DEVELOPMENT OF NOVEL ANTIMICROBIAL, ANTIOXIDANT AND BIOACTIVE EDIBLE GELS INTENDED FOR FOOD PRESERVATION AND PROMOTION OF HUMAN HEALTH

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ABSTRACT

DEVELOPMENT OF NOVEL ANTIMICROBIAL, ANTIOXIDANT AND BIOACTIVE EDIBLE GELS INTENDED FOR FOOD PRESERVATION AND PROMOTION OF HUMAN HEALTH

The aim of this thesis was the development of edible gelatin (GEL) based composite gels with antimicrobial, antioxidant, and bioactive properties. For this purpose, composite gels of gelatin with rice starch (RS), candelilla wax (CW) and soy protein isolate (SPI) were incorporated with lysozyme (LYS) and green tea extract (GTE). LYS and GTE release profiles of GEL and its composites were tested in aqueous media and cold-smoked salmon selected as a model food. GEL, GEL/RS and GEL/CW gels did not show considerably different LYS and GTE release profiles in aqueous media, but their release profiles in the model food system were different. GEL/SPI gels bound LYS and prevented its release from the gels, but they released GTE. GEL showed the most rapid LYS delivery onto smoked salmon, while GEL/RS and GEL/CW caused sustained LYS delivery profiles. The total LYS delivered from GEL and GEL/RS gels onto smoked salmon were similar while GEL/CW gels caused almost 30% lower LYS delivery onto food samples. On the other hand, GEL/CW gels caused the highest amounts of GTE delivery onto model food. In broth media at 4°C, all LYS containing gels (with or without GTE) showed antimicrobial activity with a minimum 1.5 decimal reduction in *L. innocua* that was used as a target bacterium. In smoked salmon samples, the LYS containing gels inhibited the growth of *L. innocua*, but they did not cause a significant reduction in its counts. Antioxidant capacity, antihypertensive and antidiabetic activity, and cytotoxicity tests against human colon carcinoma cells (Caco-2) conducted with released GTE proved the bioactive potential of developed gels. The gels were also tested for their water binding capacity, and physical (textural properties) and morphological (SEM images) properties. This work clearly showed the possibility of using active gels as multifunctional delivery tools (as coating material, pad or pre-cast film) to prevent microbial and oxidative changes in food and to enhance their bioactive properties.

ÖZET

GIDALARIN MUHAFAZASINDA VE İNSAN SAĞLIĞINI DESTEKLEMEK AMACIYLA KULLANILABİLECEK ANTİMİKROBİYAL, ANTİOKSİDANT VE BİYOAKTİF ETKİLİ YENİLİKÇİ YENİLEBİLİR JELLER GELİŞTİRİLMESİ

Bu tezin başılıca amacı antimikrobiyal, antioksidant ve biyoaktif özelliklere sahip jelatin (GEL) temelli kompozit yenilebilir jeller geliştirmektir. Bu amaçla jelatinin pirinç nişastası (RS), kandelilla mumu (CW) veya soya protein izolatı (SPI) içeren kompozit jellerine antimikrobiyal bir enzim olan lisozim (LYS) ve yeşil çay ekstraktı (GTE) ilave edilmiştir. Su içerisinde gerçekleştirilen salım deneyleri LYS ve GTE'nin GEL, GEL/RS ve GEL/CW´den geçiş hızlarının birbirinden farklı olmadığını göstermiştir. GEL, GEL/RS ve GEL/CW jellerden LYS'in model gıda olarak kullanılan tütsülenmiş somona geçiş hızları ise birbirinden farklıdır. Model gıda ortamında ürüne en hızlı LYS geçişi GEL´den olurken, GEL/RS ve GEL/CW jellerden gıdaya LYS geçişi nispeten daha yavaş olmaktadır. GEL ve GEL/RS jellerden model gıda olan tütsülenmiş somona salınan toplam LYS aktiviteleri benzer düzeyde ve GEL/CW jellere göre %30 daha yüksektir. Model gıdaya GTE geçişleri incelendiği zaman en yüksek düzeyde fenolik geçişlerinin GEL/CW jellerden olduğu görülmektedir. Sıvı besiyerinde 4°C 'de yürütülen antimikrobiyal testler LYS içeren tüm jellerin (GTE içerse de içermese de) *L. inoccua* üzerinde 15 gün içerisinde en az 1.5 desimal azalma sağlayacak antimikrobiyal etkisi olduğunu göstermektedir. *L. inoccua* ile inoküle edilmiş 4°C'de depolanan ve her iki yüzeyi aktif jel dilimleri içeren tütsülenmiş somonlarla yürütülen testlerde sözkonusu bakterinin gelişimi LYS içeren jellerle başarıyla inhibe edilmiş, ancak bakteri inaktive edilememiştir. Gerçekleştirilen antioksidant kapasite, antihipertensif ve antidiyabetik aktivite ve insan kolon karsinoma hücreleri (Caco-2) üzerinde yürütülen sitotoksisite testleri jellerden salınan GTE'nin biyoaktiviteye sahip olduğunu göstermiştir. Çalışmada üretilen jellerin su bağlama kapasitesi, fiziksel özellikleri (tekstür analizi) ve morfolojik özellikleri (SEM görüntüleme) karakterize edilmiştir. Bu çalışma aktif çok fonksiyonlu jellerin gıdalarda antimikrobiyal ve antioksidant değişimlerin kontrolü ve gıdaların biyoaktif özelliklerinin geliştirilmesi amacıyla kaplama, ped ve yenilebilir film şeklinde kullanılabileceğini göstermiştir.

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CHAPTER 1

INTRODUCTION

In rapidly developing food packaging market, the new approaches bringing functionality to new materials have gained an interest in recent years. Since traditional packaging do not fulfil the need for interactive technologies providing more quality and safety, the research on the development of new packaging materials has been increased. Nowadays, consumer demand for healthier and safer food products has prompted the research on novel preservation techniques.

