elliptical of the protein (Zhao, Ao, Du, & Zhu, 2011). The spectra of ADG-Gs were not significantly different from that of the control were obtained in the far-UV region. The results indicated that the addition of green tea infusion had no distinct impact on the secondary structure of protein.

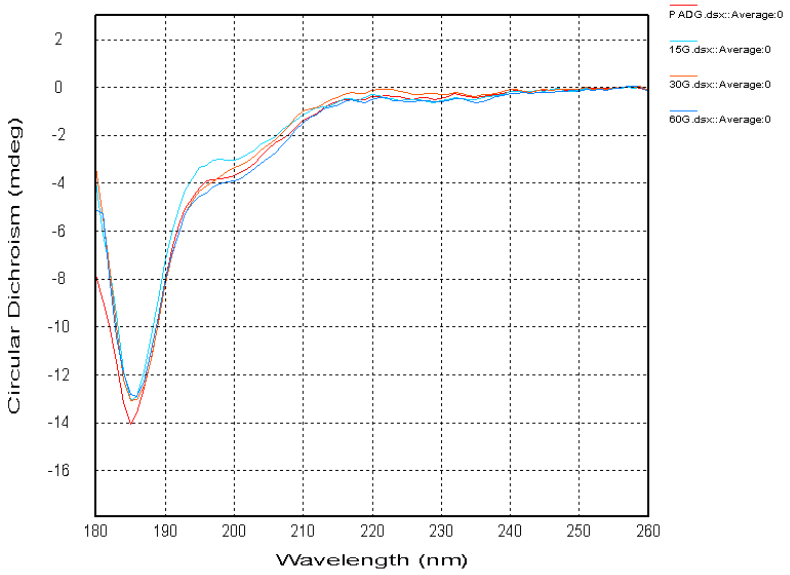


Figure 7.4 UV CD spectra evaluating the effect of green tea extracts on the secondary structure of acidified dairy gel. PADG: plain acidified dairy gel, 15G: acidified dairy gel containing 15% green tea infusion, 30G: acidified dairy gel containing 30% green tea infusion, 60G: acidified dairy gel containing 60% green tea infusion.

Figure 7.5 and **7.6** show CD spectra of oolong tea and black tea enriched ADG, respectively. The addition of oolong tea and black tea changed the negative peak at about 185nm which indicated the an increase of Type I β-turn (Ranjbar & Gill, 2009).

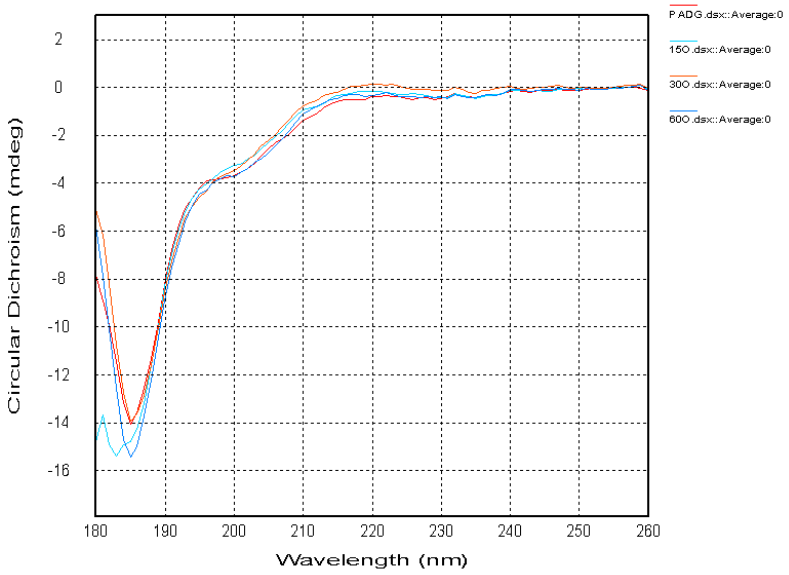


Figure 7.5 UV CD spectra evaluating the effect of oolong tea extracts on the secondary structure of acidified dairy gel. PADG: plain acidified dairy gel, 15O: acidified dairy gel containing 15% oolong tea infusion, 30O: acidified dairy gel containing 30% oolong tea infusion, 60O: acidified dairy gel containing 60% oolong tea infusion.

