EFFECT OF PROTEIN POLYPHENOL INTERACTION ON THE PHYSICOCHEMICAL PROPERTIES OF SET TYPE YOGURT AND ON THE DIGESTIBILITY OF MILK PROTEINS

PROTEİN POLİFENOL İNTERAKSİYONUNUN SET TİPİ YOĞURDUN FİZİKOKİMYASAL ÖZELLİKLERİ VE SÜT PROTEİNLERİ SİNDİRİLEBİLİRLİĞİ ÜZERİNDEKİ ETKİSİ

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ETHICS

In this thesis study, prepared in accordance with the spelling rules of Institute of Graduate Studies in Science of Hacettepe University,

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- I did not do any distortion in the data set
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09.01.2017

ÖZGE DÖNMEZ

ABSTRACT

EFFECT OF PROTEIN POLYPHENOL INTERACTION ON THE PHYSICOCHEMICAL PROPERTIES OF SET TYPE YOGURT AND ON THE DIGESTIBILITY OF MILK PROTEINS

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The subject of protein-polyphenol interaction is of great interest to food science because of causing the physicochemical changes in protein structure, which results possibly in not only enhance the food product quality but also effect human health in the presence of polyphenols. The main focus of this thesis was to reveal the probable consequences of milk protein-polyphenol interactions in both the food and simulated human gastrointestinal system.

In the first part, the effect of added different concentrations of green coffee powder and green tea powder, as polyphenol sources, on syneresis behavior which is described as a defect due to release serum phase from the gel matrix, and consistency of set yogurts were investigated. The results showed that the interaction between milk proteins and polyphenols improved the acid-induced gel network of set yogurts as confirmed by decreased syneresis rate and increased consistency during storage. But, green tea powder and green coffee powder behaved differently as a concentration-dependent manner in acidified gel networks of set yogurt,

modifying its rheological behavior, as they have different profiles and concentrations of polyphenols.

In the second part, the effect of the interaction of the milk proteins beta-lactoglobulin and micellar caseins (as micellar casein isolate) with either the polyphenol epigallocatechin-3-gallate or alternatively with a polyphenol-rich green tea extract on the modulation of protein digestibility promoted by gastrointestinal enzymes and free radical scavenging capacity of phenolic compound was studied. The results showed that the polyphenol binding is likely to alter protein structure leading to increased protein stabilization through gastrointestinal tract. In addition, the free radical scavenging capacity for polyphenols gradually decreased resulted in interaction with protein, especially casein, from beginning to the end of the digestion.

Keywords: Protein-polyphenol interaction, set yogurt, syneresis rate, rheological behavior, free radical scavenging capacity, in-vitro digestion.

ÖZET

PROTEİN POLİFENOL İNTERAKSİYONUNUN SET TİPİ YOĞURDUN FİZİKOKİMYASAL ÖZELLİKLERİ VE SÜT PROTEİNLERİ SİNDİRİLEBİLİRLİĞİ ÜZERİNDEKİ ETKİSİ

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Protein-polifenol interaksiyonu konusu, proteinin fizikiokimyasal özelliklerinde meydana getirdiği değişim ile gıda ürünlerinde kalitenin iyileşmesi ve de polifenol varlığında öngörülen insan sağlığı üzerindeki etkileri sebebiyle gıda bilimi için ilgi çekici hale gelmektedir. Bu tezin ana odak noktası, süt proteinleri ile polifenoller arasındaki etkileşimin hem gıda sisteminde hem de simüle edilmiş insan sindirim sistemindeki muhtemel sonuçlarını ortaya koymaktır.

Birinci bölümde, polifenol kaynağı olarak farklı konsantrasyonlarda eklenen yeşil çay tozu ve yeşil kahve tozunun set tipi yoğurtlarda, jel matrisinden serum fazının salınması sonucu bir kusur olarak tanımlanan sineresis hızına ve akış davranışları üzerine etkileri araştırılmıştır. Sonuçlar, süt proteinleri ile polifenoller arasındaki interaksiyonun depolama sırasında sineresis hızındaki azalma ve kıvamdaki artışa sebep olarak, asidifikasyonla meydana gelen yoğurt jel yapısını iyileştirdiğini göstermektedir. Fakat, yeşil çay tozu ve yeşil kahve tozu yoğurt jelinin reolojik

davranışını değiştirirken konsantrasyona bağlı farklı davranışlar sergilemişlerdir. Bu durum farklı konsantrasyon ve tipte polifenollere sahip olmalarına bağlanmıştır.

İkinci bölümde, süt proteinlerinden beta-laktoglobulin ve kazein izolatı ile epigallokateşin-3-gallat veya polifenolce zengin yeşil çay ekstraktı interaksiyonunun, sindirim sırasında protein stabilitiesine ve fenollerin serbest radikal yakalama kapasitesi üzerine etkisi incelenmiştir. Sonuçlar, polifenol bağlanmasının protein yapısındaki muhtemel değişimi sonucu sindirim boyunca protein stabilitesini arttırdığını göstermektedir. Ayrıca, özellikle kazeinle interaksiyonun genel olarak sindirimin başından sonuna kadar fenollerin serbest radikal yakalama kapasitesini düşürdüğünü göstermektedir.

Anahtar Kelimeler: Protein-polifenol interaksiyonu, set yoğurt, sineresis hızı, reolojik davranış, *in-vitro* sindirim.

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SYMBOLS AND ABBREVATIONS

Symbols

τ Shear stress of the material

τ^o Apparent yield stress

K Consistency coefficient

γ Shear rate

n Flow behavior index

ΔE Color differences

L* Luminance or lightness

a* Green to red

b* Blue to yellow

Abbrevations

AA Amino acid

β-Lg Beta Lactoglobulin

BSA Bovine serum albumin

α-La Alfa Lactalbumin

Ig Immunoglobulin

Lf Lactoferrin

Lp Lactoperoxidase

Xo Xanthineoxidase

C Catechin

EC Epicatechin

ECG Epicatechin gallate

EGCG Epigallocatechin gallate

5-CQA Chlorogenic acid

α_{s1}-CN α_{s1} casein

α_{s2}-CN α_{s2} casein

β-CN Beta casein

κ-CN Kapa casein

GTP Green tea powder

GCP Green coffee powder

GAE	Gallic acid equivalent
GTE	Green tea extract
GIT	Gastrointestinal tract
d	Day
UHPH	Ultra high pressure homogenization
FRSC	Free radical scavenging capacity
ITC	Isothermal titration calorimetry

INTRODUCTION

Polyphenols, the secondary plant metabolites, have ability to interact with proteins, resulting in the formation of protein-polyphenol complex [1-3]. Interactions between polyphenols and proteins are mostly based on multiple weak interactions, mainly hydrophobic, van der Waals, hydrogen bond, and ionic interactions formed between AA side chains and polyphenol aromatic rings, indicating that the association of polyphenols with proteins is principally a surface phenomenon. Hasni et al. examined that the interactions between tea polyphenols and α-casein and β-casein using Fourier transform infrared, UV-visible fluorescence spectroscopic methods and found that the binding mechanisms were both hydrophobic and hydrophilic interactions [4]. The formation or precipitation of protein-polyphenol complex was modeled by many researchers [5-10].

Such interaction could cause alteration of the functional properties of proteins and polyhenols as well as the food microstructure. Rawel and Kroll have published several studies about the interactions of specific phenolics with proteins found in food, food products and with enzymes of the digestive pathway [11-14].

The main aim of this thesis was to focus on what changes occur in actual food system and in model human gastrointestinal system as a result of the milk proteins-polyphenols interaction.

The first chapter of the thesis covers the fundamental literature (**Chapter 1**) regarding the studies topics, and thereafter each study is given in separate chapters (**Chapter 2-3**).

1. GENERAL INFORMATION

1.1. Milk Proteins

About 80% of the milk protein is constituted by casein micelles, a group of phosphoproteins, in bovine milk. The micelle comprises four fractions (the α_{s1}-, α_{s2}, α- and β- caseins, in approximate ratios 4: 1: 3.5: 1.5) that are interacted with calcium phosphates bridges, as well as, van der Waals, hydrophobic interactions, and hydrogen bonds [15]. The structure of casein micelles tried to be explained by different models which have shown one aspect of the casein micelles on an individual base. Nevertheless, none of them seem to completely describe either the measured physicochemical properties or the functionality of the micelles. In a recent time, the proposed model has shown that the micelle structure is not a continuous aggregate of protein. In addition to this, it also contains less protein dense areas, water channels, and clefts [15].

The mean diameter of the micelles, as spherical colloidal particles, is 120 nm (range 50-600 nm). There are about 5000 casein molecules (20-25 kDa) in an average micelle [16].

Both polar and apolar residues in caseins evenly distribute along their sequences, creating hydrophobic and hydrophilic patches. This structure creates a very good surface activity so that caseins have good emulsifying and also foaming properties. Furthermore, all caseins, especially β-casein, contain high level of proline, which disorders α- and β-sheets. In addition, caseins are sensitive to proteolysis due to their open, flexible, mobile conformation, leading to surface hydrophobicity on micelles [16].

Besides caseins, serum proteins comprise about 20% of the total protein in bovine milk. There are two main proteins present in the whey fraction of milk, α-lactalbumin (α-La)and β-lactoglobulin(β-Lg) [17].

About 50% of the total whey proteins in milk is (β-Lg), exists as a dimer form. It has 162 amino acid residues per monomer and a molecular weight of about ~18kDa [18]. The most important characteristic of β-Lg is the presence of one mole of cysteine (thiol group) per monomer and two intramolecular disuphide bridges. In milk, 30% free thiol groups are from β-Lg. But, these thiol groups are not very

reactive since they normally buried in the dimer complex. After heat denaturation, its cysteine residues arise and form complexes with other cysteine containing proteins, such as α-lactalbumin, Bovine serum albumin (a fraction of whey proteins, BSA) or casein, leading to change their functional properties such as the gel strength of yogurt [19].

In conclusion, these proteins have great importance in many dairy products due to their processing functionality such as gel forming, emulsifying abilities etc. in terms of dairy technology.

1.2. Phenolic Compounds

Phenolic compounds are a major category of bioactive compounds and are abundant micronutrients in fruits and vegetables [20, 21]. They are also known as secondary plant metabolites and involve more than 8000 phenolic structures. These compounds are classified into different categories on the basis of either their simplicity or complexity of their chemical structures [21]. Harborne divided polyphenols in at least 10 classes in which the most common polyphenols are flavonoids including more than 5000 components. All polyphenols' skeletons comprise at least one phenolic ring and are commonly conjugated with saccharides, organic and carboxylic acids, lipids, and amines. Phenolic acid and highly polymerized cyclic compounds like tannins could exemplify for their structure from simpler to more complex, respectively [21].

Phenolic compounds have been attracted attention in recent years due to a growing body of evidence regarding their health benefits and multiple biological activities, such as anti-cancer, anti-microbial, and anti-inflammatory [20, 22]. They also have ability to interact with other food components including protein, lipid and carbohydrates. Therefore, these compounds have raised a new research area for food product developers to solve certain industrial problems.

Some polyphenols are very sensitive in changing environmental conditions and hence their nutritional functionality when incorporated in food matrices is often put into question [23]. This is why their applications in food products have been limited.

1.3. Interaction of Polyphenols with Proteins

Phenolic compounds are the major representatives of the flavonol subclass, are very strong antioxidants and are regularly consumed by humans. They have strong binding affinities to proteins, especially proline rich proteins, such as salivary proteins and caseins. Such interaction may cause an alteration in functional properties of both proteins and polyhenols as well as in the food microstructure [6].

The phenolics and poylphenolics become reactive when by transforming into quinones where hydroxyl groups are adjacent (ortho-quinoses), or opposite each other (para-quinoses) on the phenolic ring. Quinones react with other compounds such as proteins, anthocyanins, polysachharides, sulphur-containing compounds, reducing agents such as ascorbic acid via covalent binding depending on the reaction conditions. Beside covalent bonds, the interaction between phenolics and other compounds, such as proteins, are also based on weak interactions, mainly hydrophobic, van der Walls-, hydrogen bonds and ionic interactions, which are formed between AA side chains and polyphenol aromatic rings, indicating that the association of polyphenols with proteins is principally a surface phenomenon [24, 5-10].

Covalent bonds are formed between phenolics and AAs, by oxidation of phenolic compounds to quinones via enzymatic, alkali or acid activation. Protein modification can occur by reversible associations (via hydrogen bonding, hydrophobic interactions and van der Waals forces) between proteins and either simple phenolic compounds or higher polymeric polyphenols. These associations may or may not result in protein precipitation, depending on the factors: ionic composition of solution, pH and the ratio of proteins to polyphenols [24].

Rawel and Kroll have published several studies about the large body of research on this topic that are interactions of specific phenolics with proteins found in food, food products and with enzmyes of the digestive pathway [11-14]. In most of these published studies, the increasing number of interactions was ensured at high phenolic to protein ratios and also at pH of about 9 by inducing o-quinone production. On the other hand, the incubation of chlorogenic acid with BSA at high temperature and at pH 7 has been shown to induce covalent modification preferentially via interacting quinones or phenolic dimers with proteins containing

hydrophobic AAs such as tryptophan. AAs such as cysteine, containing thiol groups, form covalent interactions via a quinone-mediated mechanism, whereas proline is an imino acid, which has tertiary amine and carbonyl group, is responsible for hydrophobic interactions. Hasni et al. [4] examined that the interactions between tea polyphenols and α-casein and β-casein using FTIR, UV-visible fluorescence spectroscopic methods and found that the binding mechanisms were both hydrophobic and hydrophilic interactions.

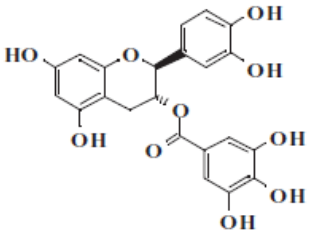
In this study, we also aimed to approach comprehensively the different ratios of polyphenol to protein how effects on food quality.

1.3.1. Interaction of Polyphenols with Whey Proteins

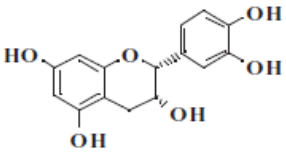
The major whey protein, in milk, consists of β-Lg and α-La. A minor part of the proteins come from the blood: BSA, Immunoglobulins (Ig), and enzymes as Lactoferrin (Lf), Lactoperoxidase (Lp) and Xanthine Oxidase (Xo) .The whey proteins are present in the portion of 9.8: 3.7: 12 for β-Lg, α-LA and BSA [25]. β-Lg is present as two homodimers, (A) and (B).

There are some studies about the interaction of whey proteins with polyphenols. One of the reasons for this is the application of proteins in coffee, tea and other phenol containing plant beverages. When the binding of tea polyphenols (shown in Figure 1.1.) ((+)-catechin (C); (-)-epicatechin (EC); (-)-Epicatechin gallate (ECG); (-)-epigallocatechin gallate (EGCG)) to β-LG were studied, binding occured through hydrophobic and hydrophilic interactions and this binding increased β-sheet and α-helical structure that all has been demonstrated by Kanakis et al. [26]. Treatments like heating, may affect the structure of β-Lg via unfolding the protein, leading to more non-polar and inner part of the proteins exposes. An another study showed that addition of EGCG to β-LG hetero and homodimers lead to differences in reactivity causing oligomerization and aggregate formation in the order of β-LG A > B > AB [27]. Rawel et al. [11] also reported that when whey proteins react with the plant phenolic substances (ferulic-,chlorogenic-, caffeic-, gallic,- quinic acids, and p-quinone), this binding influenced physicochemical properties of proteins by means of changing in hydrophilic/hydrophobic character, solubility, and isoelectric points of whey proteins. However, the characterization of the non-covalent interactions between the 5-o-caffeoylquinic acid (chlorogenic acid, 5-CQA) and BSA, lysozyme,

and α-La indicated that non-covalent binding had no effects on the functional properties of these proteins in the food system [28].



ECG



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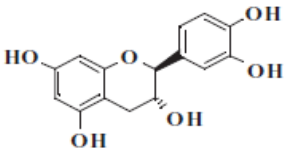
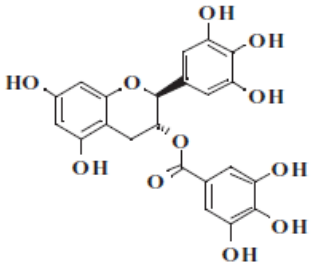


EC



EGCG

Figure 1.1. Structures of some tea phenolics (adapted from [29]).

1.3.2. Interactions of Polyphenols with Caseins

Milk proteins consist of 80% caseins (consisting of α_{s1}-casein (α_{s1}-CN)), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN), κ-casein (κ-CN)). The casein are compenents of the casein micelles which are essentially spherically shaped micelles associated with calcium phosphate nanoparticles [30]. The caseins are present in the proportion 31: 8: 28: 10 for α_{s1}-CN, α_{s2}-CN, β-CN and κ–CN [31].

Caseins have large proportions of proline residues, especially in α- and β- CNs, and hydrophobic AAs, which have been reported to interact with polyphenol hydroxyl residues and phenolic ring structures [32]. The binding affinity of polyphenols to proline rich proteins has been reported to increase with the molecular weight and the number of hydrophilic hydroxyl groups [33, 34]. When the binding of tea polyphenols ((+)-catechin (C); (-)-epicatechin (EC); (-)-Epicatechin gallate (ECG); (-)-epigallocatechin gallate (EGCG)) to α-CN and β-CN were studied, binding occured through hydrophobic and hydrophilic interactions. In this study, casein conformation altered in the presence of catechins with reducing of β-sheet and α-helix and increasing of random coil turn structure [4]. Caseins are proteins which have a very

disordered open structure and it can be somewhat restored by calcium binding. Calcium sensitive caseins are α_{s2}, α_{s1} and β-CN, but κ-CN is nonsensitive to calcium. Thus, although all caseins may bind EGCG, κ-CN is likely to bind most strongly, based on its grater disorder [35]. Additionally, the casein-polyphenol complexation leads to the changes of the antioxidant activity of polyphenols in solutions [4].