Active packaging, which has a wide application scale, is one of the most promising alternatives to traditional packaging. Oxygen and ethylene scavenging, CO_2 scavenging and emitting, moisture regulating, release or adsorption of flavors and odors, incorporation and release of antioxidant and antimicrobial components are the examples of active packaging systems that have high application potential (Appendini and Hotchkiss 2002). To prevent oxidation and control the microbial growth, antioxidant and antimicrobial packaging systems are promising form of active packaging, especially for the meat products since the microbial contamination occurs primarily at the surface. A vast number of research has been done on development of antimicrobial or antioxidant coating materials and films. However, there are limited research made for developing materials showing both antimicrobial and antioxidant properties in the same body. Also, a little attention was paid to the food application studies of the developed antimicrobial systems (Han 2005). Recently, in the light of research done on active packaging, a novel approach has risen termed as “bioactive packaging” in which the consumer´s health is aimed to be improved through the foods with enhanced functionality by bioactive packaging materials (Lopez-Rubio, Gavara and Lagaron 2006).

Active agents are delivered onto food surface via different polymers prepared as packaging materials. Among these materials, edible films and coatings are one of the most popular polymeric systems due to their outstanding physical properties and possibility of being produced from different biodegradable materials including agricultural wastes. However, edible films have several limitations of application. First, self-standing films are not suitable for large-scale production and are produced discontinuously. Also, hydrophobic or hydrophilic films produced from proteins or carbohydrates are not very

compatible with dissimilar active agents, and may cause undesirable release kinetics. Another restriction of using edible films is incorporation of active agents at limited levels. So, it is essential to overcome these constrictions and propose alternative active biodegradable and edible polymeric materials that have better application possibilities.

The aim of this study is to develop antimicrobial, antioxidant and bioactive gels from gelatin and its mixtures with different polymers, and bring the main properties of active packaging concept namely improving safety against *Listeria monocytogenes*, can be found sporadically in ready-to-eat foods, such as cold smoked salmon. As well as antimicrobial activity, bioactive properties were aimed to be obtained by the active gels, such as delivery of health-supporting phenolic compounds into food systems, and the effects of the phenolics delivered by the gels on the human health has been also aimed to be investigated. Additional to the active and bioactive features of the gels, their mechanical properties were the target of improvement.

In this purpose, gelatin (Gel) based gels blended with rice starch (RS), candelilla wax (CW), and soy protein isolate (SPI) were incorporated with lysozyme (LYS) and green tea extract (GTE). Antimicrobial, antioxidant and bioactive properties of LYS and GTE agents released from Gel, Gel/RS and Gel/CW were determined. Developed gels were tested on cold-smoked salmon, and the released GTE were tested against human colon carcinoma cells (Caco-2 cell line) to see its cytotoxic effect on the cells in case of its consumption with the gel-coated food. Since no LYS release was obtained, Gel/SPI gel was evaluated as an antimicrobial absorbent gel.

CHAPTER 2

ANTIMICROBIAL, ANTIOXIDANT AND BIOACTIVE GELS

2.1. Active Packaging

The consumer demand for fresh, safe, and minimally processed foods with high quality and long shelf life increased the research on active packaging development and applications. Active packaging systems interact with the food and the environment to extend the shelf life, and to improve the quality and safety (Kuorwel et al. 2011). Examples to active packaging systems are oxygen and ethylene scavengers, CO_2 scavengers and emitters, moisture regulators, adsorbers or releasers of flavors and odors, and antimicrobial packaging systems. Among these active packaging tools, antimicrobial packaging is a promising active packaging type to maintain food quality, and to increase safety and shelf life of fresh food products.

2.1.1. Antimicrobial Packaging

Antimicrobial packaging is a novel application that can inhibit or suppress the growth of spoilage and pathogenic microorganisms contaminated on the food surfaces (Han 2005). Antimicrobial packaging systems are advantageous since they reduce the amount of preservatives used to prevent spoilage (Ahvenainen 2003). The most important advantage of antimicrobial systems is their activity on the food surface, the most susceptible area for spoilage and contaminations (Appendini and Hotchkiss 2002). Antimicrobial packaging systems include antimicrobial compounds and a carrier material to deliver or immobilize the antimicrobial agents. Main role of an antimicrobial packaging system is to control microbial growth rather than meeting the traditional packaging roles such as providing moisture and gas barrier. However, in a well-designed packaging system with antimicrobial features, maintaining other functions besides antimicrobial activity would be desired to obtain more than one benefit.

While designing an antimicrobial coating, some of the factors that may affect the impact of the antimicrobial system should be considered. Stability of the antimicrobial

agent during the formation of coating material and their compatibility with the coating polymer are the main factors. Additionally, interactions between the antimicrobial and the target food system at the storage temperature of that food should be considered (Perez-Perez et al. 2006). It is also challenging to add effective amounts of antimicrobials that does not negatively change the sensorial characteristics but provides microbiological safety.

2.1.1.1. Food Applications of Antimicrobial Packaging Systems

In designing and antimicrobial system, food composition and the target microorganism(s) are the most important considerations. There are various ways of application of antimicrobial packaging systems on food materials. Main approaches are summarized in Figure 2.1. The conventional packaging material can be modified to show antimicrobial properties, or antimicrobial materials can be combined with the conventional packaging material. Antimicrobial agents can be immobilized on the plastic packaging surfaces. In these systems the antimicrobial surface must contact to the food surface. The most researched application method of antimicrobial packaging materials is coating the food products with these materials, especially with antimicrobial edible films and coatings.

Edible and biodegradable coatings are mostly preferred due to the health and the environmental concerns (Han 2005). Edible films and coatings can be produced from hydrocolloids, lipids and composites. Hydrocolloids used in edible coating production are mostly proteins (i.e. gelatin, zein, whey protein, soy protein, wheat gluten) and carbohydrates (i.e. cellulose, alginate, pectin, starch, chitin). Composite materials are advantageous since the mechanical, barrier and release properties can be improved by adopting the best features of two different polymers to obtain biocomposite materials.