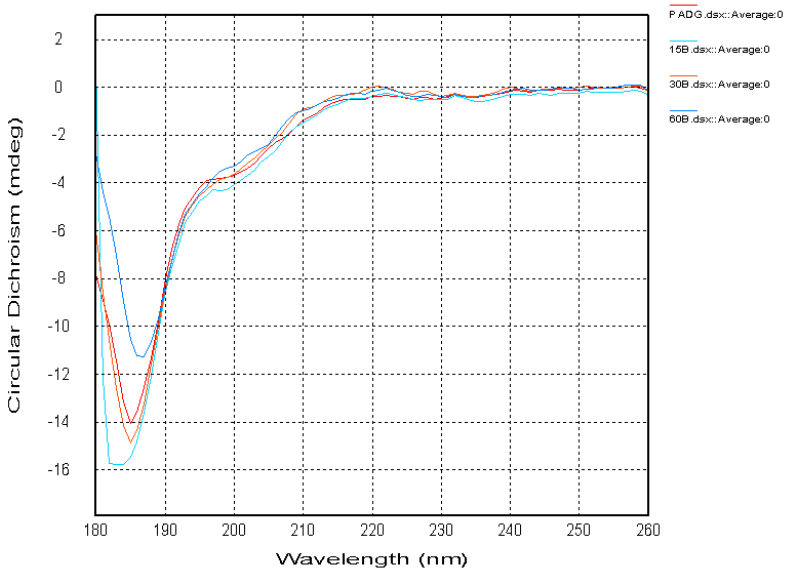


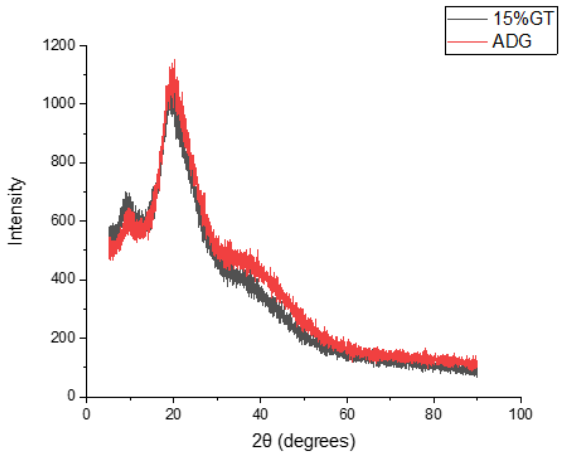
Figure 7.6 UV CD spectra evaluating the effect of black tea extracts on the secondary structure of acidified dairy gel. PADG: plain acidified dairy gel, 15B: acidified dairy gel containing 15% black tea infusion, 30B: acidified dairy gel containing 30% black tea infusion, 60B: acidified dairy gel containing 60% black tea infusion.

The possible explanation of the difference between CD spectra of ADG-G, ADG-O and ADG-B samples could be due to the different constitution of polyphenols among different types of tea. Lee, Lee, and Lee (2002) reported that theaflavins (2–6% of extracted solids) and thearubigens (>20%) are high in black tea, whereas green tea is higher (30–42%) in catechins. Therefore, the interaction between theaflavins and protein, or thearubigens and protein, has more impact on the secondary structure compared to catechins and protein.

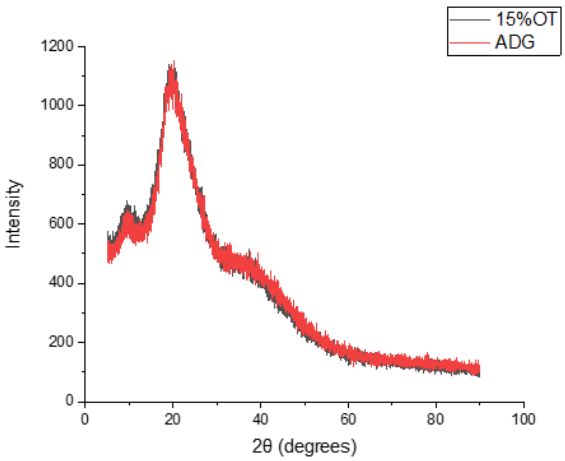
In general, CD spectra in the far-UV (180–260 nm) region reflect the secondary structure of a protein (Zhao et al., 2011). In the far-UV region, the spectrum of ADGs depended on the different constitution of phenolic compounds in different types of tea. The spectra of the native soy protein showed one strong negative peak in the vicinity of 185 nm.

7.3 X-ray diffraction (XRD) of tea enriched ADGs

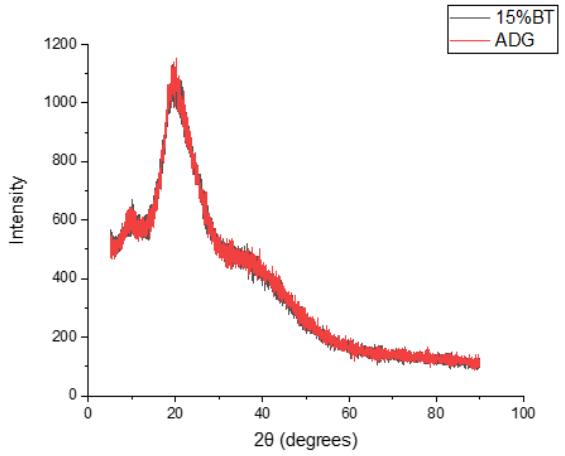
The structural properties of ADGs were studied by XRD and the comparisons of the control and probiotic ADGs are shown in **Figure 7.7**.


15%GT
ADG
Intensity
2θ (degrees)

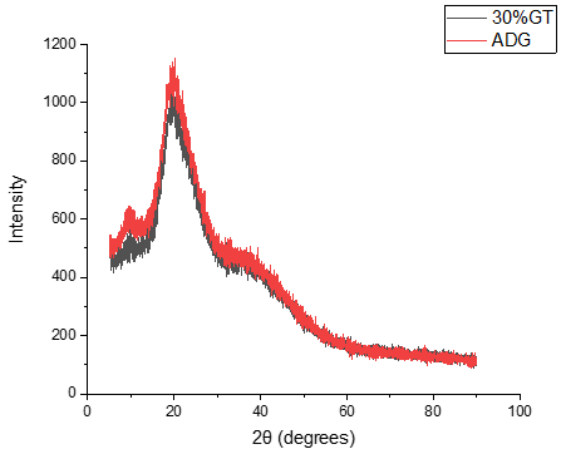
a


15%OT
ADG
Intensity
2θ (degrees)