The previous studies including in vitro digestion and model system, have already shown that the interaction of milk proteins and polyphenols reduces the number of hydroxyl groups in polyphenols, resulting to a lower antioxidant capacity in the solution [4, 26, 36-39]. But, there are also the inconsistent results in the literature about changes in antioxidant capacity of polyphenols after addition of milk to tea. For instance, milk has no significant effect on antioxidant capacities of tea polyphenols according to the results of Leenen et al. [40], whereas both Kilmartin and Hsu [41] and Dubeau and Samson [39] reported that the free radical scavenging capacity of tea polyphenols is adversely affected by adding milk.

1.3.3. Role of the Milk Proteins-Polyphenols Interactions on Changes in Gastroinetstinal Tract

Essential amino acid contents and digestibility in gastrointestinal tract (GIT) are two major parameters for protein source quality in the view of nutrition [42]. Maintaining protein conformation and disulfide bonds play a major role on the stability of proteins during digestion. Due to the changes in protein conformation, such as denaturation of protein by shifting the position of disulfide bonds in the three dimensional structure of the protein, the chemical reactivity of disulfide bonds may change [43].

Each of the whey proteins, including β-Lg, α-La, BSA, and Ig are major milk proteins rich in disulfide bonds [44]. Several studies have shown that β-Lg, the main fraction of whey proteins, is quite resistant to proteolysis at low pH (pH<3). Because its stable globular tertiary structure, that is highly hydrophobic β-barrel, results in diffucult accesibility of target peptide bonds for enzymes [45, 46]. But, the tertiary structure of native β-Lg could be altered by certain treatments, such as high pressure, emulsification, and foaming, via unfolding partially or completely. This alteration could improve the digestibility of native β-Lg as more susceptible peptide bonds expose for enzyme hydrolysis [47-49]. In addition, heating is also one of the

susceptible treatments, resulting in increasing accesibility of specific peptide bonds for digestive enzymes, especially pepsin, as a result of conformational changes in protein structure [50]. On the other hand, β-Lg is hdyrolysable by both tyripsin and chymotryripsin [51].

The other main classes of milk proteins: caseins, especially β-CN, are well digested under the same conditions. This feature has been related to their poor secondary structure [44]. The results of Benede et al. [52] have supported that the early stages of gastric digestion occured quickly when β-CN cleavage by pepsin, whereas oral digestion had no effect on β-CN proteolysis. In addition to this, they demonstrated that the rate of casein degradation with human gastric fluids was significantly higher compared to commercial enzymes.

The previous *in vitro* studies have reported that the complexes formation of green tea polyphenol-milk protein (EGCG- β-Lg) in the stomach (acidic pH), resulted in conformational changes to the protein. They have also suggested that the rate and extent of pepsin hydrolysis significantly decreased during gastric phase as a result of the alteration which stabilize the protein due to increase in β-sheet and α-helix structure of the protein [26, 36, 53-55]. Van der Burg-koorevaar et al. [56] found the consistent results with other researchers in his study of monitoring protein digestion of black tea with or without milk during pepsin digestion well. However, the large increase in the pH after stomach causes the casein dissociation, leading to favorable structure for the pancreatic enzymes (trypsin and chymotrypsin),while green tea do not have remarkable effect on delaying hydrolysis of milk protein at the intestinal phase [36, 53]. In κ-CN, it has been speculated that residued 98-111 (HPHPHLSFMAIPPK) around the chmyosin cleavege site may be involved in the binding of EGCG and when both casein micelles and sodium caseinate were cleaved by chmoycin in the presence of EGCG, a slower release of caseinoglycomacropepetide resulted, suggestive of interaction of this polyphenol around the chymosin cleavege site between Phe 105 and Met 106 [57]. Nevertheless, He et al. [58] demonstrated that addition of chlorogenic acid and catechins to the whey protein reduced significanty in the number of free amino groups in the whey protein and delayed the intestinal digestion of protein, which was attributed to the strong affinity of polyphenols to the protein at neutral pH. Some

authors also suggested that the delay digestion of protein might be due to the interaction between the polyphenol and enzyme that caused to changing in enzyme molecular configuration and the loss of catalytic activity. Polyphenols such as epigallocatechin gallate, epicatechingallate, epigallocatechin, and gallic acid are some of the ones that causes the enzyme (pepsin, trypsin, and α-chymotrypsin) inhibition [53, 59]. In the contrary, the improvement in enzyme activity of pepsin by polyphenolic compounds including epigallocatechin gallate, resveratrol, and quercetin, in simulated digestion model was shown [60, 61].

If dietary polyphenols which have a pronounced effect on protecting the gasrointestinal tract from oxidative damage, interact with milk proteins via increasing binding affinity, this renders decrasing the total andioxidant activity of polyphenols [36, 37]. Likewise, the polyphenols, as alone, exhibit the lower antioxidant activity due to their degradation in the intestinal environment. But, the simultaneous consumption of green tea and dairy products (cheese and milk) provided maintaining the stabilities and antioxidant activities of polyphenols during digestion in a simulated gastro intestinal environment has been indicated by [53].

This contradictory literature results presented that protein-polyphenol interactions need to further research with supported by in-vivo studies in order to broadly elucidate changes in both proteins and polyphenols during digestion.

1.4. Fermented Milks-Yogurt

Yogurt is very popular fermented dairy product that has widely consumed all over the world [62]. The rapid market growth of yogurt over the past few decades is particularly related to its healthy food image, as a means of probiotic effects, such as protection against gastrointestinal upsets, enhanced digestion of lactose, decreased risk of cancer, lower blood cholesterol, improved immune response, enhanced short chain fatty acids (SCFAs) production assimilation of protein and calcium [63]. Yogurt is also rich in protein, fat, calcium, potassium, B vitamins (B1, B2, B6, nicotinic and pantothenic acids) but is deficient in iron, vitamin C, carotenes and dietary fibers [63].

Different processing steps are needed to produce industrial yogurt according to set and strried types as shown in Figure 1.2. [64]. From past to present, the effect of each steps on yoghurt properties including macroscopic or microscopic aspects has

been important research areas for dairy technology [65]. The gel formation is one of the critical steps while producing yogurt in order to maintain the stability of structure through storage and also to be accepted by consumers. Casein plays the most important role in the formation of gel matrix via aggregation of casein micelles as the pH approaches 4.6 as a result of lactic acid production during fermentation [66, 67].

As shown in Figure 1.2., the first and also one of the essential step of yogurt production is milk standardization in order to obtain a standard product, such as yogurts which contain 1.5 g / 100 g (medium fat yogurt) or 0.5 g / 100 g (low fat yogurt) [68]. Following homogenization treatment (temperature & pressure), chemical and physical changes occur in the milk fat globules. There have been various studies, which reviewed these alteration affecting the quality of dairy products [69-71]. In general, the size of fat globules decreases in the sub-micro range and even if serum proteins is not remarkably affected, the interaction between the micro sized fat globule and some of the casein micelle occurs after disintegrated of casein particles as a result of homogenization [68].

Schmidt and Bledsoe [72] demonstrated that the yogurts produced by using homogenized milk at 0, 10.3, and 34.5 mPa showed different syneresis behavior and water holding capacity. Besides, it was proposed that the increase in homogenization pressure led to an increase in the rate of acidification of the milk during fermentation. It is an important point that the desirable effects of homogenization on yogurt can be achieved by not only the correct level of fat content in the milk but also by the appropriate temperature and pressure conditions [68].

Moreover, depending on required texture and viscosity, adding of whey protein or skim milk powder or concentrating the milk are commonly used to obtain 100 and 200 g/kg of non-fat solid content in the milk for yogurt production [73]. After homogenization, milk heated to 80 °C for several minutes and this results in whey protein denaturation and interaction between caseins (κ-CN and α_{s2}-CN) and whey proteins, especially β-Lg, via covalent and non-covalent bonds [74, 75]. However, only minor changes are observed in casein micelles, leading to binding β-Lg to surface of normal intact micelles, when the heat treatment applies at 110 °C [73].

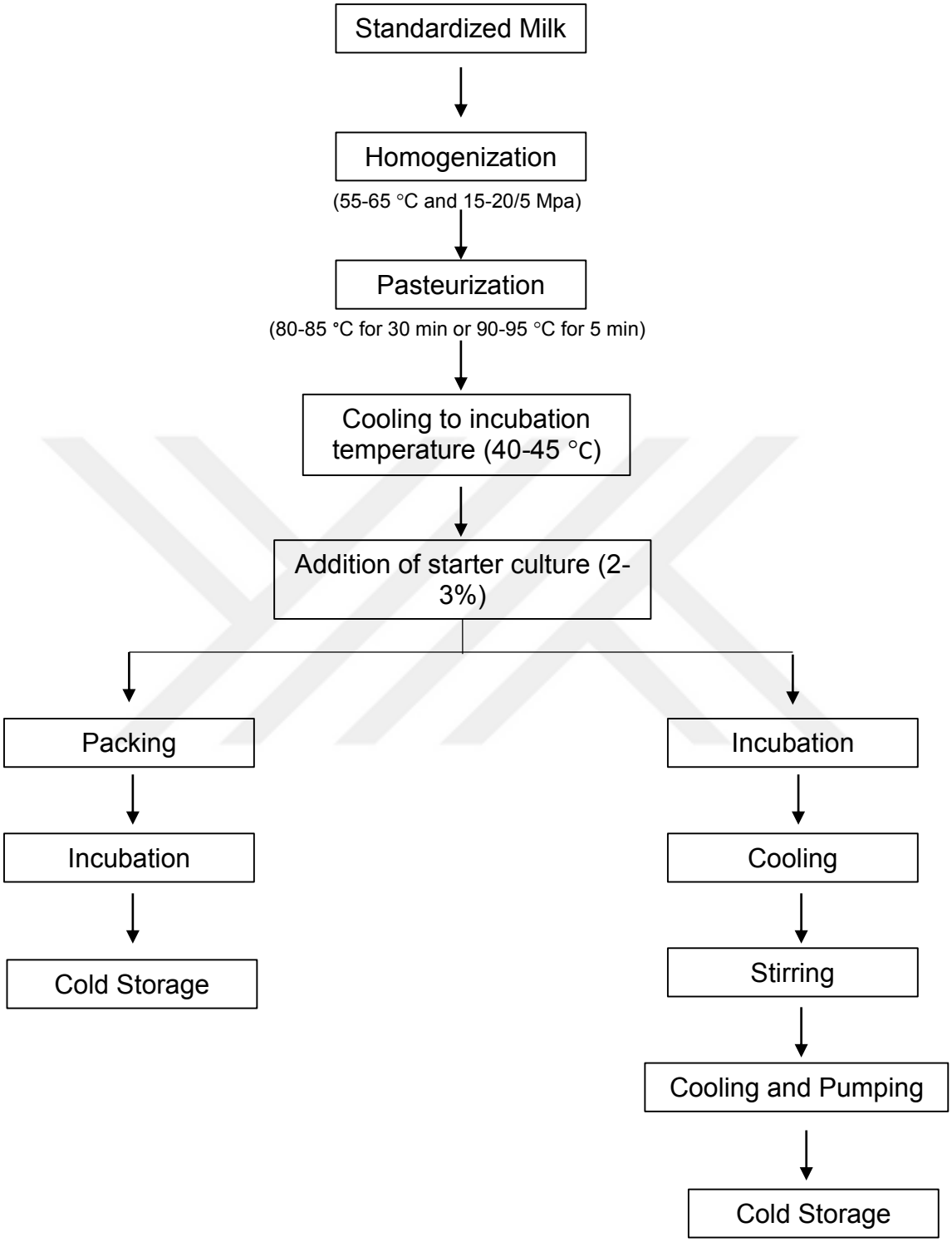


Figure 1.2. The scheme of main processing steps for yogurt production in the manufacture of set and strirred type (adapted from [64]).

The heat treatment is also necessary to destroy potential competition for starter bacteria mainly as *Lactobacillus delbrueckiie sups bulgaricus* and *Streptococcus thermophilus* [64]. The milk is cooled to the inoculation temperature, around 40-45 °C, in order to growth of the starter culture which must remain viable in the product, followed by heat [64]. Tamime and Robinson [68] also indicated that they should be present approximately equal numbers in the milk in order to obtain a satisfactory flavor, which are obtained by acetic acid, diacetyl, acetaldehyde.

While lactose is being degraded to lactic acid by starter bacteria, the pH of the milk reduces from pH 6.7 to pH 4.6. Meanwhile, some chemical changes occur in the milk at certain pH intervals, which led to formation of the yogurt three dimensional network.

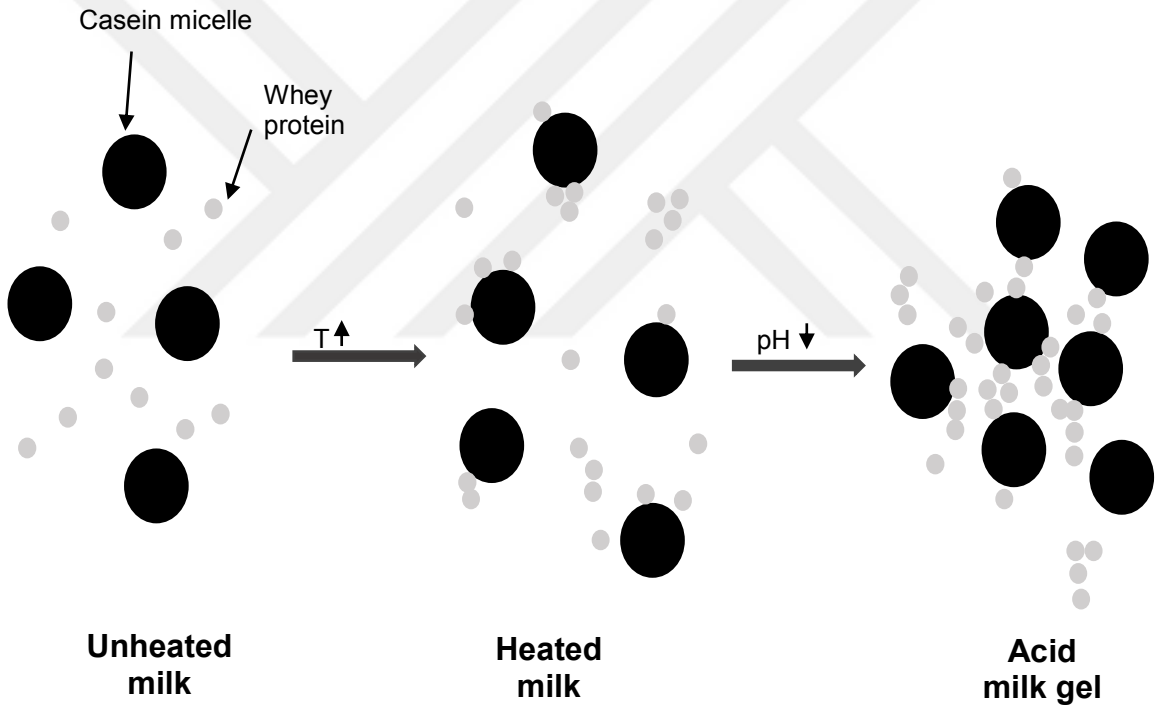


Figure 1.3. Schematic representation of the formation of protein network which occurs in the acidification phase after heating the milk. The large particles represent the casein micelle, the small particles represent the whey protein (adapted from [76]).

For instance, while the pH of the milk decreases from pH 6.6 to pH 5.0, the net negative charge of casein micelles starts to decrease and then their internal structure disrupts due to increase in solubilization of CCP. All result in a decrease in electrostatic repulsion and steric stability of casein micelles in the milk [64].

When the pH of the milk approaches to the pH 4.6, the isoelectric point of casein, the aggregation of casein micelles occurs that leads to the formation of yogurt gel network [77]. Furthermore, the product is stored at 4 °C following by incubation in order to complete the formation of the three dimensional gel network which involve in clusters and chains of caseins [64].

As a consequence, the dissociation of caseins from the micelles for the gel formation is both temperature and pH dependent as shown in Figure 1.3.

1.4.1. One of the Quality Parameters of Yogurt Texture 'Syneresis'

Syneresis, water releases from the yogurt gel network, is a major defect in yoghurt production that could limit the shelf life and acceptability because of undesirable appearances [63]. Therefore, enhancing of physical properties of set type yoghurts during storage by means of either improving the gel strength or reducing the syneresis are desired in dairy industry. Many studies have been performed on evaluating the textural quality of yoghurts, including increment of the total solid contents, changing heat treatment and homogenization conditions, usage of different type or quantity of starter culture, and enzymatic cross-linking by transglutaminase etc. [68, 78-82].

Tamime and Robinson [83] revealed that importance of proteins on the formation of network structures in fermented milk products, mainly yoghurt. By increasing protein content, an improvement on the firmness and the resistance of yogurt gels against syneresis were reported by Schkoda et al. [84]. In order to enrich the protein content, skim milk powder is commonly used. However, if the addition of this supplement is higher than 3-4%, the recommended range, it may lead to a 'powdery' taste in the yogurt [81].

As mentioned above, changes in process conditions, such as heat treatment or homogenization, is another way to decrease the syneresis. Although heating to milk at 85^{o}C for 30 min or 95^{o}C for 5 min is a critical time-temperature point in yogurt production to obtain sufficient firmness as well as minimal syneresis, high heat treatment results in high levels of whey separation and also a weak-bodied yoghurt [85]. Serra et al. [80] investigated the effect of ultra-high pressure homogenization (UHPH, 200 and 300 mPa) on the quality of yogurt compared to producing yogurt via conventional homogenization conditions. Their results showed that yoghurts

made from UHPH treated milk existed higher firmness values and water holding capacity than conventional yogurts.