In the application of antimicrobial packaging systems on food products, the important point is the antimicrobial agents used in the packaging systems. Due to the changing consumer preferences with the demands of more natural and healthy products, natural antimicrobial agents have been employed in the development of antimicrobial systems. An ideal natural antimicrobial used in the food systems would be effective at low concentrations, inhibit a wide spectrum of microorganisms, be economically suitable and would not negatively affect the sensorial properties of food. Antimicrobials used in

active packaging are selected based on their activity spectrum, chemical composition, mode of action, and the growth rate of the target microorganism.

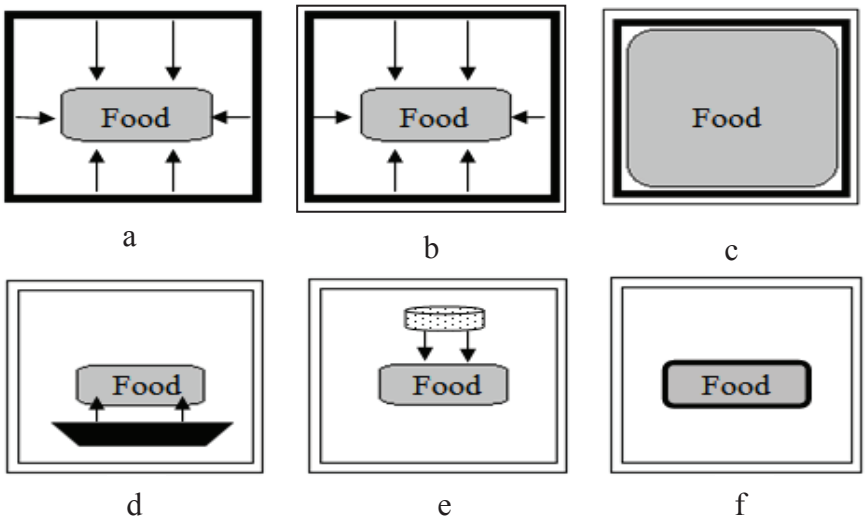

Figure 2.1. Different antimicrobial film packaging systems; (a) antimicrobial packaging, (b) antimicrobial coating on conventional packaging material, (c) immobilization of antimicrobial agents in packaging material, (d) antimicrobial tray or pad, (e) antimicrobial sachets, (f) antimicrobial edible coating on food (Source: Han 2005)

Another important issue in application of the antimicrobials in food systems is the regulatory approval of the food safety authorities. In the USA, Food and Drug Administration (FDA) and USDA-FSIS have the regulatory oversight. According to these authorities, the compounds having GRAS (generally recognized as safe) status are safe to use as antimicrobial in foods. For the ingredients that are not considered as GRAS, the regulatory statement must be taken into account while designing any antimicrobial packaging system. Lastly, while selecting an antimicrobial compound, its effect on the sensorial characteristics of the food should be considered. It is highly possible to alter the taste and the flavor of the food by antimicrobial essential oils (Davidson, Critzer and Taylor 2013).

Antimicrobial packaging systems are mostly applied on meat, poultry, fish, cheese, bread, fresh fruits and vegetables (Vermeiren et al. 2000). In particular, to reduce moisture loss, to eliminate dripping juice from meat, poultry and seafood (fish cuts), to reduce the oxidation of lipids and myoglobin in meat, to prevent flavor loss, to reduce the microbiological load and prevent growth on the surface, the barrier and delivery properties of edible coatings had an active role in active packaging applications

(Gennadios, Hanna and Kurth 1997). Some food products cannot be preserved against microbial spoilage by the traditional methods like freezing, drying, thermal or non-thermal processing. Especially ready-to-eat products are processed before packaging, and consumed without any pretreatment such as cooking. Therefore, these type of food products have high potential for food spoilage and food-borne outbreaks. Moreover, some microorganisms such as *Listeria monocytogenes* survives and maintains its biological activity even at low storage temperatures and MAP or vacuum packaging conditions (Tsigarida, Skandamis, and Nychas 2000; Yilmaz et al. 2009). Additionally, *L. monocytogenes* is tolerated to sodium chloride and low pH (Thomas, Prior and O´Beirne 1999; Rorvik 2000). For these reasons, the use of antimicrobial packaging on foods, especially on fresh and minimally processed meat products, gained an increasing attention (Coma 2008).

2.1.1.2. Evaluation of the Antimicrobial Activity of Antimicrobial Packaging Materials

There are various techniques to evaluate the efficacy of the antimicrobials employed in antimicrobial packaging systems. Mainly the antimicrobial activity can be determined in microbiological media (in vitro) or in a real food system (in situ).

In vitro methods give the best results to determine the most effective antimicrobial type and concentration of one or more antimicrobials and their combinations against target microorganisms. In these tests, a microorganism inoculated in a growth medium is challenged for a period of time in the presence of the tested antimicrobial, and the effect of a certain concentration of an antimicrobial on a determined microorganism can be evaluated. Agar plate method, minimally inhibitory concentration (MIC) and dynamic shake flask tests are in vitro methods to evaluate the antimicrobial activity. In the agar plate test, antimicrobial films or sheets are placed on a solid agar medium inoculated by the test microorganism, and the clear zones appear around the films after the incubation time as a result of the inhibitory effect. Zone diameters correlate with the antimicrobial activity. This method simulates the contacting of the antimicrobial film with the food surface and the diffusion of the antimicrobial. MIC determination gives information about the strength of the antimicrobial on the test microorganism. This test is held in the series of tubes containing the microbiological growth media seeded with the test microorganism, and the culture is treated with different concentrations of antimicrobial.

MIC is the lowest concentration of an antimicrobial causing the total inhibition of the test microorganism.

Dynamic shake flask test is performed in the inoculated liquid microbiological media, placed in flasks together with the antimicrobial material, and incubated with mild shaking. Samples are taken and enumerated over time, and the reduction in the growth rate is measured. The surface area/volume ratio of the antimicrobial polymer is proportional to the antimicrobial activity in the shake flask test (Davidson, Critzer and Taylor 2013; Appendini and Hotchkiss 2002). Another type of in vitro assay is descriptive tests such as turbidimetric assays or growth curves. Descriptive tests give information about the effect of a single concentration of an antimicrobial against a single microorganism over time. Thus, the extent of growth or death can be determined.