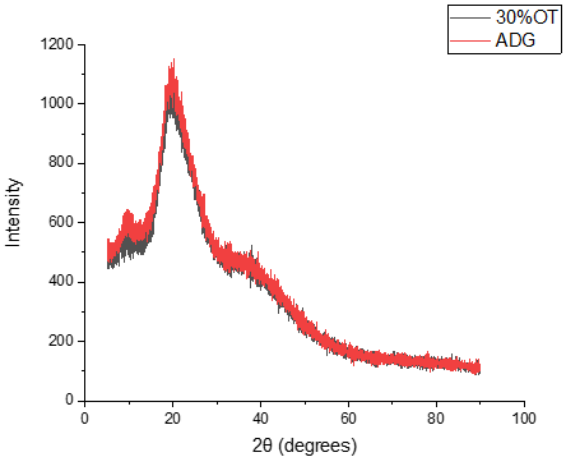
b


15%BT
ADG
Intensity
2θ (degrees)

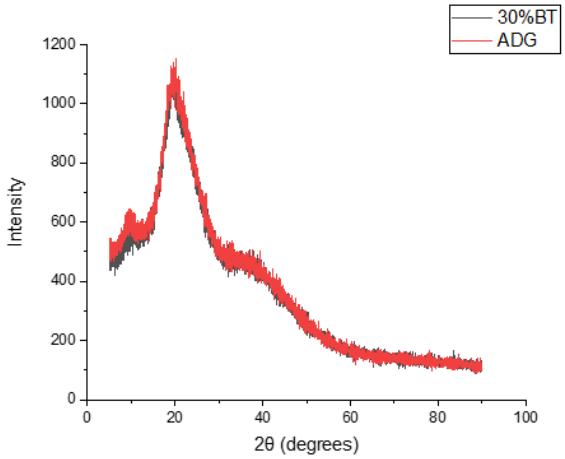
c


30%GT
ADG
Intensity
2θ (degrees)

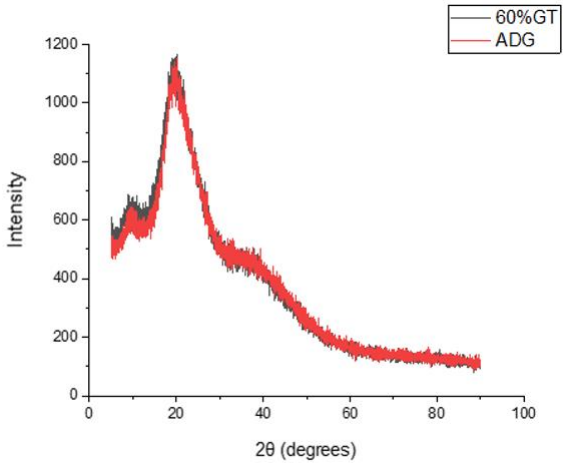
d


30%OT
ADG
Intensity
2θ (degrees)

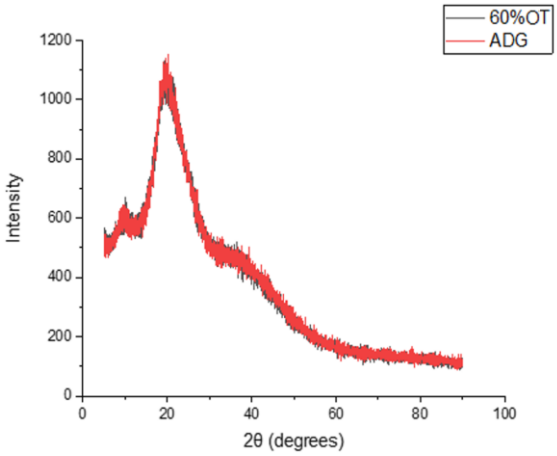
e


30%BT
ADG
Intensity
2θ (degrees)

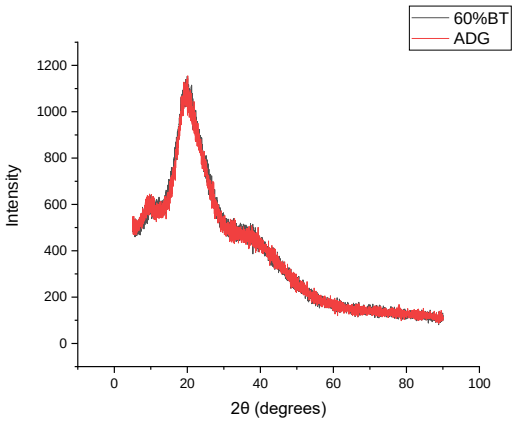
f



g



h



i

Figure 7.7 X-ray diffraction pattern of ADG powders from green oolong and black tea at concentration of 15, 30 and 60%.

Table 7.1 2θ value of X-ray diffraction of powders of ADG enriched with green oolong and black tea infusion at concentration of 15, 30 and 60%.

Samples	Angle 2θ (°)
ADG	10.49
	20.11
$ADG_{15\%}$-G	9.84
	20.20
$ADG_{15\%}$-O	9.52
	20.11
$ADG_{15\%}$-B	9.93
	19.22
$ADG_{30\%}$-G	9.71
	19.45
$ADG_{30\%}$-O	9.34
	19.19
$ADG_{30\%}$-B	9.92
	19.48
$ADG_{60\%}$-G	10.09
	19.00
$ADG_{60\%}$-O	9.59
	19.51
$ADG_{60\%}$-B	19.98

The control sample and probiotic ADGs showed a high crystalline peak at a 2θ value of about 20.00° and a small peak at a 2θ value of about 10.00°. The XRD results indicated that the structure of ADG was not significant changed by the addition of tea extracts (**Table 7.1**). Zhao et al. (2015) reported that the peak angles of α-helix and β-sheet structure in proteins obtained by X-ray were at 2θ around 10° and 20°, respectively.

Although the characterized peaks were not significantly changed by the addition of tea extracts, the intensity of the peaks were changed in $ADG_{30\%}$-GT with a lower peak at 9.71° compared to the control, which in turn indicated a slightly decrease for α-helix.