Water holding capacity is explained as a protein network potential for retaining water in the structure of yoghurt, but casein micelles may break yielding casein rearrangements and increment in the syneresis and decrement in the water holding capacity during the storage [86]. One of the reasons is the reduction of pH during the storage, which have constriction effect on the casein micelle network resulting in more serum release [87]. An increment in density of the protein matrix in the microstructure due to increase in total solid contents causes higher water holding capacity, which then could reduce the syneresis [88]. Similar observations have been promoted by Shaker et al. [89].

Addition of hydrocolloids have shown to increase water holding capacity and yogurt viscosity because of their functionality to react with the milk constituents, bind water and stabilize the protein network [68]. Earlier studies have also reported that using of exopolisaccarides producing starter culture and using of transglutaminase enzyme strengthened the gel network and suppressed the syneresis due to the increased water binding capacity in set yogurt [82, 90, 91]. Transglutaminase effect have been explained by means of more evenly distributing proteins in gel network due to the formation of cross links between proteins [82].

In conclusion, as producing acid gels without added stabilizers that do not show syneresis during storage has a growing interest [85], so modifing of yoghurt texture via innovative approaches needs further research in view of both industry and consumer acceptance.

1.4.2. Rheological Characteristics of Yogurt

Although yogurt is a widely consumed dairy product owing to its positive health effects, it has limited shelf life of about 20 days under refrigeration [18]. Rheology builds a bridge between structural microscopic aspects and continuous macroscopic parameters [92]. Knowledge of the microstructure and the rheological properties of set yogurts are considerably critical to the design and operation of the processing equipment used in industry, product development, enhancing of quality of the material and shelf life [65, 75, 93-95].

A food colloid, such as yogurt, is an example of particle gels, and the casein plays an important role in the formation of gel matrix via aggregation of casein micelles as the pH approaches 4.6 as a result of lactic acid production during fermentation [66, 67].

The rheological properties of acid casein gels have been studied extensively by [96]. Yogurt, is an example of thixotropic material, belong to non-Newtonian fluids and its rheological properties can be characterized using both the viscous and elastic components. [93, 97]. Viscoelastic indicates that the material has some of the elastic properties of an ideal solid and some of the flow properties of an ideal (viscous) liquid [64]. Two common models, Power Law and Herschel-Bulkley, are used to represent the flow characteristics of yogurt, given in Eq. 1.1 and Eq. 1.2, respectively [98, 99].

$$(\tau = K\gamma^n) \qquad \text{Eq 1.1.}$$

$$(\tau = \tau^o + K\gamma^n) \qquad \text{Eq 1.2.}$$

where τ is the shear stress of the material, τ^o is the apparent yield stress, *K* is the consistency coefficient, γ is the shear rate, and *n* is the flow behavior index of the material. It is clear that the power law is a particular case of the Herschel-Bulkley model when the yield stress is zero. Barnes and Walters [100] argued that the yield stress is only introduced as a consequence of not being able to measure what happens for low values of shear rate. The set yogurt system is a dispersion system comprising of many particles and, it causes to form a yield stress, which is defined as the required initial force to initiate the yoghurt to flow [67]. Factors that contribute the yield properties of gels include the strength of protein-protein bonds, the number of bonds per cross-section of the strand, relaxation times for the network bonds, and the orientation of strands in the matrix [65, 97]. Furthermore, yogurt exhibits time-dependency and shear thinning behavior i.e. the viscosity decreases with a shear rate increase [93].

One of the most important attributes for yogurt quality is texture. The composition of processed milk, especially dry matter and protein content, fat content, homogenization conditions, the type and amount of starter culture, incubation temperature, cooling conditions, usage level and type of stabilizers, storage time

and handling of product post manufacture i.e. physical and temperature abuse are the main processing parameters that influence the yogurt texture [65, 101, 102].

Fiszman et al. [103] showed that addition of milk solids, is one way of enhancing the texture of some milk products, which increase firmness and prevent syneresis. However, some people believed that these additives affect the taste, aroma and mouthfeel of the true yoghurt in the negative way [104]. On the other hand, flavorings or fruit concentrates, as additives, are commonly used with stabilizing agent such as starch or pectin not to tend to reduce consistency of the product [105].

As a consequence, it is crucial to know relationship between the shear rate and the shear stress as it assist to determine the facility of production itself and the quality of the final product.

2. SYNERESIS AND RHEOLOGICAL BEHAVIORS OF SET YOGURTS COMPRISING GREEN TEA AND GREEN COFFEE POWDERS

2.1. Introduction

The microstructure and the rheological properties of set yoghurts are considerably critical to product quality and shelf life [75]. Syneresis, serum release from the gel matrix, is regarded as a technological defect of set yogurts.

Polyphenols, secondary plant metabolites have ability to interact with proteins resulting in the formation of protein-polyphenol complex [1-3]. Even when compared to other polyphenol-rich, plant-based foods, green tea and green coffee contain high levels of polyphenols. Such that, chlorogenic acid represents 4.1-11.3 g/100 g of the green coffee seeds, where catechins constitute 30-42% of green tea extract solids [106, 107]. Within this regard, this study aimed to investigate the effect of protein-polyphenol interaction on the syneresis of set type yoghurts by using green coffee and green tea powders. The effects of different amounts of green tea or green coffee powders were determined on syneresis rate and consistency of set yoghurts using centrifugal acceleration test and rheological measurement, respectively, during 3 weeks of storage at 4^{o}C.

The text of this chapter is under in press as a research article as follows:

Dönmez Ö., Mogol B. A., Gökmen V., Syneresis and rheological behaviors of set yogurt containing green tea and green coffee powders, *Journal of Dairy Science*, http://dx.doi.org/10.3168/jds.**2016**-11262.

2.2. Materials and Methods

2.2.1. Chemicals and Consumables

Pasteurized (85°C and 5 min) and homogenized milk (3% protein, 3% fat), green coffee beans (*Coffea canephora* var. *robusta*), and green tea leaves were supplied from a local market in Turkey. A freeze-dried lactic acid culture YF-L812, containing a mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, was obtained from CHR Hansen (Victoria, Australia).

Sodium carbonate, sodium hydroxide, potassium sulfate, boric acid, hydrochloric acid (37%), and sulfuric acid (95%) were purchased from Merck (Darmstadt,

Germany). Ethanol (96%) and Folin-Ciocalteu Reagent (2 *N*) were obtained from Sigma-Aldrich (Steinheim, Germany). Cupric sulfate pentahydrate was purchased from Fluka Chemie AG (Buchs, Switzerland). Gallic acid (98%) was from Acros (Geel, Belgium).

2.2.2. Preparation of Green Tea and Green Coffee Powders as a Yogurt Ingredients

Green tea brew was prepared by the extraction of coarsely ground green tea leaves. Thirty grams of green tea was extracted into 1 L of boiling water by keeping it at 90°C in a water bath for 30 min. Green tea leaves were removed by using a filter paper (Macherey-Nagel 751/60). Then, the green tea extract was immediately lyophilized to obtain green tea powder (GTP). The freeze-drying was performed for 48 h (Christ Alpha 1–2 LD+, Osterode, Germany) operated at 0.1 Pa and ice condenser temperature of 76°C.

Six grams of finely ground green coffee was weighted into an espresso cap and the first 25 mL of extract was collected from the espresso machine (Ecov 311. BK Icona Vintage, DeLonghi, Treviso, Italy). The same procedure was repeated until enough extract was obtained. The combined green coffee extract was lyophilized to obtain green coffee powder (GCP). Both GCP and GTP were stored at −18°C until the yogurts would be prepared.

2.2.3. Preparation of Yogurts Comprising GCP and GTP

Pasteurized milk was heated to 42°C and then rapidly inoculated with direct vat set starter culture (3 g/100 mL). As soon as the inoculation was performed, GCP (0, 1, and 2%) or GTP (0, 0.01, 0.02, 1, and 2%) was immediately added to the milk. The samples were incubated at 42°C until the pH reached to 4.6 in 3 h and then at 4°C for 18 h. The GCP-added yogurt (GCP yogurt) and GTP-added yogurt (GTP yogurt) samples were stored at 4°C for 21 d and the analyses were performed on d 1, 7, 14, and 21 of storage. All yogurts were prepared in duplicate with one lot of milk.

2.2.4. Methods

2.2.4.1. Total Phenolic Content Analyses

The total phenolic content of the GTP and GCP was determined according to the Folin-Ciocalteu colorimetric method [108]. One hundred milligrams of powders was mixed with 10 mLof ethanol-water (50:50, vol/vol) in a test tube. Then, the samples

were vortexed for 1 min and centrifuged at 5,500 x *g* for 3 min at room temperature. Then, 0.2 mL of the supernatant was transferred to another test tube and mixed with 0.8 mL of 0.2 *N* Folin-Ciocalteu reagent and 0.8 mL of 20% aqueous Na_2CO_3, consecutively. The reaction mixture was subsequently incubated at 25°C for 2 h. Then, the absorbance of the samples was measured at 765 nm using a Shimadzu model 2100 variable wavelength UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). Standard calibration curve was prepared by using gallic acid between the ranges of 0 to 100 mg/L. Three independent measurements were performed and the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of sample.

2.2.4.2. Determination of Syneresis Rate for Yogurt Samples

The syneresis rates of yogurts were determined by a centrifugal acceleration test. Five grams of yogurt sample was placed in a test tube and centrifuged at 1,200 *g* for 0, 3, 6, 9, 12, and 15 min at room temperature. At each time interval, the volume of the serum separated from the samples was measured to estimate the initial rate of syneresis, which was expressed as milliliters of serum released per gram of sample per unit of time. The average of the 5 times (except 0) tested was reported to evaluate the syneresis rate for that day.

2.2.4.3. Analyses of the Rheolocigal Properties for Yogurt Samples

Rheological property of yoghurts was specified with a Brookfield RVDV-II+P **(**Middleboro, MA) under controlled temperature by using Cone CP-40. The yogurt samples were gently stirred 30 times for homogenization. Then, 0.5 g of yogurt sample was placed into the cap and the temperature of the water bath is set to 20°C to prevent the temperature fluctuations during measurements. The samples were exposed to high shear rate (500 s^{-1}) for 60 s to obtain a better homogenization and make same starting conditions for all yoghurts. Following this procedure, samples were maintained for 300 s without shear rate to rebuild structure of yoghurt samples as described by Purvandari et al. [109]. The flow curve was generated by measuring the shear stress as a function of shear rates from 0.1 to 400 s^{-1} by using DVLoader software. The Herschel-Bulkley model, is given Eq. 2.1, where τ is the shear stress of the material, τ^o is the apparent yield stress, *K* is the consistency coefficient, γ is the shear rate, and *n* is the flow behavior index of the material, was fitted to

measurement results. The consistency coefficients of GCP- and GTP-Yogurts were normalized to use the control as base of 100 Pa.s.

$$(\tau = \tau^o + K\gamma^n) \quad \text{Eq 2.1.}$$

2.2.4.4. The pH and Color Measurement for Yogurt Samples

The pH values (PHM210 MeterLab, France) and the color information in CIE L*a*b* space (Minolta colorimeter CM3600d, Tokyo, Japan) were monitored to evaluate quality characteristics of yoghurts during cold storage. The pH and color measurement were performed at room temperature. The color values of the control sample were taken as the reference to calculate color differences (ΔE) of GCP- and GTP-Yogurts, by using the equation given in Eq. 2.2. In CIE L*a*b* space, L* represents luminance or lightness. The a* and b*, chromatic components, represent colors from green to red, and blue to yellow, respectively.

$$\Delta E = \sqrt{(L_1 - L_2)^2 + (a_1 - a_2)^2 + (b_1 - b_2)^2} \quad \text{Eq 2.2.}$$

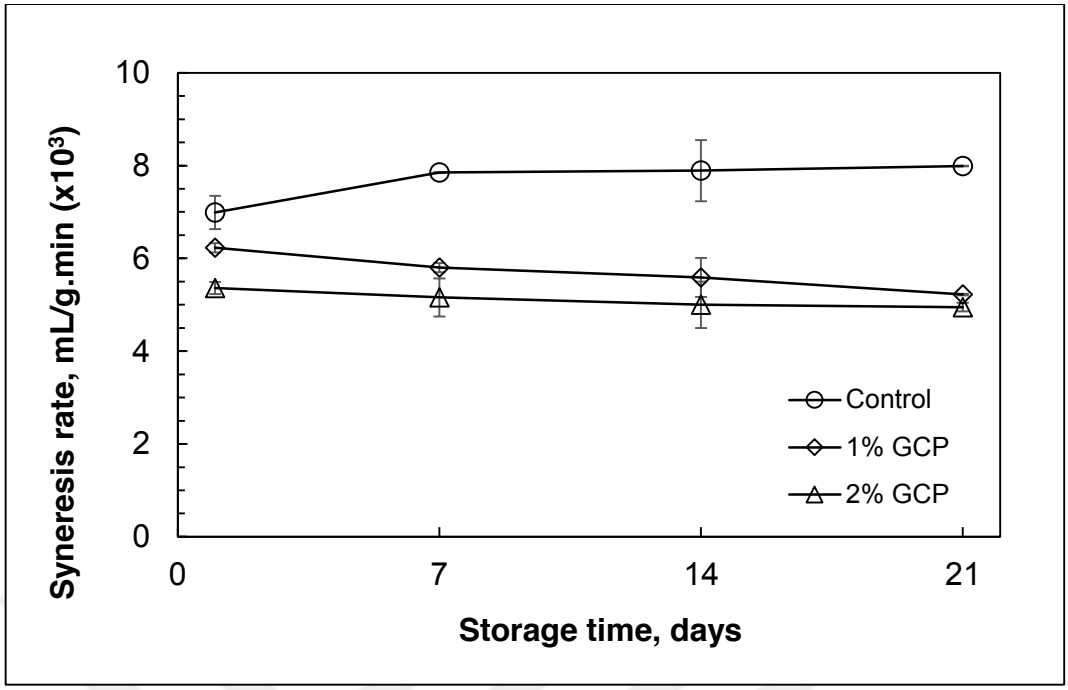
2.2.5. Statistical Analyses

After preliminary experiment, those concentrations were chosen due to the sensorial acceptance. The Tukey post-hoc test was employed to determine the significance of the difference within treatments for each analysis. A total of 2 replicates were performed and the mean values were calculated. Values were considered significantly different when $P < 0.05$. The results were reported as mean ± standard deviation. All statistical analyses were performed by using the SPSS 13.0 statistical program package (SPSS Inc., Chicago, IL).

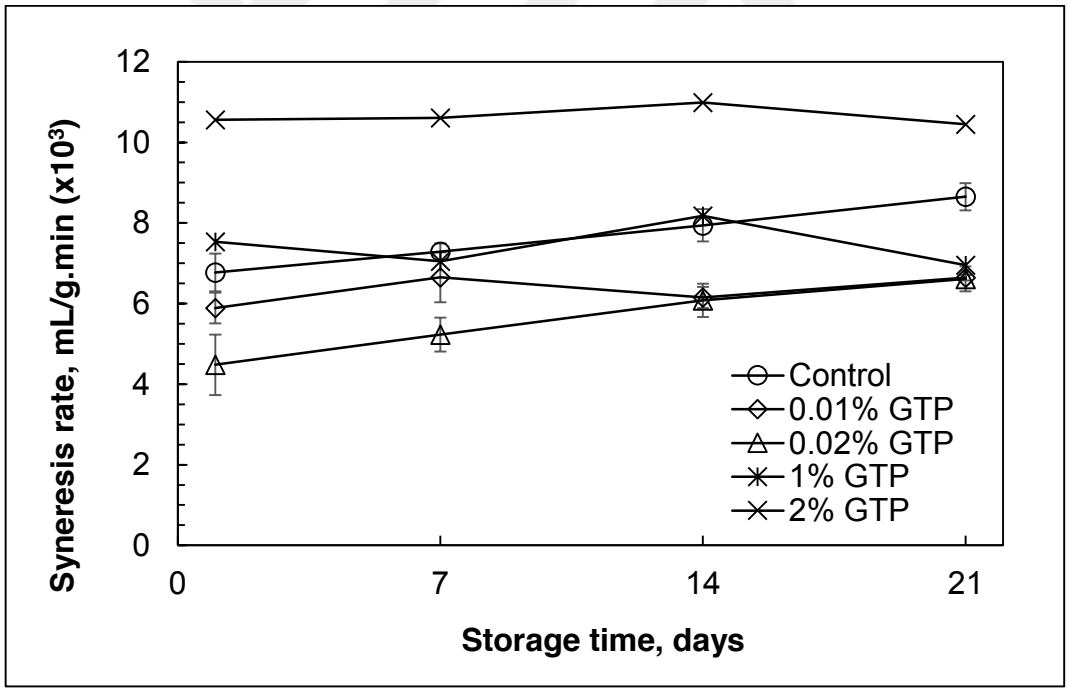
2.3. Results and Discussion

2.3.1. Syneresis Rate

Serum release, known as syneresis, is considered as one of the most important parameters indicating the quality of yogurt during storage. Figure 2.1 shows the changes in the syneresis rates of yogurts added with different amounts of GCP and GTP, respectively. Here, the syneresis rate was expressed as milliliters of serum phase released per gram of sample per unit of time.



(A)



(B)

Figure 2.1. Changes in syneresis rate for yogurts added with (A) GCP, (B) GTP during cold storage.

Addition of GCP was found to decrease the syneresis rate of yogurts compared with control during storage (Figure 2.1.A). The decrease in the syneresis rate was

proportional to the increase in coffee concentration, so that the serum separation was significantly restricted when 2% GCP was added ($P < 0.05$). In the case of GTP yogurts, adding 2% GTP concentration resulted in increase in the syneresis rate, unlike GCP yogurts (Figure 2.B). Şengül et al. [110] also reported that increased concentrations of sour cherry as the polyphenol source in yogurts led to an increase in the serum separation. The syneresis rate was found to be significantly lower for 0.02% GTP yogurts compared with control ($P < 0.05$), whereas it was higher for 2% GTP yogurts. Moreover, storage time showed no statistically significant effect on the syneresis rate of the GCP and GTP yogurt samples ($P > 0.05$).