For antimicrobial packaging materials, not an agreed standard has been developed to test the effectivity of the antimicrobial packaging (Appendini and Hotchkiss 2002). Only to determine the self-sterilizing effect of antimicrobial films and sheets and their resistance to microbial growth, a method called “film contact method” was developed. The method is based on inoculating the bacteria on the surface of the material, and incubating for a determined period under certain conditions. The final bacterial count is compared to the initial number. This method is mostly applied for inorganic antimicrobial packaging materials (Singh et al. 2015).

After screening the efficacy of an antimicrobial against a certain microorganism, it is important to apply the same antimicrobial system to a food or model food system. For food challenge tests, a model food system or a whole food can be evaluated with the developed antimicrobial system at a determined storage temperature and period under an appropriate storage atmosphere. Few factors should be clarified for a food system application; the type of the food, storage conditions, target microorganism(s), inoculation level, type of the processes used and the success criteria (inhibition or inactivation). The selected target microorganism should represent the safety concerns on the tested food system and the inoculation level of the microorganism should be at a realistic level. The inoculum level should be appropriate to monitor both the growth and the inhibition caused by the antimicrobial (~4 log per unit weight or volume). It is also useful to test the different strains of the same organism individually, and a mixture of these strains or the most resistant system should be used in the food challenge studies (Davidson, Critzer, and Taylor 2013). However, there exists various challenges in an effective food application. Firstly, the selected antimicrobial and its upper limit that could be found in

the food must be defined by the regulatory authorities such as FDA or Codex Alimentarus. The effects of storage and temperature on the activity of the antimicrobial compound should be known. The selection of food system is another challenge since the activity of natural antimicrobial may alter greatly when incorporated in the food. Proteins, lipids, carbohydrates, simple sugars and cations have been addressed to diminish the activity of antimicrobial compounds. Also, the diffusion of antimicrobials incorporated into antimicrobial packaging materials can be altered by the food components (Appendini and Hotchkiss 2002; Suppakul et al. 2003). It should be considered that the possibility of resistance development of the microorganisms against the antimicrobial and the influences of the natural microflora on the antimicrobial activity. On the other side, low pH of some food systems such as fruit juices may positively interact with the antimicrobial compound and inhibit the target microorganism more effectively compared to the activities obtained from the tests performed in culture growth media. Resulting from their nonhomogeneous nature high in lipid and proteins, meat, poultry and seafood products are the most difficult food systems to be employed in the challenge test of a natural antimicrobial (Davidson, Critzer, and Taylor 2013).

According to a research by Mendoza-Yepes et al. (1997), minimum inhibitory concentration (MIC) of rosemary, sage and citrus essential oils were determined as 500 mg/L in microbiological growth media, however 2500 mg/L of essential oils did not control *E. coli* O157:H7 in fresh cheese. Similarly, 0.2 mg/mL rosemary extract showed good inhibitory effect in vitro, but showed no antimicrobial activity against *Campylobacter jejuni* in chicken meat (Piskernik et al. 2011)

2.1.1.3. Natural Antimicrobials

Antimicrobials are chemical compounds that naturally present or added into foods, food packaging materials, or surfaces contacting to food to inhibit the growth or to inactivate the pathogenic or spoilage microorganisms. There is an increasing search for the alternatives for application of natural antimicrobial preservation in foods, especially in minimally processed foods. Due to the safety concerns of the consumers, a great interest has been raised for incorporating natural antimicrobials such as enzymes (lysozyme, lactoperoxidase), bacteriocins (i.e. nisin), essential oils (i.e. thymol, carvacrol) and phenolic compounds into natural polymers, especially into edible films (Suppakul et al. 2003).

Natural antimicrobials may be produced from animal, plant, or microbial sources. Animal-origin antimicrobials, except chitosan, are found in polypeptide form and obtained from mammals or avian. The most common studied animal-origin antimicrobials are chitosan, lysozyme, lactoferrin, lactoperoxidase and conalbumin (ovotransferrin). They show antimicrobial activity against bacteria and fungi with different mode of actions such as destabilization of outer membrane, hydrolysis of peptidoglycan, sequestration of essential nutrients and enzymatic formation of antimicrobial components from the naturally occurring compounds in the environment. Plant-origin antimicrobial compounds are mainly the phenolic compounds found in spices, herbs, fruits and vegetables, and are strong in flavor. Examples to plant-origin antimicrobials applied in food systems are eugenol, thymol, carvacrol, vanillic acid and cinnamaldehyde found in spices, onion and garlic extracts, and polyphenols and phenolic acids found in berries, pomegranate, grape seed, olive products and tea (Tiwari et al. 2009; Tajkarimi, Ibrahim and Cliver 2010; Gyawali and Ibrahim 2014). Microbial-origin antimicrobials are mainly the bacteriocins produced by gram-positive bacteria during their growth and fermentation. Natamycin, produced by *Streptomyces natalensis,* mostly effective against fungi, and nisin, produced by *Lactococcus lactis,* effective against gram-positive bacteria are used in the food systems. Also bacteriophages are the viruses used to control of *Listeria monocytogenes*, *E.coli* O157:H7 and *Salmonella* (Davidson, Critzer, and Taylor 2013).

2.1.1.3.1. Lysozyme

Lysozyme is one of the most frequently used natural antimicrobial agents in antimicrobial packaging (Han 2003; Güçbilmez et al. 2007; Bower 2006). This non-toxic hydrolyase-type enzyme splits the bonds between N-acetylmuramic acid and N-acetylglucosamine found in the peptidoglycan layer, the constituent of cell wall of Gram-positive bacteria (Benkerroum 2008). Therefore, lysozyme is only effective against Gram-positive pathogens and spoilage bacteria such as *Bacillus* species*, Listeria monocytogenes, Clostridium tyrobutyricum* and lactic acid bacteria (Abdou et al. 2007; Hughey, Wilger and Johnson 1989; Wasserfall and Teuber 1979; Chung and Hancock 2000). Gram-negative bacteria are not sensitive to lysozyme because of their protective lipopolysaccharide layer (Mecitoğlu and Yemenicioğlu 2006; Bower 2006). All mammals, birds and fishes are the source of lysozyme; but mainly hen-egg white is used

to produce lysozyme (Bower 2006). Approximately 3-5% of hen egg albumin is made of lysozyme, and 3400-5840 mg/L lysozyme can be produced from it.