The XRD spectra of the ADGs showed a similar pattern to the UV CD results which indicated that the addition of tea extracts had little impact on the secondary structure.

7.4 Particle size of tea enriched ADGs

The distribution of particle sizes in milk was obtained by dynamic light scattering (DLS) and the distributions are shown in **Table 7.2**.

Table 7.2 Summary of particle size of studied ADGs. D_{10}, D_{50}, and D_{90} are the diameters (μm) where 10%, 50%, and 90% of all powder particles have smaller size, respectively. n = 3.

	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)	Mean ± SD (μm)
ADG	3.61 ± 0.07^{A}	25.30 ± 2.10^{C}	45.99 ± 5.10^{D}	26.14 ± 3.28^{C}
$ADG_{15\%}$-G	3.58 ± 0.02^{A}	28.19 ± 0.25^{C}	57.85 ± 1.36^{E}	29.57 ± 0.28^{C}
$ADG_{15\%}$-O	3.90 ± 0.13^{B}	11.61 ± 0.35^{B}	31.29 ± 1.03^{C}	15.21 ± 0.39^{B}
$ADG_{15\%}$-B	4.60 ± 0.07^{C}	9.66 ± 0.02^{A}	18.29 ± 0.40^{B}	11.06 ± 0.49^{A}
$ADG_{30\%}$-G	3.95 ± 0.03^{B}	25.92 ± 1.80^{C}	59.42 ± 6.40^{E}	29.05 ± 2.26^{C}
$ADG_{30\%}$-O	4.14 ± 0.03^{B}	10.51 ± 0.18^{B}	25.81 ± 1.90^{C}	13.62 ± 1.53^{B}
$ADG_{30\%}$-B	4.80 ± 0.02^{C}	9.21 ± 0.09^{A}	15.55 ± 0.26^{A}	11.46 ± 2.61^{A}
$ADG_{60\%}$-G	4.11 ± 0.06^{B}	10.25 ± 0.23^{B}	28.41 ± 0.13^{C}	15.52 ± 1.04^{B}
$ADG_{60\%}$-O	4.58 ± 0.04^{C}	9.83 ± 0.28^{B}	18.79 ± 0.84^{B}	11.07 ± 0.35^{A}
$ADG_{60\%}$-B	4.71 ± 0.49^{C}	8.47 ± 1.26^{A}	14.63 ± 0.14^{A}	9.91 ± 0.70^{A}

$^{A-E}$ Means ± SD within a column with different superscripts differ ($P \leq 0.05$).

The particle size of the green tea enriched ADGs was similar to the control sample when 15% or 30% green tea infusion was added but the degree of their dispersion was greater in green tea enriched ADG control ($P < 0.05$). The addition of oolong tea and black tea into ADG showed a decreasing effect on particle size regardless of the concentration ($P < 0.05$). The $ADG_{60\%}$-G sample showed similar results.

Ahmed, Al-Jassar, and Thomas (2015); Ullah et al. (2019) and Ahmed, Al-Attar, and Arfat (2016)reported that the particle size significantly affected composition, hydration and microstructural properties which partly agree with our results. The negative correlation between particle size and WHC could be because the greater surface area is exposed to water molecules binding in fine particles, inducing higher water intake.

7.5 Conclusion

In this chapter, the secondary structure of tea enriched ADGs was estimated by FTIR, CD, XRD and DLS.

The FTIR fingerprint can discriminate the changes of secondary structure of proteins, identify specific absorption peaks for α-helix (1657–1651 cm^{-1}), β-sheet (1634–1608 cm^{-1}), turn (1670–1667 cm^{-1}), and β-antiparallel (1691–1686 cm^{-1}), 1660 cm^{-1}. Main differences were observed in bands: 1695 cm^{-1} (aggregated β sheets), 1670 cm^{-1}, 1663 cm^{-1} (β turns), 1653 cm^{-1} (α helix), 1645 cm^{-1} (unordered structures), 1600 – 1500 cm^{-1} region and derives (mainly from N–H bending and from the C–N stretching vibration) and ~1242 cm^{-1} (O-H bending vibration).

Sun et al. (2016) gained similar plots when exploring the interaction between starch and protein. The possible explanation is that hydrogen bond, electrostatic adhesion and steric stabilization were involved both in the interaction between starch and protein as well as polyphenols and protein.

As CD measurement, the possible explanation of the difference between CD spectra of ADG-G, ADG-O and ADG-B samples could be due to the different constitution of polyphenols among different types of tea. The compositions of three types of tea are very different such as theaflavins (2–6% of extracted solids) and thearubigens (>20%) are high in black tea, whereas green tea is higher (30–42%) in catechins as Lee et al. (2002) reported. The CD plots showed that the interaction between theaflavins and protein, or thearubigens and protein, has more impact on the secondary structure compared to catechins and protein.

There are small changes appeared at the small peak at a 2θ value of about 10.00° in XRD measurement. Chen et al. (2013) also noticed differences at 2θ value of about 10.00° when determining the domain structure of the 7S and 11S globulins from soy proteins using different

extracting methods indicated the position of 2θ = 10.00° is sensitive to structure changes of protein and can be a characteristic peak.

Particle size is highly related to hydration, microstructural and textural properties of food matrix. In our experiments, green tea infusion had no significant impact ($P < 0.05$) on particle size at lower level (15 and 30%). The addition of oolong tea and black tea infusion decreased the particle size ($P < 0.05$) of the ADG regardless of concentration along with $ADG_{60\%}$- G. Poppitt et al. (2018) found that dairy fat can significantly increase the particle size of yoghurt which could partly explain the small particle size of our results.