Considering the addition of increased amounts of GTP and GCP, which means increased total phenolic content, one would expect to retention of more serum phase in the yogurt structure. However, having different total phenolic content of GCP (35 mg GAE/g sample) and GTP (61 mg GAE/g sample) added yogurts showed different behaviors depending on their concentrations, which could be explained by the protein-polyphenol interaction model proposed by Siebert et al. [5] (Figure 2.2). According to this model, our goal was to fix the number of polyphenol binding sites of protein molecules and the number of polyphenols, which leads to create new cages that could have a role in limiting the serum release from the gel network (Figure 2.3.B). The interaction of polyphenols with proteins in 2% GCP yogurts was found to be well enough to strengthen the gel structure of yogurt, which led to decreased syneresis rate. On the other hand, the reason of the increased syneresis rates, observed in 2% GTP yogurts, could be explained with the model shown in Figure 2.3.C. Excess green-tea polyphenol concentrations increased the number of cages, but decreased the volume of the individual cage, leading to a reduction of the time of serum trap in the gel matrix. The syneresis of GTP yogurts at lower concentrations (0.02 and 0.01%) was similar to the 1 and 2% GCP yogurts. This is the result of different amount and profile of polyphenols present in GTP and GCP.

When increasing the number of particle-particle junctions in the gel structure, the network then tends to shrink, thereby dismissing interstitial liquid [111]. Undoubtedly, the tendency to exhibit syneresis also depends on the changing in pH, which affects the gel structure, which is a casein micelle network containing heat-denaturated whey proteins bound to the surface of the casein micelles [112].

Continuing to grow the lactic acid bacteria and also to produce lactic acid through the storage is responsible for the reduction in the pH [101]. The change in pH values of yogurts during storage is given in Table 2.1. The pH values of yogurts differed between 4.58 and 4.89.

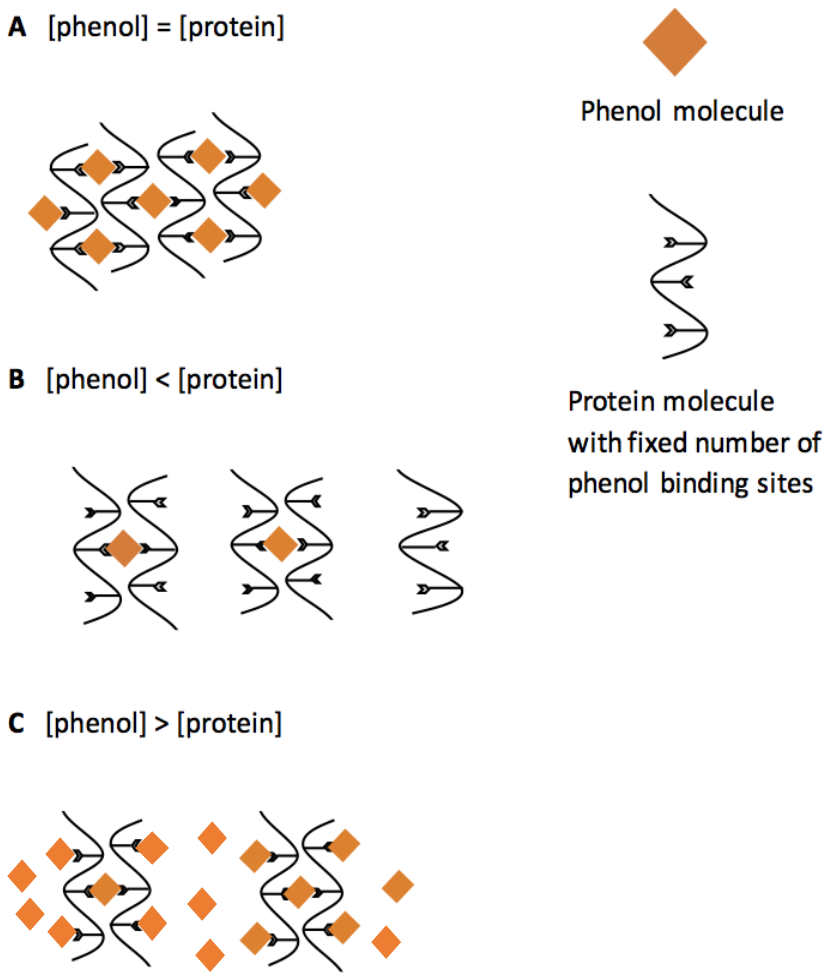


Figure 2.2. Proposed model to explain the gel structure stability of yoghurts for different ratios of [protein] and [polyphenol] (adapted from [5]).

The results showed no statistically significant difference between control and GCP yogurts during first 7 d of storage ($P > 0.05$). However, the pH value of control was different from GTP and GCP yogurts after d 7 and 14, respectively ($P < 0.05$). Compared with control yogurts, 1% and 2% GCP yogurts showed significantly higher pH values between 14 and 21 d.

The results of syneresis rate in this time period could also be affected by pH in a similar manner. This phenomenon might be due to rearrangement of the forces keeping the structural elements of a micelle together. Acidification causes several changes such as dissolving the calcium and inorganic phosphate gradually and decreasing the net negative electric charge of the casein micelles, including that of the hairy layer. As the layer collapses, the casein itself becomes insoluble near its isoelectric pH (about 4.6). Altogether, this results in aggregation. Even a small decrease of the pH leads to a decreased charge, which weakens colloidal stability [111].

Table 2.1. Changes in pH of yogurt samples during cold storage

	pH			
	Day 1	Day 7	Day 14	Day 21
Control	$4.66\pm0.06^{a,A}$	$4.66\pm0.01^{a,A}$	$4.66\pm0.01^{a,A}$	$4.65\pm0.00^{a,A}$
1% GCP	$4.71\pm0.00^{a,A}$	$4.71\pm0.03^{a,A}$	$4.71\pm0.01^{a,B}$	$4.74\pm0.04^{a,B}$
2% GCP	$4.69\pm0.00^{a,A}$	$4.72\pm0.04^{a,b,A}$	$4.75\pm0.01^{a,b,C}$	$4.76\pm0.00^{b,B}$
Control	$4.89\pm0.01^{b,B}$	$4.67\pm0.03^{a,A}$	$4.58\pm0.02^{a,A}$	$4.59\pm0.01^{a,A}$
0.01% GTP	$4.88\pm0.04^{b,B}$	$4.71\pm0.04^{a,A,B}$	$4.81\pm0.00^{b,C}$	$4.80\pm0.03^{b,B}$
0.02% GTP	$4.79\pm0.01^{a,A}$	$4.72\pm0.04^{a,A,B}$	$4.72\pm0.01^{a,B}$	$4.75\pm0.04^{a,B}$
1% GTP	$4.82\pm0.04^{b,A,B}$	$4.80\pm0.01^{a,b,C}$	$4.74\pm0.01^{a,B}$	$4.73\pm0.03^{a,B}$
2% GTP	$4.79\pm0.04^{a,A}$	$4.76\pm0.03^{a,B,C}$	$4.73\pm0.01^{a,B}$	$4.71\pm0.06^{a,B}$

Superscript lower letters in each row indicate statistically significant difference ($p<0.05$) during storage. Superscript upper letters indicate statistically significant difference ($p<0.05$) between yoghurt samples in each column within the type of the product. Data are expressed as mean ± standard deviation. GTP: green tea powder, GCP: green coffee powder.

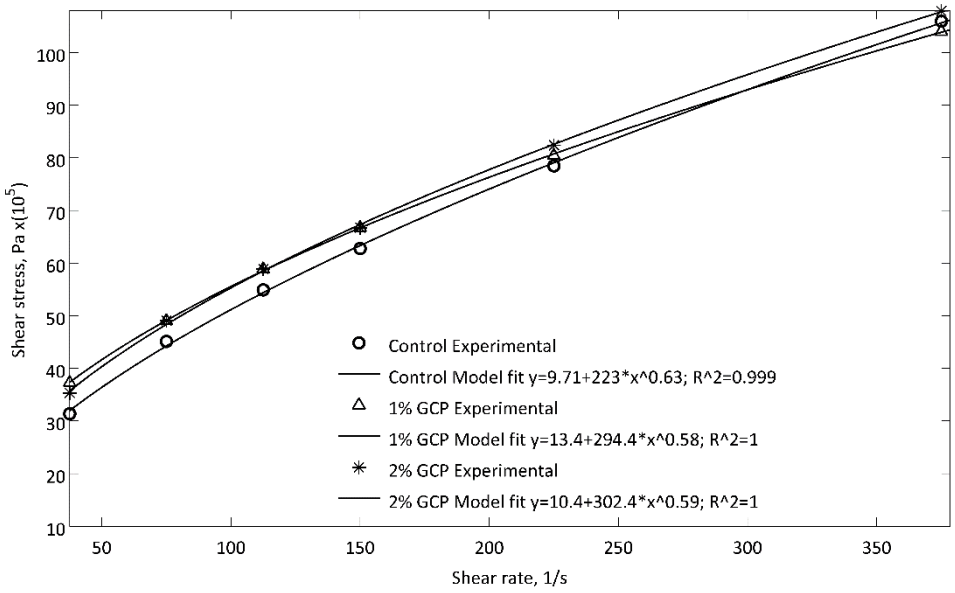
It could enhance serum releasing from the gel matrix. Moreover, Lucey [113] explained that postacidification is one of the factors that can increase the production of whey in yogurts.

2.3.2. Rheological Behavior

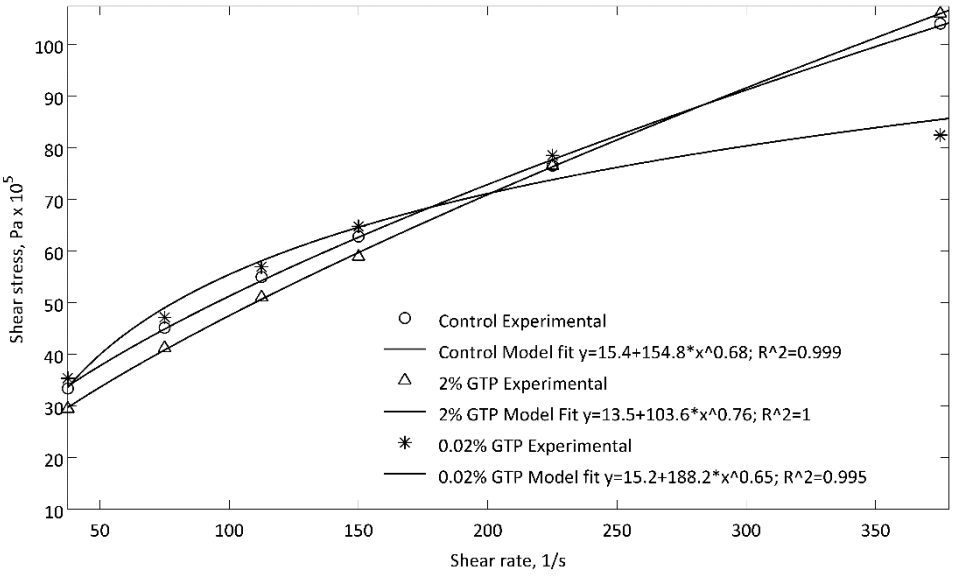
Flow characteristics of yogurt attempted to be explained by using different rheological models such as Power Law model and Herschel-Bulkley model [114, 115]. Figure 2.3 shows the examples of the relationship between the shear stress (τ) and shear rate (γ), explained by Herschel-Bulkley model, for GCP- (0%, 1% and 2%) and GTP-Yogurts (0%, 0.02%, 2%), respectively. The model parameters, namely consistency coefficient, flow behavior index and, yield stress, were calculated from the model. The standard deviations for coefficient of determination (R^2) higher than 0.994 indicated that the Herschel-Bulkley model successfully described the rheological behavior of set yogurt samples.

The model parameters indicated that the consistency coefficient of control yogurt increased significantly ($P < 0.05$) during 21 d of storage, but no significant differences ($P > 0.05$) were found in 1 and 2% GCP yogurts up to 14 d of storage (Figure 2.4.A). On the other hand, the consistency of control was considerably lower than GCP yogurts during 14 d, whereas it was found to be higher at the end of storage. The GTP yogurt results showed that the consistency coefficients of GTP yogurts were significantly different from the control samples until 14 d of storage ($P < 0.05$, Figure 2.4.B). Lower concentrations (i.e., 0.01 and 0.02%) caused increased consistency compared with control during storage ($P < 0.05$). However, lower consistency coefficients were obtained in GTP yogurts when the GTP concentration increased to 1 or 2%. Highest and lowest consistencies were obtained in 0.02 and 2% GTP yogurts, respectively.

The lactic acid bacteria continue to produce lactic acid over shelf life, this phenomenon is known as post acidification. Post acidification causes to several adverse effects on yogurt quality such as strong acid taste, increase of whey separation [116]. Xu et al. [117] also reported that when yogurt samples were fermented by different acidifying strains of *Lactobacillus delbrueckii subps. bulgaricus,* both weak post acidification and higher viscosity were observed in yogurt samples, during storage. The effect of changing in pH to the yogurt structure has already been discussed above. As a result, strengthening of protein-protein complexes, forming yogurt structure, via protein-polyphenol interaction and the weak post acidification may have been responsible for the increase in consistency.

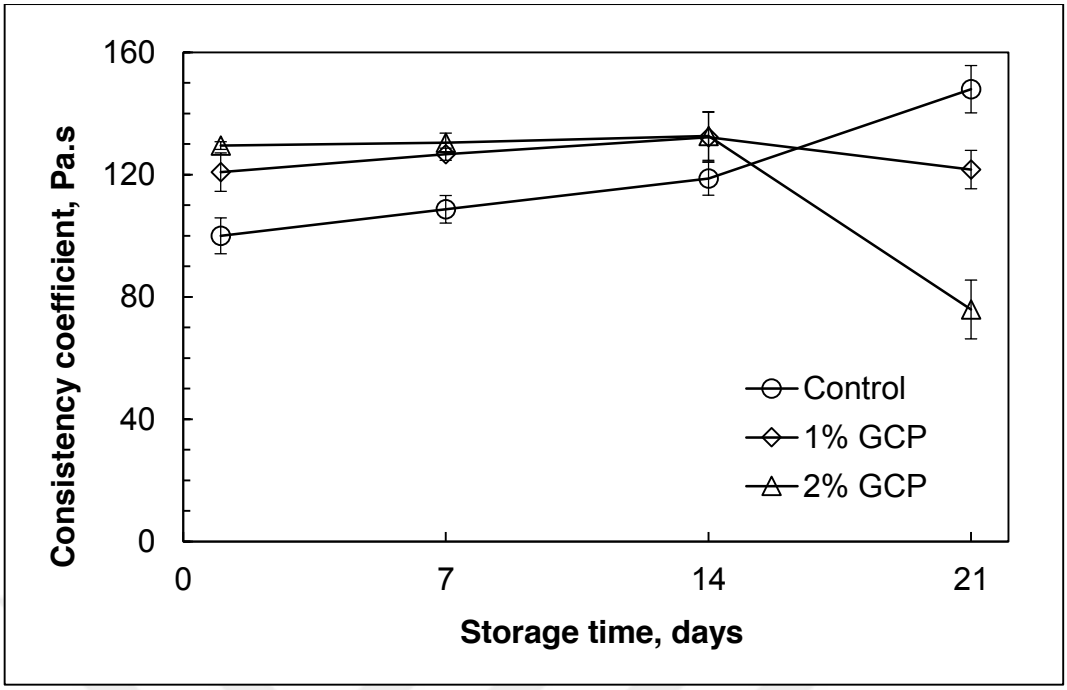


(A)

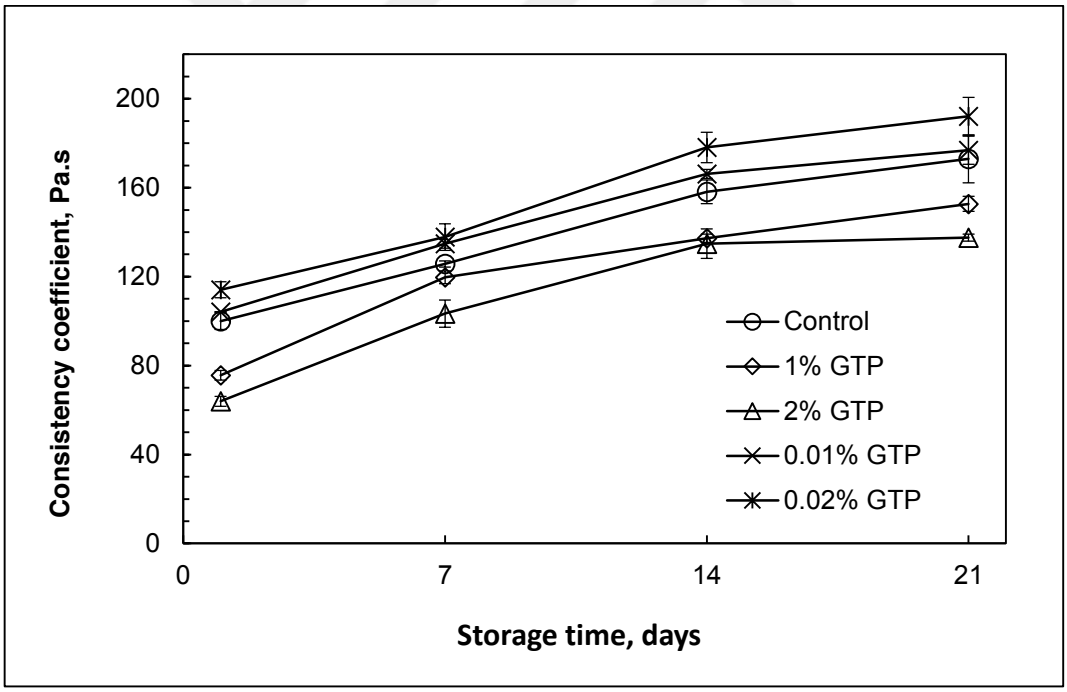


(B)

Figure 2.3. Examples of the plots of the Herschel-Bulkley model fits for yoghurts added with (A) GCP, (B) GTP.