Lysozyme has a GRAS (Generally Recognized As Safe) status, and its use as a direct food additive is approved by regulations, and is widespread in foods such as cheese, vegetables and salads (Gemili, Yemenicioglu and Altinkaya 2009). The allowed concentration of lysozyme in cheese is maximum 500 ppm (Codex General Standard). Many edible film structures were incorporated with lysozyme such as corn zein (Mecitoğlu et al. 2006), chitosan (Park, Daeschel and Zhao 2004), whey protein isolate (Min, Rumsey and Krochta 2008) alginate (Cha et al. 2002) and sodium caseinate (De Souza et al. 2010). Lysozyme and nisin are effective natural antimicrobials against gram positive bacteria, and their combination with ethylenediamine tetracetic acid (EDTA), a metal chelator, increases their antimicrobial effect (Padgett, Han and Dawson 1998). The activity of lysozyme can be significantly affected by temperature and pH (Han 2005).

On food applications lysozyme showed significant antimicrobial activity against various spoilage and pathogenic gram-positive bacteria. Gill and Holley (2000) added mixture of lysozyme and nisin (25.5 g/L, 1:3), and EDTA (25.5 g/L) to pork bologna sausages on the surface and obtained inhibition of *L. monocytogenes*. Similarly, nisin and lysozyme together kept the number of *L. monocytogenes* under detection levels for 10 weeks of refrigerated vacuum storage in pasteurized turkey bologna (Mangalassary et al. 2008). LYS caused lower number of *Brochotrix thermospacta* and *Carnobacterium* on pork tissue (Nattress et al. 2001).

2.1.1.3.1.1. *Listeria monocytogenes* as a Risky Pathogen in Minimally Processed Foods

The consumption of a wide range of products such as meat, poultry, sea food and dairy products, stored refrigerated and consumed as ready-to-eat food commonly associated with *L. monocytogenes* if the prepared food is undercooked or becomes cross-contaminated with this pathogen. *L. monocytogenes* ingested in contaminated foods normally causes flu-like symptoms in healthy adults. The listeriosis disease is more serious in elderly, immunocompromised adults and unborn fetuses, causing meningitis and meningoencephalitis, abortions, and stillborn deaths. In the United States 208 listeriosis cases since 2010, and in Europe around 2200 listeriosis cases in 2015 were reported (Buchanan et al. 2017; efsa.europa.eu)

Food-borne transmission of listeriosis to humans probably occurs mainly through environmental contamination because of the uncommon ability of *L. monocytogenes* to colonize animals and food contact surfaces from environmental sources. This pathogen can be spread through the food processing plants. It has been isolated generally from raw milk, cheeses, eggs, vegetables, salads, meat and poultry products, and sea foods (Sanaa et al. 1993; Ryser and Marth 2007). The preservation of food from *L. monocytogenes* depends on eliminating this pathogen through processing with thermal or non-thermal treatments, avoiding recontamination and addition of antimicrobial protection when the intrinsic protection is not sufficient. Especially in the case of recontamination, a preservation tool is critical to prevent foodborne outbreaks. Since *L. monocytogenes* is Gram-positive, lysozyme (LYS) is one of the most frequently used natural antimicrobial compound alone or in edible films (Appendini and Hotchkiss 1997; Benkerroum 2008; Min, Rumsey and Krochta 2008).

Listeria monocytogenes can survive and grow on ready-to-eat meat products at refrigeration temperatures. Hughey, Wilger and Johnson (1989) studied the activity of LYS against *L. monocytogenes* inoculated on vegetables, fresh sausage and cheese. In the vegetables 100 mg/kg LYS generally showed listeriastatic effect where the growth throughout the storage at 5 °C was neither declined nor increased significantly. It showed less success on animal-based food products. However, when LYS was combined with 5mM EDTA, the inhibitory effect increased for all food samples inoculated with 10^4/gram of food *L. monocytogenes*.

Cold-smoked salmon is one of the most susceptible ready-to-eat product to *L. monocytogenes*, since this pathogen sporadically found in the processing plant, and the postprocessing contamination risk during slicing and packaging is relatively high (Peiris et al. 2009; Rorvik 2000; Serio, Chaves-Lopez and Paparella, 2011). Eventhough liquid smoke treatment on salmon fillets can reduce or eliminate the *Listeria* number present in the fish (Vitt 2001) post-contamination following smoking process may occur due to the inevitable presence of *L. monocytogenes* widely spread through the processing plant. Additionally, antimicrobial effect of liquid smoke provided by the phenols found in it may reduce after the treatment due to the complexation of phenolics with the proteins of salmon (Vitt 2001). It was not reported any *Listeria* outbreak caused by cold-smoked salmon consumption, however the presence of *L.monocytogenes* in vacuum packed cold-smoked salmon is confirmed by many sources (Rorvik 2000; Buchanan et al., 2006; Tocmo et al., 2014; EFSA 2009; Dass et al. 2011).

L. monocytogenes could survive and grow in vacuum packed smoked salmon stored at 4° C, for 5 weeks and in the various range of pH and salt concentration of the product (Rorvik, Yngestad and Skjerve 1991). In Estonia, 11% of the 370 ready-to-eat food products was contaminated with *L. monocytogenes*. The contamination in fish products (17%) was higher than ready-to-eat meat products (6%) (Kramarenko et al. 2016). The regulation set by EU Commision allows prevalence of *L. monocytogenes* less than 100 cfu/g in food products (EU Comission, 2073/2005). Listeriosis caused by smoked salmon consumption became a significant threat for public health. It was estimated that 15-17.5 % of smoked fish in USA is contaminated with *L. monocytogenes* (Heinitz and Johnson 1998).