Chapter 8

Correlation coefficient and principle component analysis

8.1 Pearson correlation coefficients of ADGs

To ascertain the significant relationships between the physicochemical properties and antioxidant capacity, firmness, cohesiveness, pH value, content of calcium ion, EOS, WHC, ABTS, DPPH, FRAP and TPC were used to perform a Person's correlation coefficients analysis. These Pearson correlation coefficients of different ADG samples were showed in **Figure 8.1** to **8.3.**

One common correlation shared by the three types of tea enriched ADG is that antioxidant related parameters (DPPH, FRAP and ABTS) were significantly correlated with TPC (for ADG-Gs, r = 0.996, 0.983 and 0.995, respectively; for ADG-Os, r = 0.967, 0.979 and 0.972, respectively; for ADG-Bs, r = 0.851, 0.795 and 0.881, respectively, $P < 0.005$). These correlations indicate that the polyphenols provide the most of antioxidant capacity in tea. Zhao et al. (2008) reported that TPC was strongly correlated with DPPH and ABTS on malting barley samples and Bozdogan et al. (2018) also found that TPC strongly correlated with DPPH and FRAP value on edible mushroom. Another common correlation was that the content of calcium ions was significantly negatively correlated with antioxidant related parameters (DPPH, FRAP and ABTS) and TPC (for ADG-Gs, r = 0.-670, -0.637, 0.-689 and 0.672, respectively; for ADG-Os, r = -0.850, -0.866, -0.879 and -0.808, respectively; for ADG-Bs, r = -0.626, -0.747, -0.694 and -0.526, respectively, $P < 0.005$) which indicated that the calcium ions played an important role in the interactions related to polyphenols. The firmness is significantly and negatively correlated with cohesiveness among

three types of ADG (r = -0.888 for ADG-Gs, $P < 0.01$; -0.873 for ADG-Os, $P < 0.005$ and -0.899 for ADG-Bs, $P < 0.005$)

The content of calcium ions was significantly correlated with EOS for ADG-G and ADG-O samples (r = -0.524 and -0.490, respectively, $P < 0.005$) whereas they have no significant correlation for ADG-B samples. WHC was significantly negatively correlated with antioxidant parameters (DPPH, FRAP and ABTS) and TPC for ADG-O samples (r = -0.607, -0.588, -0.611 and -588, respectively, $P < 0.005$) while the WHC was also correlated with antioxidant parameters and TPC for ADG-G and ADG-B samples ($P < 0.01$).

Table 8.1 Pearson correlation coefficient of green tea enriched ADGs

	Firmness	COH	pH	Ca^{2+}	EOS	WHC	ABTS	DPPH	FRAP
COH	-0.888*								
pH	0.070	-0.059							
Ca^{2+}	0.279	-0.316*	0.512**						
EOS	-0.225	0.176	-0.479*	-0.524*					
WHC	-0.063	0.127	-0.346**	0.125	-0.198				
ABTS	-0.143	0.241	0.057	-0.689**	0.205	-0.321*			
DPPH	-0.150	0.249	0.059	-0.670**	0.194	-0.302*	0.996**		
FRAP	-0.075	0.195	0.067	-0.637**	0.151	-0.272	0.987**	0.985**	
TPC	-0.145	0.242	0.061	-0.672**	0.193	-0.314*	0.995**	0.996**	0.983**

* = $P < 0.01$(coefficient significant at 1% significance level), ** = $P < 0.005$ (coefficient significant at 0.5% significance level)

COH: Cohesiveness; EOS: Extend of syneresis; WHC: Water holding capacity; TPC: Total phenolic content.

Table 8.2 Pearson correlation coefficient of oolong tea enriched ADGs

	Firmness	COH	pH	Ca^{2+}	EOS	WHC	ABTS	DPPH	FRAP
COH	-0.873**								
pH	0.067	-0.020							
Ca^{2+}	0.140	-0.189	0.447**						
EOS	-0.210	0.165	-0.621**	-0.490**					
WHC	0.213	-0.262	0.189	0.534**	-0.538**				
ABTS	-0.160	0.189	-0.210	-0.879**	0.454**	-0.611**			
DPPH	-0.178	0.200	-0.175	-0.850**	0.417**	-0.607**	0.993**		
FRAP	-0.149	0.189	-0.204	-0.866**	0.453**	-0.588**	0.993**	0.981**	
TPC	-0.088	0.135	-0.165	-0.808**	0.400**	-0.588**	0.972**	0.967**	0.979**

* = $P < 0.01$(coefficient significant at 1% significance level), ** = $P < 0.005$ (coefficient significant at 0.5% significance level)

COH: Cohesiveness; EOS: Extend of syneresis; WHC: Water holding capacity; TPC: Total phenolic content.

Table 8.3 Pearson correlation coefficient of black tea enriched ADGs

	FIRMNESS	COH	pH	Ca2+	EOS	WHC	ABTS	DPPH	FRAP
COH	-0.899**								
pH	-0.599**	0.552**							
Ca^{2+}	0.055	-0.065	0.505**						
EOS	-0.474**	0.487**	0.366**	0.190					
WHC	0.497**	-0.499**	-0.307*	0.267	-0.131				
ABTS	-0.579**	0.609**	-0.025	-0.694**	0.153	-0.404*			
DPPH	-0.589**	0.598**	-0.006	-0.626**	0.254	-0.379**	0.940**		
FRAP	-0.550**	0.587**	-0.083	-0.747**	0.170	-0.446**	0.978**	0.935**	
TPC	-0.671**	0.698**	0.220	-0.526**	0.188	-0.548**	0.851**	0.795**	0.881**

* = $P < 0.01$(coefficient significant at 1% significance level), ** = $P < 0.005$ (coefficient significant at 0.5% significance level)

COH: Cohesiveness; EOS: Extend of syneresis; WHC: Water holding capacity; TPC: Total phenolic content.