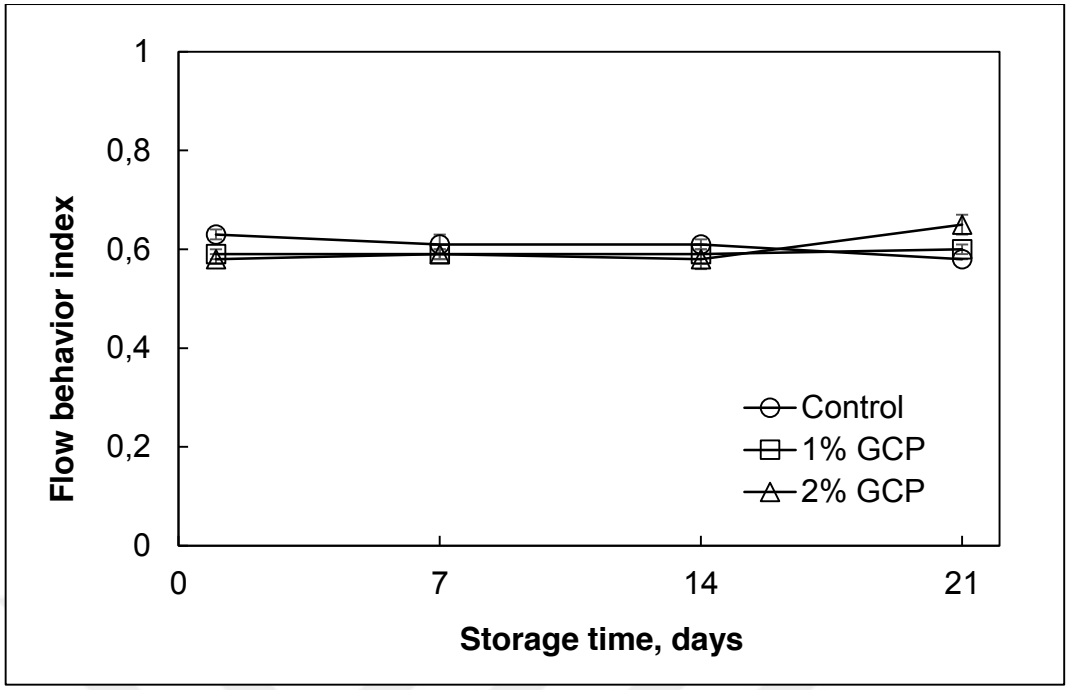


(A)

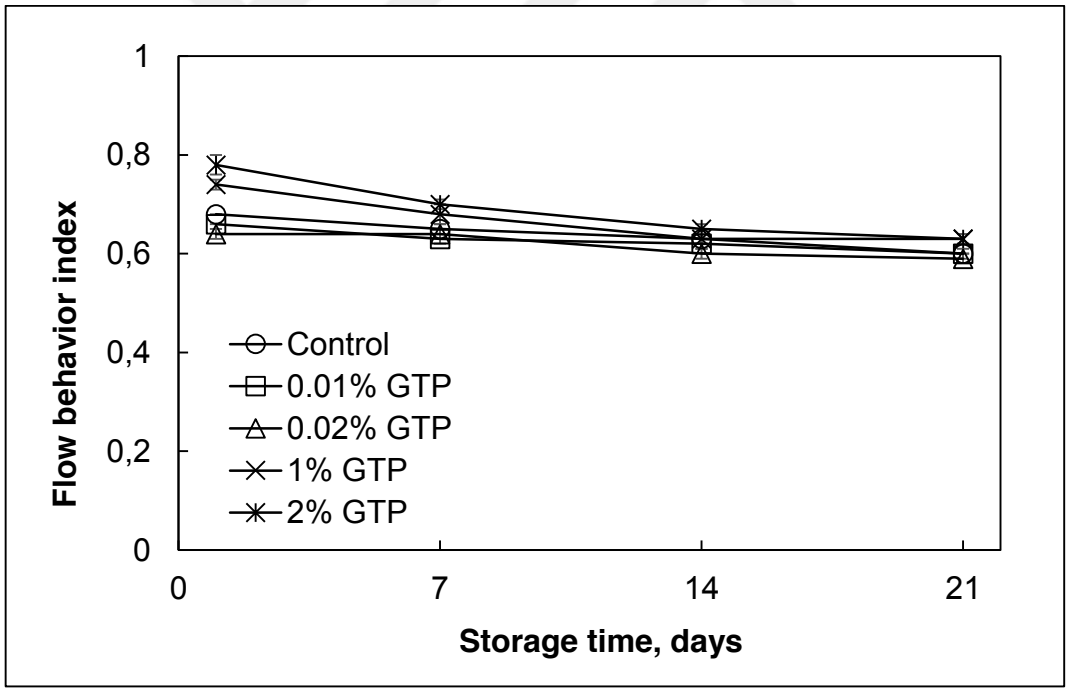


(B)

Figure 2.4. Changes in the consistency coefficients calculated from the Herschel-Bulkley model fit equations for yoghurts added with (a) GCP, (b) GTP during cold storage



(A)



(B)

Figure 2.5. Changes in the behavior indexes calculated from the Herschel-Bulkley model fit equations for yoghurts added with (a) GCP, (b) GTP during cold storage.

Meanwhile, the range of mean flow behavior index was found to be between 0.78 and 0.58, and the apparent viscosity decreased with increasing shear rate (the data

were not shown). These findings referred the shear-thinning phenomenon, which also showed the pseudoplastic behavior for each sample at all concentrations [118, 119].

Figure 2.5.A demonstrates that the flow behavior index of GCP yogurts and control, ranging between 0.58 and 0.65, did not significantly changed during 21 d of storage ($P > 0.05$). The same trend was observed in GTP yogurts in low concentrations (0.01 and 0.02%). No significant difference was observed for the parameter n for these yogurt samples, indicating that they all had similar degrees of shear thinning. However, the flow behavior index of 1 and 2% GTP yogurts was significantly higher ($P < 0.05$) than control and 0.01 and 0.02% GTP yogurts the first day of storage, whereas it decreased until 14 d of the storage (Figure 2.5.B). After 14 d, flow behavior indices of all samples remained constant until the end of the storage. The set yogurt system is a dispersion system consisting of many particles and causes the formation of yield stress, which is defined as the required initial force to initiate the yogurt to flow [67]. The yield stress of the samples changed between 8.1 x 10^5 and 23.1 x 10^5 Pa. No significant difference was observed for the yield stress for the yogurt samples during storage (data not shown).

2.3.3. Changes in Color during Storage

Color is one of the most important visual attributes in dairy products, and color differences affect storage, shelf life and color deterioration of the yoghurt [120]. The total color difference (ΔE) is important, so that all differences encountered between L*, a*, b* color values of the samples and control are taken into account. ΔE values (<1.0) indicated that there was no difference between control sample and low concentrations of GTP-Yogurts (0.01 and 0.02%) [121]. ΔE values of GTP-Yogurts at1% and 2% and GCP-Yogurts at 1%, 2% concentrations were calculated as 7.73, 12.4 and 3.07, 6.29, respectively. This result implied that an increase in the concentration of GCP or GTP caused an increase in the ΔE values. Additionally, no significant differences were found in color values (L*, a*, b*) of all samples throughout 21 d of storage ($P > 0.05$; data were not shown).

2.4. Conclusion

The modification of technological properties of dairy products such as yogurt has gained considerable interest for the development of new products. The results

indicated that green tea and green coffee powders, which are rich in polyphenols, could significantly modify the syneresis and rheological behaviors of set yogurts. These powders affected the gel matrix of yogurts differently in a concentration-dependent manner. It is thought that the differences in the polyphenol profiles of green tea and green coffee played a certain role on the observed differences of the yogurts added with their powders. Overall, the results suggest that polyphenols present in green coffee and green tea could interact with casein micelles in yogurt.

Consequently, this protein-polyphenol interaction promoted the strength of the casein network and stabilized yogurt structure by increasing the consistency and reducing the syneresis rate at certain concentrations.

Based on the results of present study, incorporation of GCP and GTP at a ratio of 2.0 and 0.02%, respectively, is recommended to improve the gel structure of set yogurts. To conclude, the powders obtained from green coffee and green tea are promising ingredients from a technological point of view. In addition, these natural ingredients draw attention due to their health-promoting properties, which make them easily adopted by consumers.

3. MODULATION OF GASTROINTESTINAL DIGESTION FOR β-LACTOGLOBULIN FOLLOWING BINDING BY (-)-EPIGALLOCATECHIN-3-GALLATE (EGCG) AND GREEN TEA POLYPHENOLS

3.1. Introduction

Milk proteins may play an important role in modulating the adsorption, distribution, metabolism, excretion and antioxidant activity of polyphenols during their passage through the gastrointestinal tract. The major part of milk proteins consist of 80% caseins (comprising of α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN), κ-casein (κ-CN)) [122]. The major whey proteins consist of β-Lg and α-La with some minor proteins being derived from the blood, such as BSA, Ig and enzymes as Lf, Lp and Xo) [25]. In the very initial stages of digestion, polyphenols bind to proline-rich randomly coiled salivary proteins in accordance with the molecular weight of the polyphenol, its hydroxyl content and the presence of esterified gallic acid. This binding has been correlated to the number of hydrophilic hydroxyls located on the polyphenol which cross-link in a polydentate manner through hydrophobic and hydrogen bonding [34, 123].

EGCG, having the greatest number of reducing hydroxyl groups, binds most strongly to proline-rich mucin proteins located in the lining of the gastrointestinal tract [124] and also to proline-rich regions occuring in the open structures of α_s-CN and β-CN. These strong binding affinities result in an alteration of secondary structure of the protein. It is unclear whether covalent attachment of polyphenols to proteins, thus forming quinones, alters protein digestibility in the gastrointestinal tract. By having the greatest number of hydrophilic groups EGCG also has the greatest antioxidative capacity [125], however antioxidant capacity may be changed following interaction with proteins. Other factors, such as the mineral content and pH environment of different regions of the gastrointestinal tract may also modulate the effects of the polyphenols.

Based on knowledge that β-Lg is partially stable during gastrointestinal digestion compared to casein [126], we hypothesised that any potential interaction between milk proteins with the main polyphenol EGCG will influence the stability of milk

protein to digestion by gastrointestinal enzymes. Moreover, this interaction may also change the antioxidative effects of EGCG. To compare the effects of processing during extraction of the polyphenols we also wanted to compare the effects of pure EGCG with polyphenols contained in a complex polyphenol matrix, green tea extract (GTE) of which EGCG represents over half of the total phenolic compounds. Therefore, we incubated both β-Lg and micellar casein isolate (MCI) in the presence and absence of both EGCG and GTE and digested both complexes with pepsin followed by pancreatin under conditions simulating gastrointestinal digestion.

3.2. Materials and Methods

3.2.1. Chemicals and Consumables

MCI was supplied from Arla Foods Ingredients, Denmark. Green tea leaves (*Camellia sinensis*) were supplied from a local market in Turkey. Sodium carbonate, sodium bicarbonate, sodium hydroxide, potassium sulfate, boric acid, hydrocloric acid (37%), and sulphuric acid (95%) were purchased from Merck (Darmstadt, Germany). NuPAGE gels and sample buffer were purchased from Invitrogen. Cupric sulfate pentahydrate was purchased from Fluka Chemie AG (Buchs, Switzerland). Gallic acid (98%) was from Acros (Geel, Belgium). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium peroxydisulfate, (-)-epigallocatechin-3-gallate (E-4143) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Beta-lactoglobulin (β-Lg) Sigma-Aldrich Chemie (L-3908) and all other chemicals were from Sigma-Aldrich Chemie.

3.2.2. Preparation of Green Tea Extract

GTE was prepared from coarsely ground green tea leaves (*Camellia sinensis*). Thirty grams of green tea was extracted into 1 L of boiling water by incubating it at 90 °C in a water bath for 30 minutes. Green tea leaves were then removed by using a filter paper (Macherey-Nagel 751/60) and the green tea infusion was immediately lyophilized by freeze-drying for 48 h (Christ Alpha 1-2 LD+, Osterode, Germany) at 0.1 Pa and an ice condenser temperature of 76 °C to obtain green tea extract (GTE).

3.2.3. In-vitro Pepsin and Pancreatin Digestion

10 g/L (w/v) protein (in 10 mM HCl, pH 2) was incubated with or without a 10-fold molar excess of either EGCG or a 10-fold weight excess of GTE at 25 °C for 4h. In vitro pepsin digestion (Sigma P-7000, 0.005% (w/w) enzyme:milk protein ratio, with

mixing) was thereafter performed at 37 °C for up to 2 h and 10 µl aliquots were removed at 0 (before pepsin digestion) 1, 2, 5, 10, 30, 60 and 120 min, respectively, mixed with non-reducing LDS sample buffer (Invitrogen) and then heat treated at 80 °C for 10 min. For intestinal digestion, pepsin hydrolysed samples were either adjusted to 15 mM sodium bicarbonate, pH 6.8 to simulate upper duodenal digestion or they were adjusted to 110 mM sodium bicarbonate, pH 8.3 to simulate distal small intestinal digestion. In both cases pancreatin digestion (Sigma P7545, 1.5% (w/w) enzyme : milk protein ratio, with mixing) was performed at 37 °C, and aliquots (200 µl) were taken at 0 (before pancreatin digestion), 1, 2, 5, 10, 30, 60, 120, 180 and 240 min respectively, mixed with non-reducing LDS sample buffer and then heat treated at 80 °C for 10 min.

3.3. Methods

3.3.1. Seperation of Protein and Nitroblue Tetrazolium Staining for Quinoprotein Detection

Proteins (5 µg) were separated by electrophoresis using NuPAGE 12% Bis-Tris, 1.0 mm gels (Invitrogen) under non-reducing conditions and stained with Coomassie Brilliant Blue R-250. Visualised proteins were analyzed by densitometry and the time taken to digest 50% of the original amount of protein before initiation of digestion was determined together with the degree of intact protein remaining following digestion with pepsin and pancreatin was calculated.

For detection of quinoproteins, separated proteins were electro-transfered onto PVDF membranes (Life Technologies) using transfer buffer containing 25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA and 10% Methanol, pH 7.2 in an Xcell II Blot Module (Novex), at 30 V for 60 min. Membranes were then stained for quinones and quinonoid compounds initiated by binding of EGCG to milk protein using redox-cycling staining resulting from the production of formazan from reduced Nitroblue tetrazolium under alkaline conditions in the presence of glycine as a reductant. Quinoproteins were visualized by a purple-blue staining [127].

3.3.2. Isothermal Titration Calorimetry

1 mM EGCG and 0.1 mM β-Lg were dissolved in 100 mM Tris buffer (pH 7.4, ionic strength of 0.16). Before dissolving EGCG and β-Lg, the Tris buffer was bubbled with nitrogen gas for 1 h to slow down the oxidation of EGCG. Then all the solutions

were degassed for 15 min. After degassing, EGCG and β-Lg solution were loaded to the syringe and isothermal titration cell using a Nano-ITC titration calorimeter with a gold sample cell (TA Instruments, New Castle, USA) Calorimetric measurements were performed at 25 °C, respectively. The cell was stirred at 300 rpm for efficient mixing. Typically, 10 µL of titrant was injected into the sample cell over a period of 25 s and a 300 s interval between the injections was allowed to ensure complete equilibration. The data were analyzed using NanoAnalyze (TA Instruments, New Castle, USA) and a one-site binding model. The enthalpy changes (ΔH), binding constant (Ka) and stoichiometry (n) were calculated from the isothermal titration curve. The heat of dilution was obtained by titrating 1 mM EGCG to 100 mM Tris buffer (pH 7.4, ionic strength of 0.16). The obtained heat as the blank control was subtracted from the final results.

3.3.3. Inhibition of Free Radical Scavenging (%)

Free radical scavenging capacity (FRSC) was determined by the ABTS radical scavenging assay using a Spectramax i3X Molecular Devices (Austria) spectrophotometer at a wavelength of 734 nm. The absorbance was measured separately for the EGCG, GTE samples and the respective buffer which was used during digestion, and the decrease in antioxidant capacity (%) of the samples was calculated with reference to the data obtained for the related buffer used in the digestion. The same experiment was performed with either EGCG or GTE incubated with their corresponding proteins and the decrease in antioxidant capacity (%) was again calculated according to the reference buffers. For this purpose, a mixture of 10 µl of sample and 140 µl of diluted ABTS* solution were loaded in a microplate and the absorbance was measured after 6 min. This experiment was performed with minor modifications according to the method described by Stojanidovic et al. [36]. All experiments were conducted in triplicate and all calculations were made by using the equation given in Eq. 3.1.

$$\text{Inhibition } \% = \frac{(\text{Abs buffer} - \text{Abs sample})}{\text{Abs buffer}} \times 100 \qquad \text{Eq 3.1.}$$

3.3.4. Statistical Analyses

All data were reported as means with their standard deviations. Comparison between groups *in vitro* and antioxidant experiments were performed using one-way

ANOVA and Tukey test by using SPSS 13.0 statistical program package. Significance was considered for *P*-values < 0·05.