2.1.2. Antioxidant Packaging

Antioxidants are used to prevent lipid oxidation in foods and extend the shelf life of oxygen-sensitive foods. Incorporation of antioxidants into packaging materials become popular since oxidation is a major problem affecting quality. Lipid oxidation, a major cause of spoilage after microbial growth, is an important quality deterioration especially in muscle foods causing various breakdown products which results in undesirable off-odors and off-flavors (Kerry, O´Grady and Hogan 2006). Other negative effects are the formation of toxic aldehydes and the loss of nutritional quality because of polyunsaturated fatty acid (PUFA) degradation (Guillen and Goicoechea 2008).

In antioxidant packaging, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were frequently used because of their low cost, stability and efficiency. There are few examples of antioxidant packaging researched, such as BHT impregnated cereal packs to extend shelf-life (Vermeiren et al. 2000). Jongjareonrak et al. (2008) reported that antioxidant fish-gelatin film loaded with α-tocopherol and BHT was coated on lard, and gelatin film alone showed preventive effect against lipid oxidation where antioxidant compounds showed negligible effect. However, the concerns about the health effects of BHT limits its release onto food. Due to the toxicological characteristics of synthetic antioxidants, natural antioxidant compounds are preferred and studied extensively to be employed in antioxidant packaging systems (Siripatrawan and Harte 2010).

The alternative approach that is being studied widely is the use of natural antioxidants, particularly tocopherol, plant extracts, and essential oils from herbs and spices. The release of these natural antioxidants can even increase the nutritional value of the food while preventing from lipid oxidation (Gomez-Estaca et al. 2014). Rosemary and oregano extracts incorporated into active films increased the oxidative stability of lamb meat stored at 1 °C while being exposed to light for 13 days (Camo, Beltran and Roncales 2008). Similarly, corn zein films laminated by LDPE and incorporated by antioxidant essential oils inhibited the lipid oxidation in beef patties stored for 14 days (Park et al. 2012).

In the literature, there are few examples of packaging materials possessing both antimicrobial and antioxidant properties tested on the food systems. Ha, Kim and Lee (2001) developed polyethylene films containing grapefruit seed extract (0.5-1%) inhibited the growth of aerobic and coliform bacteria on fresh minced beef while enhancing the color and oxidative stability. Zein based films incorporated with lysozyme, catechin and gallic acid showed inhibitory effect against *L. monocytegenes* and prevent oxidation in fresh kashar cheese (Unalan et al. 2013).

2.1.2.1. Antioxidant Activity of Phenolic Compounds

Antioxidants are the principal nutrients responsible for the prevention of various diseases, especially cardiovascular disease and some types of cancer. During normal aerobic metabolism, reactive oxygen species (ROS) that can cause damage in DNA, proteins and lipids are formed, and natural antioxidant defense system is not sufficient to repair it. Accumulation of damaged units such as oxidized LDL and free oxygen radicals in the body is critical in the development of cancer, diabetes, chronic inflammation and atherosclerosis (Knekt et al. 2002). Many researches showed the in vitro antioxidant activity of phenolic compounds present in different plant-origin foods (Ross and Kasum 2002). Flavonoids show antioxidant activity by their free radical scavenging properties that helps repairing biological units such as repairing single strand break in DNA, and by chelating transition metal ions and inhibit the initiation of lipoxygenase reaction. Free radical scavenging capacity is primarily attributed to the high reactivities of hydroxyl substituents that participate in the following reaction:

$$F\text{-}OH + R^{\cdot} \rightarrow F\text{-}O^{\cdot} + RH \quad (2.1)$$

Antioxidant capacity of phenolic compounds depends on several structural properties. Number and position of hydroxyl (-OH) groups relative to carboxyl (-COOH) group affects the antimicrobial activity. Hydroxyl group at o- and p- positions show no antioxidant activity, but -OH at m- position has antioxidant activity in phenolic acids. Also, side chains of phenolic acids, degree of hydroxylation and, presence of -OH groups at 3′, 4′and 5′positions, a double bond between C2 and C3 conjugated with 4-oxo group in ring C, and a double bond between C2 and C3 atoms combined with 3-OH in ring C gives high antioxidant activity (Fennema 1997).

It is desired to test the antioxidant activity of the compounds with more than one assay, since each assay approaches to measurement in different ways (Erkan, Ayranci and Ayranci 2008). Radical trapping methods such as DPPH, FRAP, TEAC and ORAC are frequently employed. Using these methods, it is possible to quantify an antioxidant compound with known antioxidant activity.

There are various assays to determine the antioxidant capacity of a compound. Trolox equivalent antioxidant capacity (TEAC) assay, also known as ABTS assay, can determine the radical scavenging activities of phenolic compounds and allows comparison with each other. TEAC method is based on electron transfer, and it evaluates the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) cation ($ABTS^{\cdot+}$) scavenging ability of the antioxidant compounds in aqueous phase. Antioxidant activity is generally expressed as the mM Trolox equivalent (vitamin E analogue) of the tested compound. According to Rice-Evans (1997), phenolic acids possess lower TEAC values than flavonoids. Among the tea catechins EGCG and EGC have the highest free radical scavenging activity (Henning et al. 2003). Although tea flavonoids show great antioxidant activity in in vitro studies, bioavailability of those catechins determines their in vivo antioxidant activity.

The oxygen radical absorbance capacity (ORAC) assay measures the total antioxidant power of a compound or extract, and is based on the ability to inhibit the oxidation of a fluorophore, generally fluorescein, by a potent oxidant, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH). As a result of free radical damage, fluorescent intensity reduces in time. In the presence of antioxidants, the damage of free radical on fluorescein is inhibited and the reduction in the fluorescent intensity lowers down. ORAC method can combine both inhibition time and degree of inhibition into a single quantity

(Cao, Sofic and Prior 1996). In this assay, hydrogen atom transfer mechanism is utilized. ORAC assay is an accepted method to indicate the antioxidant capacity of some antioxidant supplements and nutraceuticals on the product labels. The ORAC assay is considered by some to be a preferable method because of its biological relevance to the in vivo antioxidant efficacy (Haytowitz and Bhagwat 2010).