8.2 Principal component analysis (PCA) of ADGs

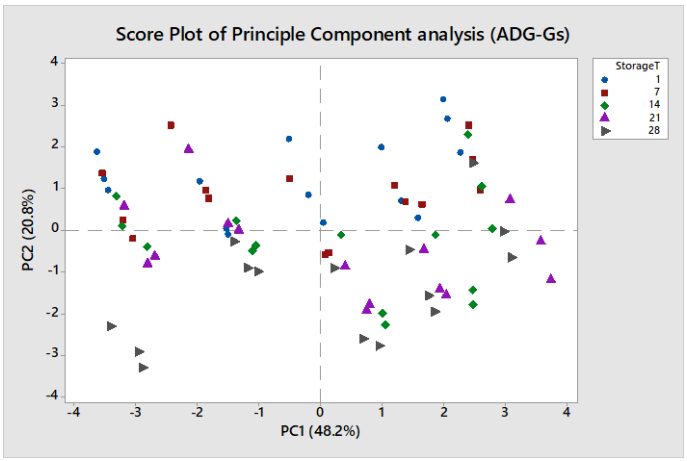
Principal component analysis (PCA) is one of the main approaches in chemometrics, and is extensively used for the classification study in the field of food research (Yi et al., 2015). To simplify the correlation between physicochemical properties and antioxidant capacity, Firmness, cohesiveness, pH value, content of calcium ion, EOS, WHC, ABTS, DPPH, FRAP and TPC were used for PCA analysis. Plots of PCA are displayed in **Figure 8.1** to **8.3**.

PCA of the physicochemical properties and antioxidant properties for green tea enriched ADG, as show in **Figure 8.1**, explained 71.0% of the variation in the first two principal components with 48.2% and 20.8%, respectively. The main difference between PC1 and PC2 is that PC1 strongly associated with the value of TPC, DPPH, ABTS and FRAP but values of EOS, pH were the dominant variables for PC2.

PCA of the physicochemical properties and antioxidant properties for oolong tea enriched ADG, as show in **Figure 8.2**, explained 75.0% of the variation in the first two principal components with 58.2% and 16.8%, respectively. The PC1 mainly represented the value of TPC, DPPH, ABTS and FRAP and Ca^{2+} while the PC2 mainly represented the value of cohesiveness and pH.

PCA of the physicochemical properties and antioxidant properties for black tea enriched ADG, as show in **Figure 8.1**, explained 79.1% of the variation in the first two principal components with 54.9% and 24.8%, respectively. The characteristic parameters for the first principal component are the value of TPC, DPPH, ABTS and FRAP and for second principal component are the value of EOS, pH and Ca^{2+}.

The results of different formulations could be better differentiated in **Figure 8.1a**, **8.2a** and **8.3a** and the results of different storage periods could be better differentiated in **Figure 8.1b**, **8.2b** and **8.3b** which indicated that the antioxidant properties were mainly influenced by the different concentrations of tea infusion, but the physicochemical properties were mainly influenced by storage period. The distributive points in the plots grouped by storage time (**Figure 8.1a**, **8.2a** and **8.3a**) are hard to split one group from another which suggested the cold storage had little impact on the parameters involved whereas the distributive points in the plots sorted by the different formulas (**Figure 8.1b**, **8.2b** and **8.3b**) are easily differentiated from other groups which indicated that the concentration of tea infusion severe impacts on the parameters involved.



a

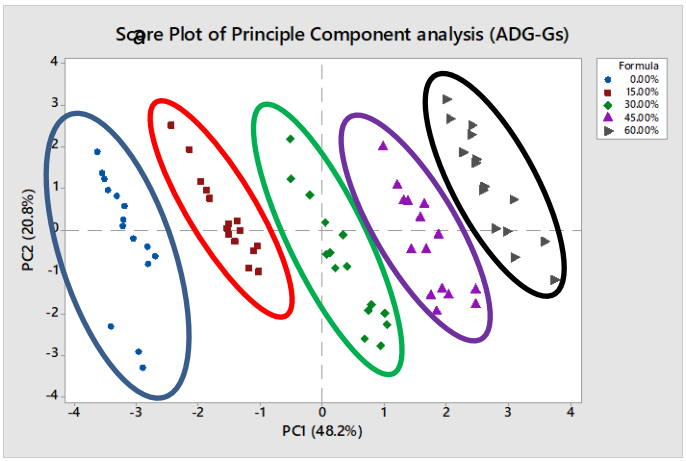
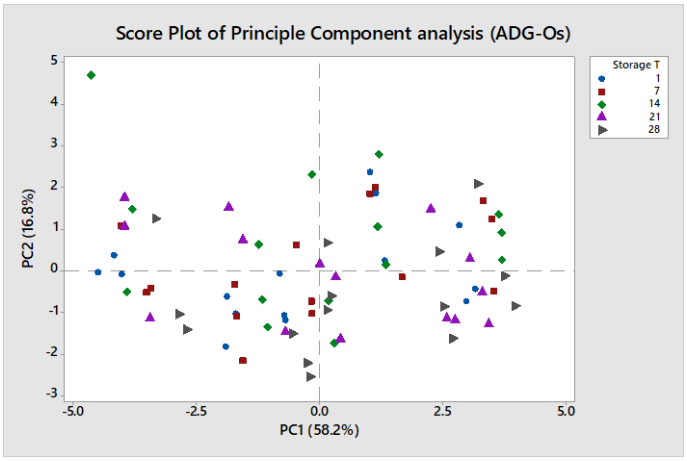


Figure 8.1 PCA of green tea enriched acidified dairy gels. PC1 strongly associated with TPC, DPPH, ABTS and FRAP; PC2 strongly associated with values of EOS, pH value. Figure 8.1a is PCA plot based on different storage time; Figure 8.1b is PCA plot based on different formula.