3.4. Results and Discussion

3.4.1. Digestion Stability of Beta-Lactoglobulin

Under reducing conditions, the molecular weight of β-Lg monomer is 18.4 kDa, and that of pepsin is 34 kDa. In this study polyacrylamide gel electrophoresis was performed under non-reducing conditions to analyse aggregated proteins contained in aliquots removed during the digestion of β-Lg by pepsin. Under these conditions the structure of β-Lg is more compact than under the reducing conditions which are usually applied during electrophoresis and this increased compactness resulted in a faster migration and lower apparent molecular weight of all proteins compared to under reducing conditions. The slower migrating band corresponds to β-Lg dimer, and not pepsin, as it is present in the 0 min sample, where pepsin is not added.

β-Lg was quite resistant to cleavage by pepsin at pH 2.0 and digestion reduced the levels of intact protein by 11.5% at 2 hours of incubation (Figure 3.1.A). This resistance to digestion by β-Lg has been previously reported [126, 128], and is likely due to the compact structure this protein, which contains a hydrophobic lipocalin β-barrel disulphide-bonded structure which renders it a poor substrate for pepsin [129]. When β-Lg was incubated with EGCG, staining of both β-Lg monomer and dimer were reduced in intensity, and compared to the 0 min sample, levels of intact protein were reduced by 28.5% at the end of digestion (Figure 3.1.B), both observations may be indicative of EGCG binding to β-Lg. To elucidate this, the binding of EGCG to β-Lg was determined using isothermal titration calorimetry (ITC) and the results demonstrated that the binding of EGCG to β-Lg

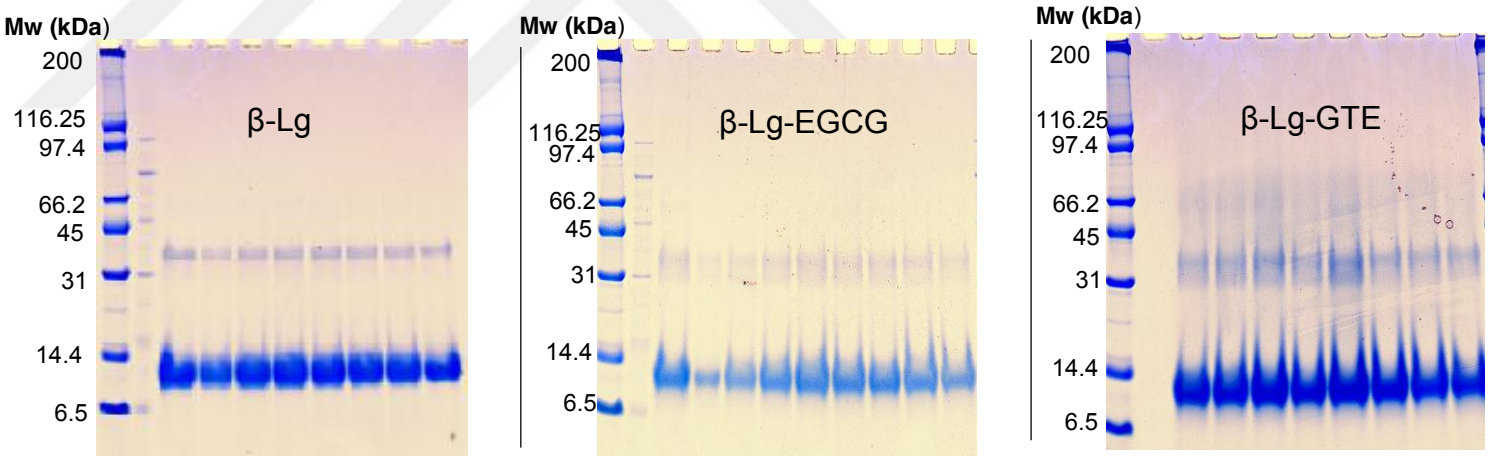


Figure 3.1. Digestion of β-Lg by pepsin, (A) β-Lg, (B) preincubated with EGCG, (C) preincubated with GTE. Lanes 1: Molecular weight markers; Lane 3: 0 min (before pepsin digestion) Lanes 4-10: 1, 2, 5, 10, 30 min, 1, and 2h after digestion treatment with pepsin.

with a binding constant of 19550 ± 3716 L/mol (n=0.97), an enthalpy of -100 ± 7.925 kJ/mol and the entropy of -253.2 J mol/K (Table 3.1). Our results from isothermal titration calorimetry, verified this binding and are in fair agreement with previously obtained data (K_a 1.3 ± 0.8 × 10^4) obtained using a fluorescence quenching technique [26].

Table 3.1. Thermodynamic binding parameters for the interaction of EGCG to β-Lg.

Ka (L/mol)	n	ΔH (kJ/mol)	ΔS (J mol/K)
19550±3716	0.97±0.04	-100±7.925	-253.2

During incubation with EGCG, we noted two points. First, when β-Lg was incubated with EGCG an increased cleavage by pepsin was observed. Secondly, compared to β-Lg incubated with pepsin in the absence of EGCG, a decreased staining intensity by Coomassie Blue was also observed. Combined, these observations may indicate that EGCG binds to β-Lg inducing a structural change in the protein thus increasing its susceptibility to pepsin cleavage by adding a particular flexibility and extended confirmation to the protein structure. A previous study has shown that the green tea polyphenols C; EC; ECG; and EGCG bound to β-Lg via both hydrophobic and hydrophilic interactions and this binding increased the β-sheet and α-helical structure in the protein [26]. In contrast, it has also been proposed that EGCG may bind to pepsin, reducing its activity [130]. However, the data in this present study support the notion of a structural change occurring in the protein, increasing susceptibility to proteolysis at least when EGCG is used.

Binding of GTE to β-Lg slightly decreased the rate of protein digestion compared to β-Lg alone, as shown in (Figure 3.1.C) and there was a gradual increase in β-Lg dimer formation up to 10 min. Levels of intact protein were reduced by 2.4% at the end of digestion.

Results from scanning densitometry of the polyacrylamide gel electrophoresis gels were in agreement with visual inspection of the effects of EGCG and GTE on digestion of β-Lg by pepsin. Pepsin digestion lead to a reduction in levels of β-Lg and this effect was significantly greater in the presence of EGCG, however co-incubation with GTE significantly reduced digestibility (Figure 3.2). The different effects of EGCG and GTE may be related to the oxidation of GTE which occurs during its extraction and processing. When pepsin digestion was followed by

pancreatin digestion at pH 6.8 in the presence of 15 mM sodium bicarbonate to simulate upper duodenal digestion [128], β-Lg was gradually degraded by pancreatin resulting in a gradually fall in levels of intact protein within 240 min, and 5.6 % intact protein remained after 4h of digestion.

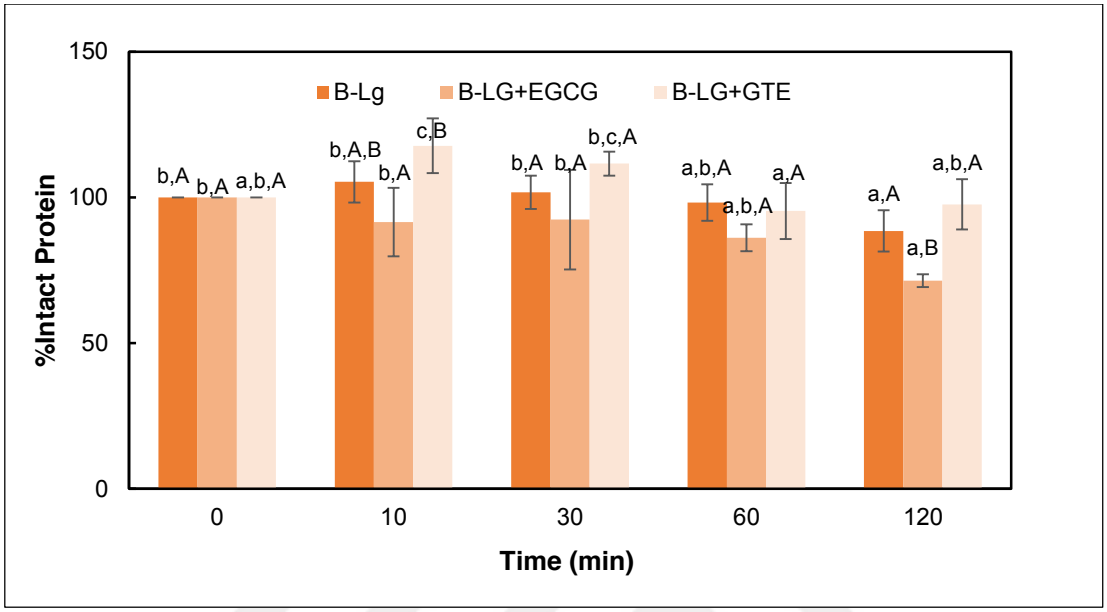


Figure 3.2. Changes in the percentage of intact β-Lg remaining during pepsin digestion in the presence and absence of either EGCG or GTE. Superscript lower letters indicate statistically significant difference ($p<0.05$) during digestion for each sample. Superscript upper letters indicate statistically significant difference ($p<0.05$) between protein and complexes of milk protein with polyphenols within the time of digestion. EGCG: Epigallocatechin-3-gallate; GTE: Green Tea Extract; β-Lg: Beta Lactoglobulin. The error bars represent the standard deviation of the mean.

β-Lg dimer was also cleaved by pancreatin (Figure 3.3.A). In the presence of EGCG, staining of β-Lg was reduced, which may be an indication of binding of EGCG and β-Lg dimer was not observed after 30 min of digestion and almost no β-Lg monomer was observed at 4h of pancreatin digestion (Figure 3.3.B). To elucidate whether EGCG bound to β-Lg under these conditions, NBT staining of electrotransferred proteins was performed. The results indicated that EGCG bound to β-Lg monomer and dimer forming a quinoprotein under these conditions and that this binding was maintained through most of the pancreatin digestion and was not observed after 3h of digestion, although binding gradually reduced in accordance with digestion (Figure 3.3.C). Scanning densitometry of the Coomassie-stained gels were in agreement with visual inspection of the gels that the half-life of β-Lg monomer was reduced by almost 60 min in the presence of EGCG in the upper duedonal enviroment (Figure 3.4). As a result, it can be

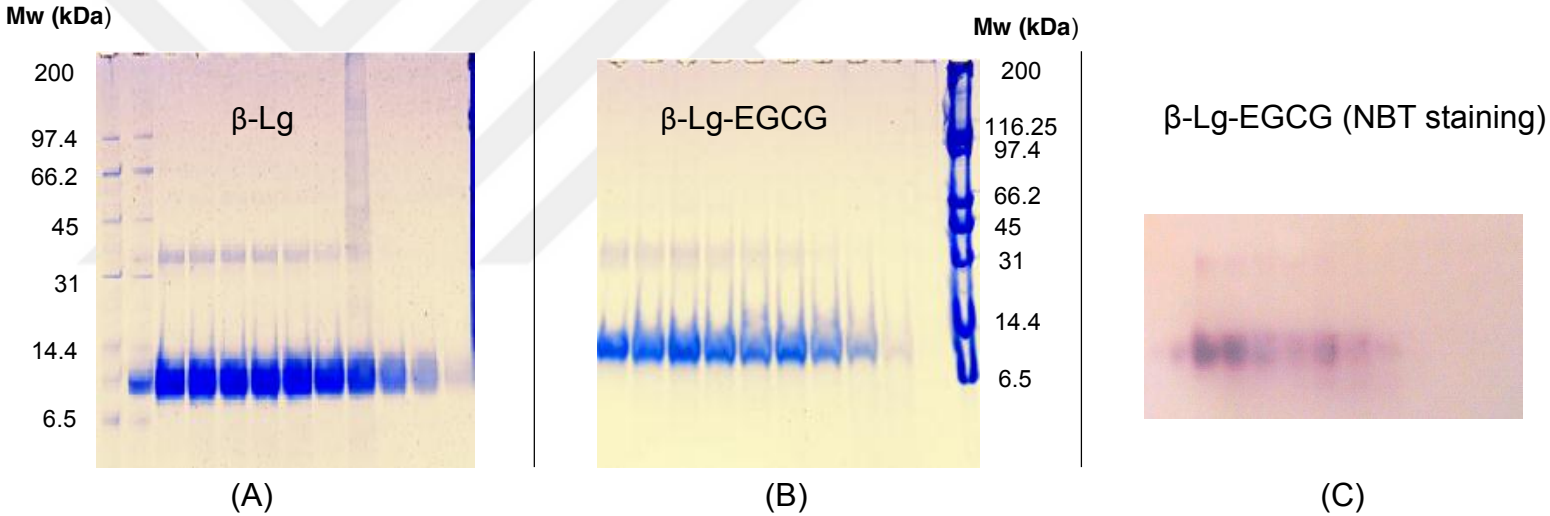


Figure 3.3. Digestion of β-Lg by pepsin, followed by pancreatin under conditions simulating digestion in the upper duodenum (A) β-Lg, Lane 1, 2: Molecular weight markers, Lane 3: 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion); Lanes 4-12: 1, 2, 5, 10, 30 min, 1, 2, 3 and 4h after digestion treatment with pancreatin, (B) preincubated with EGCG, (C) preincubated with EGCG(NBT), Lane 11: Molecular weight markers Lane 1: 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion before pancreatin digestion); Lanes 2-10: 1, 2, 5, 10, 30 min, 1, 2, 3 and 4h after digestion treatment with pancreatin. Quinoproteins are demonstrated by purple staining.

hypothesized that complexation with β-Lg either conveys EGCG for later release in the distal parts of the small intestine, or results in the release of peptides, such as dipeptides which can be transported across intestinal epithelial cells by the PepT1 transporter for later uptake and transport to other organ.

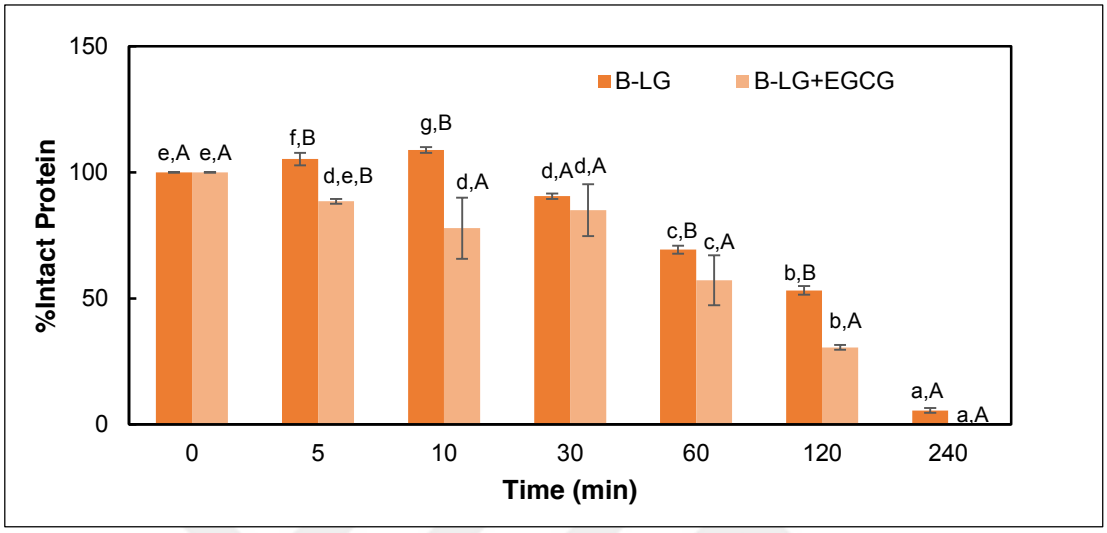


Figure 3.4. Changes in the percentage of intact β-Lg remaining during pancreatin treatment at pH 6.8 simulating conditions simulating digestion in the upper duodenum. Gels were quantified using densitometry and the data were normalized to the 0 min sample which was assigned 100%. Superscript lower letters indicate statistically significant difference ($p<0.05$) during digestion for each sample. Superscript upper letters indicate statistically significant difference ($p<0.05$) between protein and complexes of milk protein with polyphenols within the time of digestion. EGCG: Epigallocatechin-3-gallate; β-Lg: Beta Lactoglobulin. The error bars represent standard deviation of the mean.