Another important activity of phenolic compounds is their metal chelation properties. Prevention of metal redox cycling, blocking the interaction sites of metals, and forming insoluble complexes with metals can be attributed as metal chelation (Hider, Zu and Hicham 2001). Transition metals have a major role in the generation of oxygen free radicals in living organisms. There exist two different forms of oxidized iron; ferrous and ferric irons. The ferric iron (Fe^{3+}) is the relatively biologically inactive form of iron. However, it can be reduced to the active Fe^{2+}, depending on the conditions (Strlic et al. 2002). As a redox-active transition metal, iron can easily take part in free radical production. Free radicals have various damage in metabolism such as causing lipid peroxidation, protein modification and DNA damage. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes (Finefrock, Bush and Doraiswamy 2003). In human body, iron-dependent lipid peroxidation may cause serious tissue damage. Especially the damage occurs in brain tissue rich in long chain unsaturated fatty acids cause neurodegenerative diseases such as Parkinson´s disease (Guo et al. 1996). It was claimed that iron chelating properties of green tea extract has positive effects on the prevention of Alzheimer and Parkinson´s diseases (Mandel et al. 2006; Mandel et al. 2008).

2.1.2.2. Phenolic Compounds

Phenolic compounds are secondary metabolites produced by plants in three pathways; phenyl propanoid pathway, shikimate pathway and pentose phosphate pathway. Basically, phenolic compounds are classified into three groups; phenolic acids, flavonoids and tannins. The largest group of polyphenols responsible for flavor and color of the plants are flavonoids.

2.1.2.2.1. Flavonoids

Flavonoids constitute the largest group of polyphenols. Their basic structure consist two aromatic links linked via three carbons, and is defined as diphenylpropanes (C6-C3-C6) (Figure 2.2). According to the varieties in heterocyclic C-ring, flavonoids are classified under six groups, namely flavones, flavonols, flavanones, flavanols, anthocyanidins and isoflavones. Flavonoids in plants generally found as attached to sugars (glycosides), and rarely as aglycones, therefore they tend to be water-soluble (Kumar and Pandey 2013). In food, flavonoids exist primarily as 3-O-glycosides and polymers. Tannins and other complex compounds found in black tea are formed by polymerization of flavonols during fermentation. Esters of gallic acid are known as hydrolysable tannins or gallotannins. The galloyl moieties of these tannins and of the monomeric catechins in green tea are partly responsible for the chelating and radical scavenging properties of these compounds (Cook and Samman 1996; Rice-Evans et al. 1996).

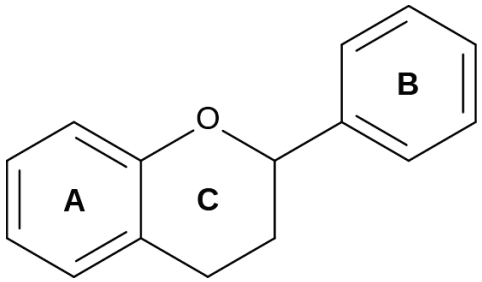

Figure 2.2. Basic flavonoid structure
(Source: Ross 2002).

The possible health benefits of flavonoids made high flavonoid containing plants very popular in nutrition. Physiological activities of flavonoids are mainly antioxidant activity, antimicrobial activity, anti-tumoral activity, anti-inflammatory activity and antiplatelet activity.

Major flavonoid sources are vegetables, fruits, tea, cacao and wine (Heim, Tagliaferro and Bobilya 2001). Tea contains mainly flavonols and flavons. Tea is one of the most consumed beverage throughout the world, and its consumption increases day by day due to the consumer preferences regarding to its bioactivity and in vitro antioxidant activity, especially of green tea. However, there are many different biological activities attributed to the flavonoids. Research about the health effects of flavonoids claimed their preventive activity against cardiovascular diseases and cancer; on the other side some

researchers reported that flavonoids have no effect or even have adverse effects (Sak 2014; Batra and Sharma 2013). Even though more studies are necessary to conclude the effect of flavonoids on human health, majority of the researches have been reported positive results for the intake of flavonoids, especially tea catechins.

2.1.2.2.1.1. Flavonoids Found in Green Tea Extract and Their Antioxidant Activity

Tea is produced from the leaves of the plant *Camellia sinensis.* Phenolic composition of tea is depended on climate, variety, season, horticultural practices and the age of the leaves. In green tea production, leaves are steamed or heated to inactivate the enzymes responsible for polyphenol oxidation. About 78% of the tea production worldwide is black tea, whereas green tea, mainly consumed in China and Japan, constitutes about 20% (Yang et al. 2002). Catechins are the tea polyphenols constitute 30-42% of the solids in brewed green tea. Due to the different analysis methods for the flavonoids, in the literature the flavonoid contents of certain foods and dietary intake levels are different from each other and sometimes contradictory.

The structures of the four major catechins, (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC) are shown in Figure 2.3. EGCG is the major catechin in tea and may account for 50% to 80% of the total catechin in tea. Catechin, gallocatechin, epigallocatechin digallates, epicatechin digallate, 3-*O*-methyl EC and EGC, catechin gallate, and gallocatechin gallate are found in smaller quantities. Flavonols, including quercetin, kaempferol, myricitin, and their glycosides, are also present in tea.

Antioxidant activity of tea extract is well correlated with its antimutagenic effect and cytotoxicity (Yen and Chen 1995). In vivo antioxidant activity of green tea was also investigated. Plasma AUC values measured with TEAC method were higher when green tea supplement was consumed compared to green tea and black tea. Consumption of green tea phenolics as supplement rather than infusion also enhanced their bioavailability (Henning et al. 2004).