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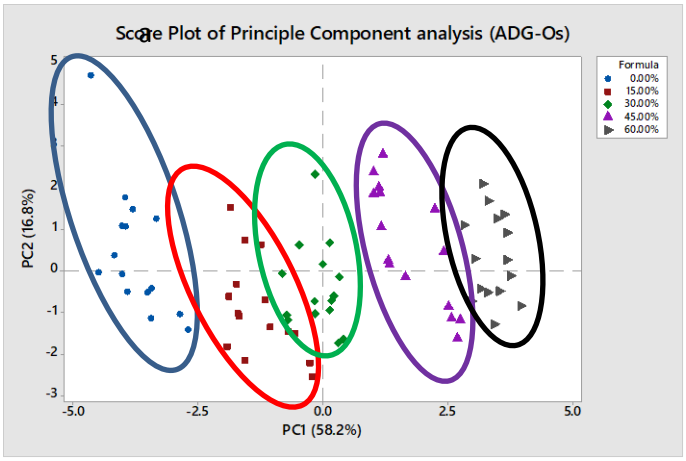

Figure 8.2 PCA of oolong tea enriched acidified dairy gels. PC1 strongly associated with TPC, DPPH, ABTS and FRAP and Ca^{2+}; PC2 strongly associated with values of cohesiveness, pH, firmness. Figure 8.2a is PCA plot based on different storage time; Figure 8.2b is PCA plot based on different formula.

Figure 8.3 PCA of black tea enriched acidified dairy gels. PC1 strongly associated with TPC, DPPH, ABTS and FRAP; PC2 strongly associated with values of EOS, pH and Ca^{2+}. Figure 8.3a is PCA plot based on different storage time; Figure 8.3b is PCA plot based on different formula.

Chapter 9 General discussion and conclusions

9.1 General discussion

The first aspect of this study investigated the effects of tea extracts on the physicochemical and textural properties of ADG. The decrease of content of calcium ion as the concentration of tea extracts increased indicated that the tea polyphenols were responsible for the reduction of calcium ions. The pH value of all ADG samples were gradually decreasing over time during cold storage. There was significant ($P < 0.05$) improvement in EOS and WHC as addition of tea infusion increased from 0% to 30% for green tea and oolong tea enriched ADG. Previous studies have found that a high content of green tea powder (2%, w/w) increased syneresis rate in yoghurt which led to an increase of serum separation (Donmez et al., 2017) whereas 1% of green tea powder could decrease the syneresis rate which is in agreement with our results. The same research also pointed out that green coffee powder (2%, w/w) could decrease the syneresis rate in yoghurt and postpone the serum separation. They also conducted experiments on 21 days of cold storage and stated that the storage time had no significant effects on syneresis. The differences in the polyphenol profiles of green tea and green coffee played a certain role on the observed differences of the yoghurts added with their powders.

The addition of oolong tea infusion undermines the hardness and adhesiveness of ADG regardless of concentration in set yoghurt and only 45% and 60% green tea and black tea infusion damaged the texture of set yoghurt in terms of hardness and adhesiveness. Similar experiments were carried out by Najgebauer-Lejko et al. (2014) with green tea and pu-erh tea infusion. They found

that low content (10% tea infusion) of tea infusion had no significant effects on firmness but improve the performance of cohesiveness which is partly agree with the results of this study.

Chapter 5 presented results on the effects of tea infusion on antioxidant capacity of ADGs. The high antioxidant capacity is the most important nutritional value of tea and it has been reported for decades and has been paid more and more attention (Bakht et al., 2019; Chen, Zhang, Qu, & Xie, 2008; Pekal, Drozdz, & Pyrzynska, 2012; Peluso & Serafini, 2017; Tanizawa et al., 1984; Xie et al., 2018; Yang et al., 2017; Zhao, 2003; Zhao, Li, He, Cheng, & Xin, 1989; Zhao et al., 2019). The tea extracts may also be used as a feed additive or a food preservation (Fang et al., 2019; Fernandez et al., 2018). The tea extracts significantly improved the antioxidant capacity of ADG in our research even in lowest concentration (15%). To assess the bioavailability, intake rate and metabolism, we utilized the CAA assay (Wolfe & Liu, 2007). The CAA results showed the oolong tea was highest on intake rate during cell culture.

Chapter 6 reported the effects of incorporating tea extracts into acidified dairy gels on microstructure. As a result, the low content (15% and 30%) of tea extracts improved the performance of ADG in terms of microstructure and high content (45% and 60%) of tea extracts destabilised the system. Nguyen, Schwendel, et al. (2018) and Nguyen, Afsar, et al. (2018) reported the similar structure. The cold storage had no severe impact on microstructure of ADGs.

In Chapter 7 and Chapter 8, the secondary structure of ADGs and the correlation between physicochemical parameters were studied. The addition of tea extracts had no significant impact of secondary structure in terms of XRD, CD and FT-IR but the addition of oolong tea and black tea extracts significantly changed the particle size of ADG which could be related to the changes of

textural properties. The results of Pearson's correlation coefficient and PCA showed the significant correlation between TPC and antioxidant parameters (DPPH, FRAP and ABTS) as well as other physicochemical parameters.