To simulate distal small intestinal digestion, β-Lg was digested firstly by pepsin, followed by pancreatin in the prescence of 110 mM sodium bicarbonate at pH 8.3 [131]. β-Lg was more rapidly digested than at pH 6.8. Under these conditions the β-Lg dimer band was barely visible and it was not observable after 2 h of pancreatin digestion. No β-Lg was observed after 3h of digestion (Figure 3.5.A). In the presence of EGCG, no dimer band was visible after 10-30 min of pancreatin treatment and no intact β-Lg was observed after 1 h of pancreatin treatment (Figure 3.5.B). NBT staining of electrotransferred proteins indicated that when EGCG was not incubated with β-Lg, where there was no quinoprotein formation (Figure 3.5.C, lanes 1-7). EGCG bound to β-Lg forming a quinoprotein and that this remained undigested for up to 2h of pepsin digestion (Figure 3.5.C, Lanes 8-10). However, under simulated

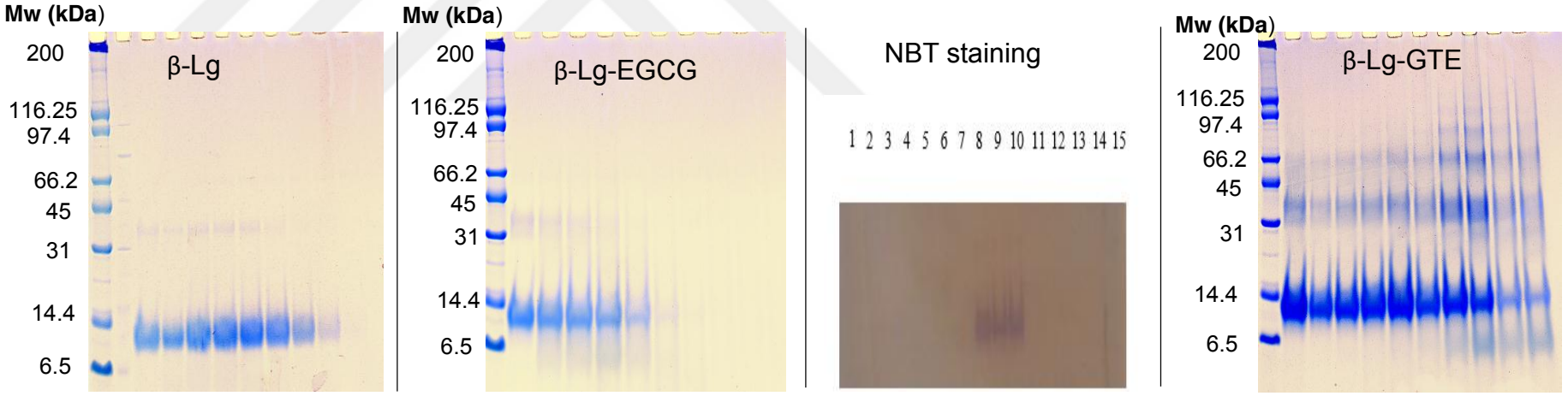


Figure 3.5. Digestion of β-Lg by pepsin, followed by pancreatin under conditions simulating digestion in the distal small intestine. (A) β-Lg, Lanes 1 & 2: Molecular weight markers; Lane 3: 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion); Lanes 4-12: 1, 2, 5, 10, 30 min, 1, 2, 3 and 4h after digestion treatment with pancreatin, (B) preincubated with EGCG, Lane 1: Molecular weight markers; Lane 2: 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion); Lanes 3-11: 1, 2, 5, 10, 30 min, 1, 2, 3, and 4h after digestion treatment with pancreatin, (C) preincubated with EGCG (NBT), Lane 1: 0 min (before pepsin digestion); Lanes 2&3: 30 min, and 2 h after pepsin digestion for β-Lg, Lanes 4-7: 1, 30 min, and 4h after treatment with pancreatin. Lane 8-10: β-Lg incubated with EGCG (before pepsin digestion) and 30 min, and 2h after pepsin treatment, respectively. Lanes 9&10: 30 min, and 2 h after pepsin treatment; Lanes 11-14: β-Lg incubated with EGCG 1, 30 min, and 4h after digestion treatment with pancreatin, respectively. Quinoproteins are demonstrated by purple staining, (D) preincucated with GTE, Lane 1: Molecular weight markers; Lane 2: 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion); Lanes 3-11: 1, 2, 5, 10, 30 min, 1, 2, 3, and 4h after digestion treatment with pancreatin.

distal small intestinal digestion, quinoprotein formation was not detected, suggesting loss of binding (Figure 3.5.C). Lanes 11- 15). This loss of binding was faster than during pancreatin cleavage of β-Lg (Figure 3.5.A). As intact protein was detected for up to 30 min of pancreatin treatment, this indicates that EGCG binding under these conditions is reversible and this polyphenol is likely released under these conditions due to changes in pH. This can be explained in two ways which may be interrelated; Firstly, that dehydrogenation and decarboxylation of EGCG are enhanced under alkaline conditions resulting in EGCG dimerization and enhancement of radical scavenging ability [132], Secondly, that it may be released due partly to electrostatic repulsion, as the pKa_1 and pKa_1 of EGCG are 7.68-7.75 and 8.0 respectively [131]. The implications for the loss of binding are that EGCG will be bound and protected by association with β-Lg and then released in dimeric form in the distal small intestine and it can be speculated that this form of EGCG will enter the colon to modulate redox conditions and growth of colonic bacteria.

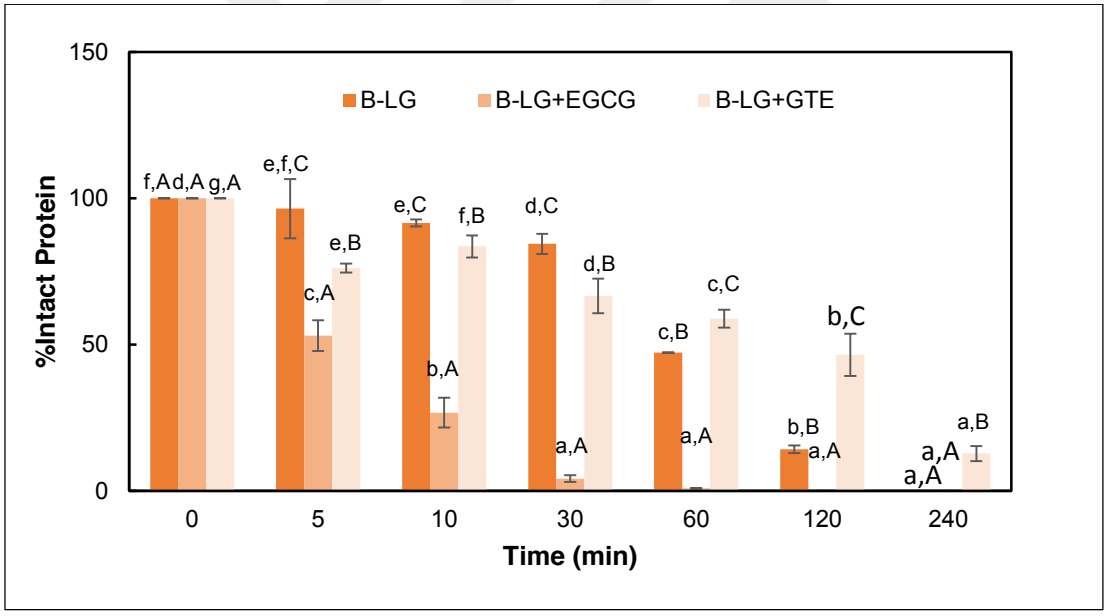


Figure 3.6. Changes in the percentage of intact β-Lg remaining during pancreatin treatment at pH 8.3 simulating digestion in the distal small intestine. Gels were quantified using densitometry and the data were normalized to the 0 min sample which was assigned 100%. Superscript lower letters indicate statistically significant difference ($p<0.05$) during digestion for each sample. Superscript upper letters indicate statistically significant difference ($p<0.05$) between protein and complexes of milk protein with polyphenols within the time of digestion. EGCG: Epigallocatechin-3-gallate, GTE: Green Tea Extract, β-Lg: Beta Lactoglobulin. The error bars represent standard deviation of the mean.

In contrast, simulated combined gastric and distal intestinal digestion of β-Lg incubated with GTE resulted in a gradual increase in the intensity of the β-Lg dimer

band peaking at 2h of digestion and after 4 hours of digestion, the level of intact β-Lg monomer was reduced by 87.3% (Fig 3.5.D). These effects are not in line with previously reported study by Stojadinovic et al. [36] who showed that GTE increased the digestibility of β-Lg, whereas other polyphenols slowed down digestibility. Scanning densitometry supported the visual inspection of the gels and that there was a faster rate of cleavage of β-Lg in the presence of EGCG. Whereas in the presence of GTE digestion was retarded (Figure 3.6).

The different digestion behavior of β-Lg-EGCG and β-Lg-GTE may be the result of the oxidation of plant polyphenols in GTE as a result of the processing steps involved during the production of this extract. This important observation may explain some of the differences noted in previous studies that have reported that polyphenols may slow proteolysis by gastrointestinal enzymes. However, caution is warranted in this explanation, as there are many particular types of polyphenols present in GTE, this being a more complex system and may be the result of complexation of several polyphenols and concomitant aggregate formation. Complexation of plant polyphenols by β-Lg may be an effective means to deliver them to the colon for modulation of bacterial growth and ensuring optimal redox conditions [133]. The indigenous microflora of the colon have the capability of metabolising some polyphenols, such as chlorogenic acid, through esterase activity into metabolites which have antioxidant and anticarcinogenic activity [134].

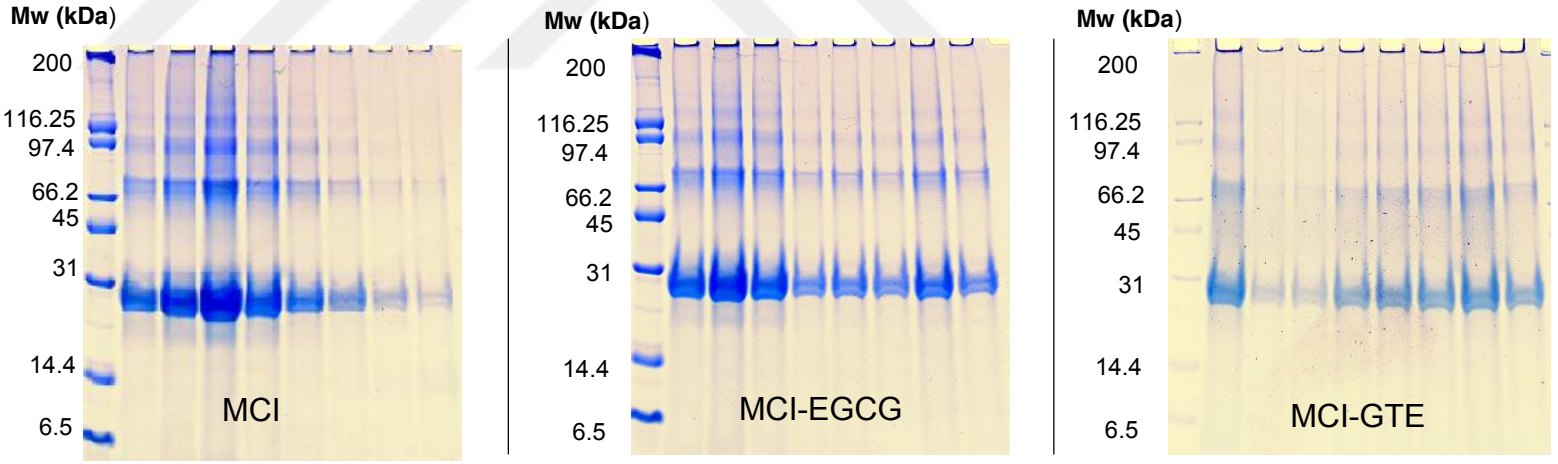
3.4.2. Digestion Stability of Micellar Caseins

To compare the effects of β-Lg with other major milk proteins, we used micellar casein isolate (MCI). The casein micelle consists of caseins which are intrinsically disordered [135], bound to nanoparticles of calcium phosphate. This formation of a micellar structure imparts order and compactness to these proteins. Under these conditions, κ-CN is generally considered to be bound to the external surface of the micelle and its hydrophilic glycosylated C-terminal portion extends out into the serum phase. Thus κ-CN may be the main casein moiety involved in polyphenol binding. However, under acidic conditions, dissociation of caseins from the micelle occurs, thereby exposing the open structure of the caseins to potential interaction with polyphenols and also to protease activity [136, 137]. When analyzed by electrophoresis under the same conditions as β-Lg, MCI showed a main protein band of molecular weight of ~23 kDa, but with also contained some higher molecular

weight casein multimers. The multimeric nature of casein has been described previously [138]. In contrast to β-Lg, pepsin digestion resulted in cleavage of most of the casein contained in MCI within 30 min of pepsin treatment. Levels of intact protein were reduced by 80.2% at the end of the pepsin digestion (Figure 3.7.A). Thus, dissociated micellar caseins are rapidly cleaved under these conditions, and the remaining caseins may likely arise from a small population of micellar bound Compared to β-Lg incubated with EGCG, pepsin digestibility of MCI incubated with EGCG was not enhanced, but rather partially inhibited by incubation with EGCG caseins. In the presence of EGCG, the rate of MCI hydrolysis was significantly reduced with the levels of intact casein remaining being 59.4% (Figure 3.7.B). This indicates that whereas binding of EGCG to β-Lg may have induced structural changes exposing potential enzymic cleavage sites in a compact hydrophobic protein, EGCG binding to MCI inhibited proteolysis by restricting access to cleavage sites located on the caseins.

Furthermore, similar effects were observed with MCI incubated with GTE. When MCI was incubated with GTE, the cleavage of caseins was less than that with MCI-EGCG, with 77.9% intact casein remaining after 2 h of pepsin digestion (Figure 3.7.C). Scanning densitometry indicated that the decrease in MCI intact protein (%) was gradually observed with both MCI-EGCG and MCI-GTE during pepsin digestion (Figure 3.8). These polyphenols may interact with the casein amino acid residues. This can be explained by the large proportions of proline residues and hydrophobic amino acids present in caseins, which interact with polyphenol hydroxyl and phenolic ring structures [32].

Under conditions simulating combined gastric and distal small intestinal digestion, the small proportion of caseins in MCI which remained intact after pepsin hydrolysis were completely digested within 1 min of pancreatin digestion (Figure 3.9.A). However, in the presence of EGCG, the rate of degradation decreased very significantly compared to pepsin-treated micellar casein alone and no intact casein remained at 1h of pancreatin digestion (Figure 3.9.B). NBT staining of electrotransferred proteins indicated that when MCI was not incubated with EGCG, there was no quinoprotein formation. In contrast, weak formation of quinoprotein was observed by NBT staining between MCI and EGCG which persisted from the



(A) (B) (C)

Figure 3.7. Digestion of micellar caseins by pepsin (A) MCI, (B) preincubated with EGCG, (C) preincubated with GTE, Lane 1: Molecular weight markers; Lane 2: 0 min (before pepsin digestion) Lanes 3-9: 1, 2, 5, 10, 30 min, 1, and 2h after treatment with pepsin. MCI: Micellar Casein Isolate.

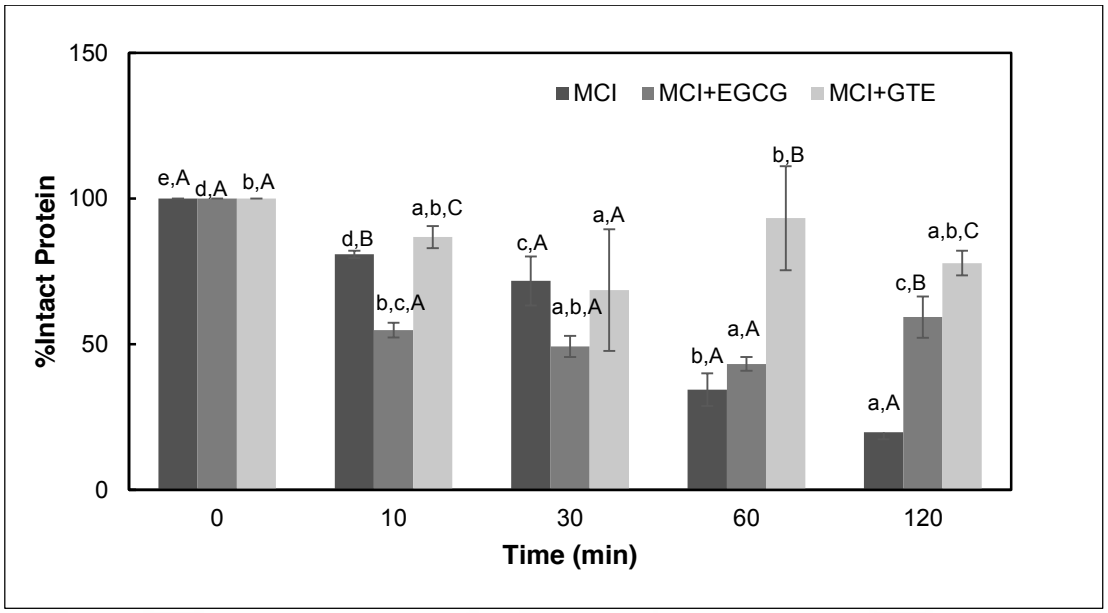


Figure 3.8. Changes in the percentage of intact micellar casein remaining during pepsin digestion. Gels were quantified using densitometry and the data were normalized to the 0 min sample which was assigned 100%. Superscript lower letters indicate statistically significant difference ($p<0.05$) during digestion for each sample. Superscript upper letters indicate statistically significant difference ($p<0.05$) between protein and complexes of milk protein with polyphenols within the time of digestion. EGCG: Epigallocatechin-3-gallate; GTE: Green Tea Extract; MCI: Casein isolate. The error bars represent standard deviation of the mean.

beginning of pepsin digestion and up to 30 min of pancreatin digestion, (Figure 3.9.C). In the presence of GTE, MCI was very resistant to digestion by pancreatin such that 7.2 % of intact casein remained undigested after 4 h (Figure 3.9.D). Scanning densitometry indicated that when MCI which exhibited susceptiblty to pancreatin digestion, incubated with GTE resulted in still remaining in colon after 4 h. Moreover, EGCG was less effective than GTE on the digestion stability of MCI, whereas both MCI-GTE and MCI-EGCG have almost the same half-life (5min) (Figure 3.10).

All results can be exlained that polyphenols have been reported to inhibit the enzyme activity of gastrointestinal enzymes and decrease proteolysis during the gastric phase, but not during the intestinal phase of digestion [53]. However, this study studied the effects of digestibility of milk, yogurt and cheese, where the major protein source is derived from caseins. Our data indicate some differences which are importantly governed by the type of protein studied. During the gastric phase, EGCG increases digestibility of β-Lg whereas GTE has no great effects. During the

intestinal phase, EGCG increases digestibility of β-Lg in both simulated upper and lower intestinal digestion whereas GTE delays digestion of this protein. In line with

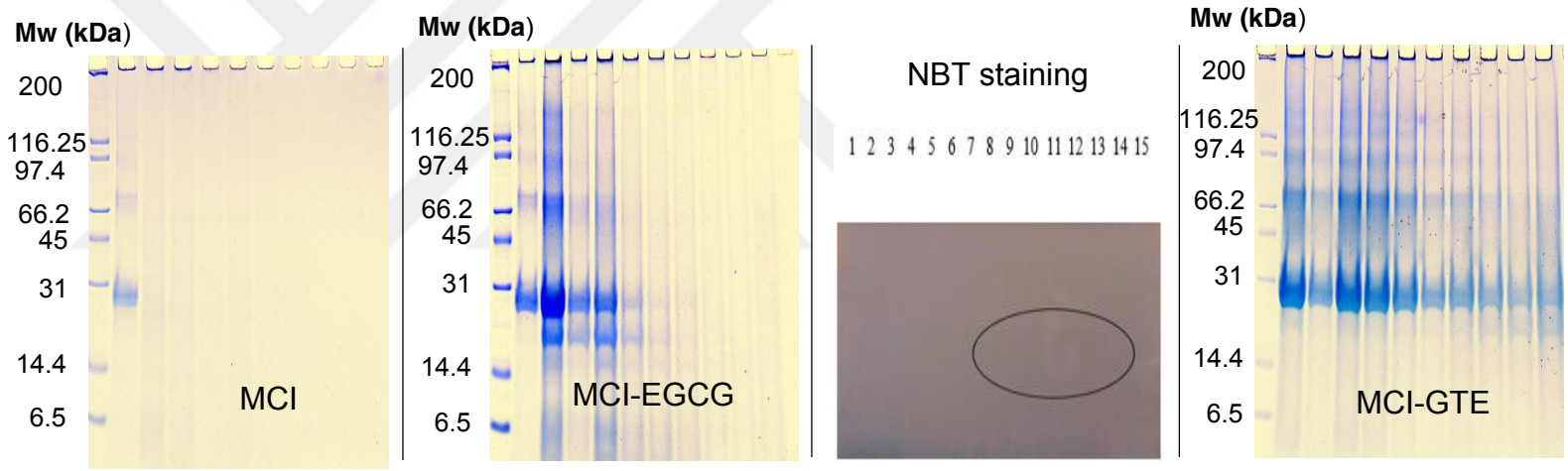


Figure 3.9. Digestion of micellar casein by pepsin, followed by pancreatin under conditions simulating digestion in the distal small intestine, (A) MCI, Lane 1: Molecular weight markers; Lane 2: 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion); Lanes 3-11: 1, 2, 5, 10, 30 min, 1, 2, 3, and 4h after digestion treatment with pancreatin, (B) preincubated with EGCG, Lane 1: Molecular weight markers; Lane 2: 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion); Lanes 3-11: 1, 2, 5, 10, 30 min, 1, 2, 3, and 4h after digestion treatment with pancreatin, (C) preincubated with EGCG (NBT), Lane 1: Molecular weight marker; Lane 2: 0 min (before pepsin digestion) for MCI; Lanes 3&4: 30 min and 2 h after pepsin digestion for MCI; Lanes 5-8: 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion); 30 min, and 4h after digestion treatment with pancreatin for MCI, respectively. Lane 9: MCI incubated with EGCG, 0 min (before pepsin digestion); Lanes 10&11: MCI incubated with EGCG at 30 min and 2 h after pepsin digestion; Lanes 12-15: MCI incubated with EGCG 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion), 30 min, and 4h after treatment with pancreatin, respectively, (D) preincubated with GTE, Lane 1: Molecular weight markers; Lane 2: 0 min (after 2h of pepsin treatment and following pH adjustment before pancreatin digestion); Lanes 3-11: 1, 2, 5, 10, 30 min, 1, 2, 3, and 4h after digestion treatment with pancreatin.

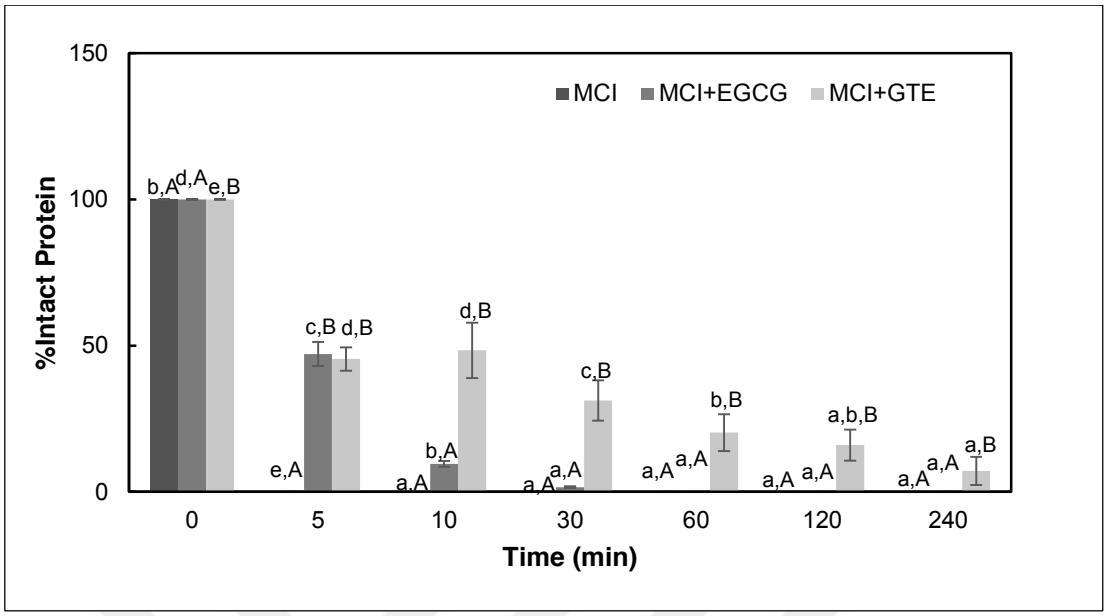


Figure 3.10. Changes in the percentage of intact micellar casein during pancreatin digestion at pH 8.3 simulating digestion in the distal small intestine. Gels were quantified using densitometry and the data were normalized to the 0 min sample which was assigned 100%. Superscript lower letters indicate statistically significant difference ($p<0.05$) during digestion for each sample. Superscript upper letters indicate statistically significant difference ($p<0.05$) between protein and complexes of milk protein with polyphenols within the time of digestion. EGCG: Epigallocatechin-3-gallate; GTE: Green Tea Extract; MCI: Casein Isolate. The error bars represent standard deviation of the mean.

the study by Lamothe et al. [53], EGCG and GTE slow the gastric digestibility of MCI, whereas in the distal intestinal phase, digestibility is slowed by both EGCG and by GTE.

3.4.3. % Inhibition of Free Radical scavenging for Milk Protein-Polyphenol Complexes

Protein binding has been reported to modulate the antioxidant effects of polyphenols by reducing their antioxidative capacity, alternatively it has been proposed that through binding or interaction with milk proteins, the polyphenols derive a stability to oxidative mechanisms present in milk [38]. The relative decrease in free radical scavenging capacity for complexes of milk proteins with either EGCG or GTE was measured during digestion by using the ABTS free radical scavenging assay (Table 3.2). Four different types of control samples (EGCG or GTE incubated with pepsin or without pepsin), were also incubated throughout the duration of the

gastric or intestinal phases of digestion, to determine any potential effects of interaction between these polyphenols and enzyme on free radical scavenging capacity. The results indicated that, there were no significant difference between the control samples, incubated with or without enzymes after 6 h of combined digestion ($p>0.05$).

In general, a decrease in FRSC was observed when protein and polyphenols were incubated together and subjected to digestion, but this was not always the case. According to our results, β-Lg-EGCG lead to only little change throughout both phases of digestion and through this study, we have demonstrated that β-Lg binds to EGCG by ITC and strong binding was observed from NBT staining during simulated upper duodenal digestion. This indicates that β-Lg appears to protect EGCG from oxidation and its FRSC was maintained throughout subsequent digestion in simulated distal small intestinal digestion, despite being released from β-Lg at the beginning of digestion. It can be speculated that released peptides may protect the protein from loss of FRSC. The FRSC of β-Lg-EGCG did not change significantly during simulated distal small intestinal digestion, whereas it was decreased in MCI-EGCG complexes from 72.6% to 41.0% in the first five minutes which demostrated a marked inhibition. Moreover, the binding of EGCG to MCI is only weak compared to that of β-Lg, based on NBT staining.

For β-Lg-GTE, the FRSC commences at a lower level than for β-Lg-EGCG, which suggests that binding to β-Lg may induce some oxidation of GTE and levels fall slightly in the early phases of gastric digestion. However, the greatest fall in FRSC occurs during the intestinal phase of digestion with levels being lower than with β-Lg-EGCG. This indicates that GTE polyphenols may be more susceptible to oxidation than EGCG under the alkaline conditions applied during simulated intestinal digestion. Even more pronounced effects were observed with MCI-GTE which showed a greater decrease in FRSC than when complexed with ECGC. As a result, MCI-GTE showed the greatest decrease in FRSC, whereas β-Lg-GTE had only a slight effect during combined digestion.

Although a reduction in FRSC was observed during pancreatin digestion, a notable increase was observed at the end (240 min), especially in the presence of GTE.

Table 3.2. Percentage inhibition of the free radical scavenging for complexes of milk proteins with polyphenols during simulated digestion (n=3)

		% Inhibition						
Pepsin Digestion	Time (min)	EGCG(with pepsin)	EGCG(without)	β-Lg+EGCG	MCI+EGCG	GTE(with pepsin)	GTE(without)	β-Lg+GTE	MCI+GTE
	0	89.27±0.2^{a,B}	89.70±0.3^{a,B}	88.65±0.2^{a,B}	69.48±13.3^{a,b,A}	87.40±0.4^{c,A}	84.39±0.9^{a,A}	65.10±16.5^{a,A}	62.29±10.3^{a,b,A}
	10	89.57±0.5^{a,B}	89.59±0.2^{a,B}	88.46±0.6^{a,B}	56.98±13.1^{a,b,A}	82.59±0.7^{a,B}	83.87±4.7^{a,B}	58.40±7.4^{a,A}	64.58±5.5^{a,b,A}
	30	89.68±0.3^{a,B}	89.18±0.5^{a,B}	88.66±0.5^{a,B}	41.30±19.8^{a,A}	85.43±0.3^{b,B}	85.33±2.9^{a,B}	61.29±10.8^{a,A}	58.81±13.4^{a,A}
	120	89.57±0.5^{a,B}	89.59±0.5^{a,B}	89.17±0.4^{a,B}	77.83±1.6^{b,A}	86.84±0.63^{c,B}	85.22±0.8^{a,B}	69.43±1.4^{a,A}	70.45±8.3^{b,A}
Pancreatin Digestion		EGCG(with pancreatin)	EGCG(without)			GTE(with pancreatin)	GTE(without)		
	0	85.08±0.5^{c,B}	86.89±0.0^{a,B}	86.60±0.4^{c,B}	72.62±3.5^{b,A}	80.96±0.9^{e,C}	78.28±1.9^{c,C}	48.88±9.1^{b,B}	23.85±9.9^{a,A}
	1	82.87±0.4^{a,b,B}	85.96±0.8^{a,C}	85.26±0.7^{a,b,c,C}	42.10±1.8^{a,A}	74.37±3.0^{d,e,C}	78.01±2.0^{c,C}	51.39±5.4^{b,B}	44.62±1.1^{b,c,A}
	5	81.54±2.0^{a,B}	86.49±0.4^{a,C}	83.40±2.2^{a,B,C}	41.04±3.0^{a,A}	64.54±5.0^{b,c,B}	73.38±5.2^{c,B}	46.35±8.8^{b,A}	48.87±7.7^{c,A}
	10	82.6±0.5^{a,b,B}	85.96±1.6^{a,B}	84.06±0.8^{a,b,B}	73.97±4.4^{b,A}	70.92±5.6^{c,d,B}	74.83±5.2^{c,B}	41.30±6.7^{a,b,A}	30.01±7.3^{a,A}
	30	84.61±0.7^{c,B}	86.09±1.1^{a,B}	85.26±0.4^{a,b,c,B}	76.76±1.2^{b,c,A}	67.20±3.4^{c,d,B}	62.65±9.9^{b,B}	39.31±4.4^{a,b,A}	33.20±9.4^{a,b,A}
	120	83.53±0.2^{b,c,B}	86.23±0.5^{a,C}	85.66±1.4^{b,c,C}	79.95±0.6^{c,A}	57.24±6.1^{a,b,B}	63.31±1.6^{b,B}	32.27±10.1^{a,A}	53.12±3.6^{c,B}
	240	83.80±0.2^{b,c,B}	85.96±0.9^{a,C}	84.99±0.5^{a,b,c,B,C}	80.08±1.4^{c,A}	56.18±2.8^{a,B}	46.36±2.0^{a,A}	67.86±5.4^{a,C}	50.33±5.0^{c,A,B}

Superscript lower letters in each column indicate statistically significant difference ($p<0.05$) during digestion. Superscript upper letters indicate statistically significant difference ($p<0.05$) between polyphenols and complexes of milk proteins with polyphenols in each row within the type of polyphenols. EGCG: Epigallocatechin-3-gallate; GTE: Green Tea Extract; β-Lg+EGCG: β-lactoglobulin incubated with epigallocatechin-3-gallate; MCI+EGCG: Micellar casein isolate incubated with epigallocatechin-3-gallate; β-Lg+GTE: β-lactoglobulin incubated with green tea extract; MCI+GTE: Micellar casein isolate incubated with green tea extract. Data are expressed as mean ± standard deviation.

Increase in the FRSC for both β-Lg-GTE and MCI-GTE can be explained by breakage of the bond between the protein and polyphenol and the release of during their interaction with micellar caseins the polyphenols could be located in the disordered structure of the caseins while being at the interface of the aqueous environment, where a reaction with an $ABTS^{\cdot+}$ radical is possible. It appears that some groups, which have antioxidant capability, are likely to bind to the protein sites after the gastric phase of digestion and some –OH groups, can be oxidized due to the alkaline conditions, negatively affecting their FRSC. Furthermore, results may vary depending on different interactions for different type of proteins and also the different polyphenols used, such as EGCG and GTE [125, 139]. By the way, it is possible that the polyphenols degradation in the intestinal environment resulted in decrease in FRSC after showed tendency to increase FRSC [53].

3.5. Conclusion

This study has demonstrated that the digestibility of two of the major protein contained in milk and their interaction and binding with polyphenols. Digestibility is affected by the binding of polyphenols and that the action of the polyphenols may be different, depending on their purity and their degree of oxidation. Polyphenols modulate digestibility differently, enhancing, or in some cases slowing proteolysis and their action differs, depending on the type of milk protein, which may be governed by structural aspects. These effects may change the bioactivity of the protein, changing release of biologically active peptides and in some cases resulting in an increased protein stabilization through gastrointestinal tract. Importantly, protein interaction affected FRSC, and FRSC was affected depending on protein structure and also the digestibility of the protein, FRSC for polyphenols gradually decreased resulted in interaction with protein, especially casein, from beginning to the end of the digestion. These interactions might allow more peptides and antioxidants absorption by the intestinal cells in general resulting in a release of bound polyphenols towards the end of digestion. Interaction of polyphenols with milk proteins may protect polyphenols during their transit to the colon, where they may modulate redox conditions and the growth of colonic bacteria, which may play important roles in the the health of the adult or infant gastrointestinal system. In conclusion, EGCG and GTE behaved differently by modifying the digestibility of milk

proteins, as GTE consists of a complex mixture of polyphenols whereas EGCG is a pure compound. Moreover, these natural ingredients draw attention due to their health-promoting properties for infants in order to transfer from plasma to breast human milk, which make them to be easily adopted by the consumer.

GENERAL CONCLUSION

The consequences of protein-polyphenol interaction were tried to comprehensively reveal in the view of both the physicochemical properties and digestion behavior of proteins throughout the thesis.

According to the results of **Chapter 2**, the rheological properties of set yoghurt could be improved by means of the addition of green coffee and green tea powders. Adding GCP (1 or 2%) decreased syneresis rate. It was confirmed that the effect of GTP on the syneresis rate was concentration dependent. In comparison to the control, GTP decreased syneresis rate when it was added at 0.02%, but it caused an increase when added at 2%. No significant difference was observed in the syneresis rates when GTP was added at amounts of 1 and 0.01%, until 14 and 7 d of storage, respectively. The Herschel-Bulkley model parameters indicated that the consistency of control was considerably lower than GCP yogurts during 14 d, whereas it was found to be higher at the end of storage. The GTP yogurt results showed that the consistency coefficients of GTP yogurts were different from the control samples until 14 d of storage. In conclusion, GTP and GCP behaved differently in acidified gel networks of set yogurt, modifying its rheological behavior, as they have different profiles and concentrations. This can be a viable strategy for the dairy industry to meet consumer demands to stabilize the gel structure of yoghurts, but also to enhance potential health benefits. Furthermore, it can be easily adapted to industrial scale processing conditions with confirmed consumer sensory acceptance.

Additionally, we reported (**Chapter 3**) comparatively how the changing in stability of both β-Lg and casein, as two major proteins in milk, the result of interaction with catechins during digestion. Furthermore, NBT-staining method was performed whether the binding occurs or disappears due to prove the interaction up to at the end of the digestion. In conclusion, EGCG and GTE behaved differently by modifying digestion behavior of milk proteins, as GTE has complex matrix in terms of polyphenols whereas EGCG is a pure compound. Also, polyphenol binding is likely to alter protein structure leading to increased protein stabilization through gastrointestinal tract. In addition, ABTS assay showed that the free radical scavenging capacity for polyphenols gradually decreased resulted in interaction with

protein, especially casein, from beginning to the end of the digestion. Based on the results of present study, intake of EGCG or GTE with milk proteins, could be recommended to be carried up the colon for both components. It is considered that these interactions might allow more peptides and antioxidants absorption by the intestinal cells from a biological point of view. Moreover, these natural ingredients draw attention due to their health-promoting properties for infants in order to transfer from plasma to breast human milk, which make them to be easily adopted by the consumers.

Overall these investigations in both actual food system and *in-vitro* system enlightened the several aspects of protein-polyphenol interaction.

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HACETTEPE UNIVERSITY
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TO THE DEPARTMENT OF FOOD ENGINEERING

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