9.2 Conclusions

9.2.1 The effects of tea extracts on physicochemical and textural properties

The pH value decreased over time during the cold storage in all samples with no significant differences ($P < 0.05$) among different formulas. The addition of tea extracts undermined the content of calcium ions suggested that the calcium ions played a certain role in the interactions related to polyphenols.

The addition of green tea and oolong tea extracts improved the performance of EOS and WHC when 15% and 30% tea infusion were added ($P < 0.05$). The black tea extracts showed negative effects regardless of concentration.

The addition of oolong tea infusion undermines the hardness and adhesiveness of ADG regardless of concentration in set yoghurt and only 45% and 60% green tea and black tea infusion damaged the texture of set yoghurt in terms of hardness and adhesiveness. Low content (15% and 30%) of green tea and black tea infusion had no significant impact on textural properties of ADG.

9.2.2 The improving effects of tea extrats on antioxidant capacity of ADG

The tea extracts significantly increased the antioxidant capacity of ADG ($P < 0.05$) from 2.5-fold to 60-fold depends on assay adopted. The green tea showed highest antioxidant capacity in terms of *in vitro* antioxidant experiments (DPPH, FRAP and ABTS). The antioxidant capacity is strongly

correlated to TPC which suggested that the polyphenols is the main source of the antioxidant capacity of tea.

9.2.3 Anti-proliferative and cell antioxidative activity of ADGs

The plain ADG and the tea enriched ADGs both showed strong anti-proliferative activity and the results of CAA assay indicated that the oolong tea had the highest intake rate which obtained similar results as green tea enriched samples in PBS wash experiment although the TPC and antioxidant capacity of oolong tea are lower than those of green tea. The black tea enriched ADG had a lower CAA results but similar intake rate compares to green tea enriched ADG.

9.2.4 The microstrcuture and secondary structure of ADG

The images of SEM and CLSM measurements suggested that the low content (15% and 30%) of tea extracts improved the performance of ADG in terms of microstructure, whilst the high content (45% and 60%) of tea extracts destabilised the system. The cold storage had no severe impact on microstructure of ADGs.

The addition of tea extracts had no significant impact of secondary structure in terms of XRD, CD and FT-IR but the addition of oolong tea and black tea extracts significantly changed the particle size of ADG which could be related to the changes of textural properties.

9.2.5 Possible interactions between tea components and casein micelles and beta-lactoglobulin (β-LG)

Protein-polyphenol interactions are mainly driven by hydrogen bridging between the phenolic hydroxyl and peptide carbonyl, as well as interactions between hydrophobic amino acid residues and the phenolic rings.

Tea catechins have a strong attraction to proline residues (Haratifar & Corredig, 2014). Hypothesis is that EGCG associated with the residues present close to the point of cleavage of κ-casein which makes residues 98–111 (His-Pro-His-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys) the most likely to interact with casein. Haratifar and Corredig (2014) also studied casein macro-peptide release to verify their hypotheses using a solution of sodium caseinate containing EGCG. Caseins will no longer be the micellar form in sodium caseinate suspensions, but are monomeric form (Ozdal, Capanoglu, & Altay, 2013). Their results confirmed that EGCG strongly interacts with caseins and the interaction also hinders the accessibility of the enzyme to the caseins.

A study carried out by Oliveira et al. (2015) suggested that (+)-catechin, (-)-epicatechin mainly interacts with β-LG. The results also indicated that the certain additive like neutral hydrocolloids (e.g. xanthan and guar) could minimise polyphenols interaction with proteins.

Charlton et al. (2002) created a 3-step model of polyphenol-peptides interaction. The initial interaction is the complexation of the peptide by molecules of polyphenol to form soluble aggregates. The aggregates are single peptide molecules with polyphenols bound by the hydrophobic face of the aromatic rings to the pyrrolidine ring of the proline residues. The second stage took place between two polyphenol-coated peptide molecules, driven by cooperative weak intermolecular bridging effects from the polyphenol. The size of the complex was doubled, which renders the complex insoluble. In the third stage, a small number of these peptide masks aggregate together until the charge repulsion between them neutralize the favourable interaction energy.

Finally, the spontaneous aggregation of these insoluble aggregates into larger complexes which is simply seen as a phase separation process in vision. The 3-step interaction model created by Charlton et al. (2002) firmly agree with our results from low level of tea infusion added samples to high level of tea infusion added samples, especially the black tea enriched samples. We can clearly see small floccules in 30% black tea enriched ADG and went through a phase separation in 60% black tea enriched ADG.

9.3 Possible future works

9.3.1 Investigation of interaction between the tea polyphenols and casein or whey protein

The present study investigated the interaction between tea polyphenols and proteins using SMP which left the deep mechanism still remain unclear.

So, the future investigation should include:

1. The secondary structure of the polyphenol-casein complexes or the polyphenol-whey complexes.

2. How the molecular weight of protein effects the polyphenol-protein interactions.

9.3.2 The effect of tea extracts on the microbial-fermented yoghurt

The present study used ADG model as a mimic of yoghurt therefore how the tea extracts affect the fermentation process should be investigated which could include:

1. The viability of microbial during yoghurt fermentation under the influence of tea extracts.

2. The effects of microbial activity during yoghurt fermentation on the antioxidant capacity of yoghurt (compare to ADG model).

9.3.3 Sensory evaluation of different types of tea enriched yoghurt

The addition of tea extracts changes the colour and flavour of food matrix. Therefore, after the microbiological profiles of tea enriched yoghurt are tested to ensure their hygiene safety for consumer, a sensory evaluation should be conducted.

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