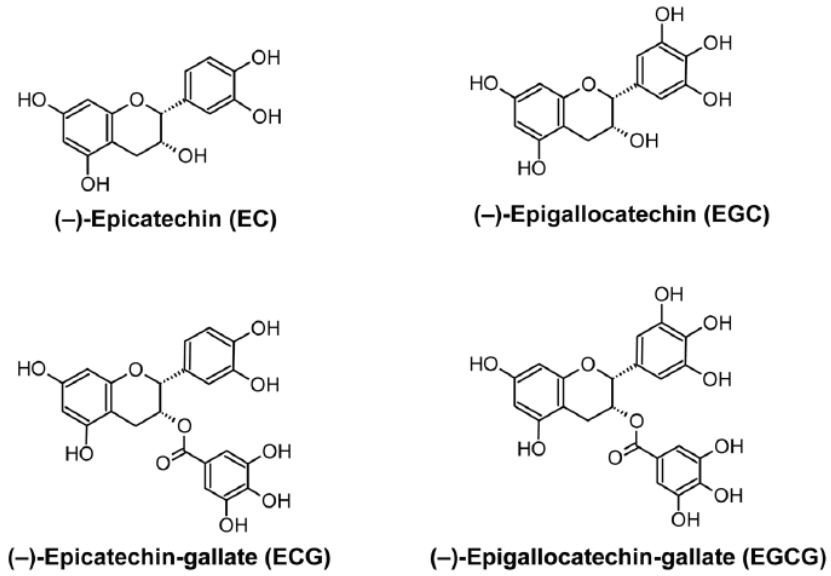

Figure 2.3. Major catechins found in green tea
(Source: Green et al. 2007).

2.1.3. Bioactive Packaging

Bioactive packaging is a novel concept in which the consumer´s health is aimed to be improved through the packaging or coating materials (Lopez-Rubio, Gavara and Lagaron 2006). To support health and to prevent diseases such as cancer, cardiovascular disease, ageing-caused disorders and gastrointestinal problems, diet is the major factor. Due to the increase in the consumer demand in functional foods, new products enhancing human health have gained a huge interest. If a food has targeted to affect one or more body functions in a positive way or reduce the risk of a disease not only by its inherent nutrients but more, that food is considered as "functional" (Siro et al. 2008). For some important nutrients, the daily recommended intake levels are not reached according to the surveys taken about nutrition (USDA,1990). So, the functional foods can be highly demanded to compensate the nutrient lack, especially in the countries where the western-type of diet is dominant. However there exist several challenges in the functional food production such as the loss of functionality during processing, storage and marketing. Also, it is important to provide the safe dose of the micronutrients carried by the functional foods. In this case, just like the antimicrobial/active packaging, functional coating materials covering the food surface may be a good solution since the release of the micronutrients into the food matrix would be slower and controlled compared to the direct addition in the food bulk. The main difference between active and bioactive

packaging is; the first aims to maintain food safety and quality but the latter one aims a direct impact on the human health. Bioactive packaging approach can be achieved by the release of the active compounds from a biodegradable (or sustainable) polymer matrix or from micro/nano-encapsulates incorporated into the packaging materials or foods. Among the functional compounds, phytochemicals, vitamins, nanofibers and probiotics are the most suitable for incorporating into the coating matrix (Lopez-Rubio, Gavara and Lagaron 2006).

Phytochemicals are plant-sourced chemicals and more than 900 different types are found in fruits, vegetables, spices and tea. They are mainly related to the prevention of cancer, cardiovascular diseases, diabetes and hypertension (Hasler et al. 2004). The major group of phytochemicals are polyphenols known for their antioxidant properties resulted from the free radical scavenging or metal ion-chelating activities (Shahidi 2000). However, naturally found polyphenols in the food products are not stable and lessens during the processing steps. Therefore, it is supportive to replenish the lost phenolic content using the functional coating materials.

In this study, the bioactive gels releasing GTE into the food materials together with LYS, have three main roles which are maintaining antimicrobial (antilisterial), antioxidant and bioactive activities.

2.1.3.1. Effects of Tea on Human Health

The possible chronic disease-preventive potential of tea has received a great attention in recent years because of its antioxidant, anticancer, antidiabetic, antiproliferative and anti-inflammatory activities demonstrated in in vitro, in vivo, and epidemiological studies (Higdon and Frei 2003). Epidemiological studies in different countries, however, have not generated consistent results concerning the cancer-preventive effect of tea in humans. Biologic activities of tea include scavenging of reactive oxygen and nitrogen species, chelation of redox active metals, inhibition of cancer-related transcriptional factors, and inhibition of oxidative enzymes (Green et al. 2007).

A clinical study showed a relationship between flavonoid intake and reduced risk of chronic diseases, especially lung and prostate cancer in men, cardiovascular diseases, and asthma (Knekt et al. 2002). Similarly, the Zutphen Elderly Study demonstrated an

inverse relationship between consumption of catechin, a predominant flavonoid in tea, and ischemic heart disease mortality in a group of 806 men (Arts et al. 2004).

There have been numerous studies reported high flavonoid intake is associated with decreased risk of cancer including colon, breast, lung, esophageal, bladder, stomach, pancreas and oral cancer. Antiproliferative activity of flavonoids helps the inhibition of cellular transformation and proliferation. Kuntz et al. (1999) showed antiproliferative activity of 30 flavonoids against colon carcinoma cells but no cytotoxicity. High daily intake (26 mg/day) of flavonoids is also related to lower risks of cardiovascular diseases (Hertog et al. 1997). Another human study conducted for 4-25 years indicated that there exist no association between flavonoid intake and cancer, which was a contradictory result (Goldbohm et al. 1995; Hertog et al. 1995). Average intake of flavonoids showed inverse correlation with coronary heart disease according to a research done with a cohort of sixteen people. Tea consumption was related with the decreased risk of stroke according to Keli et al. (1996).

As a result, flavonoids have a potential beneficial effect for human health; however, the in vitro studies and human studies have given conflicting results. Each flavonoid compound may show different health effect on human resulting from the differences in their chemical structure, bioavailability, and metabolism.

2.1.3.1.1. Antidiabetic Activity of Green Tea

Starch takes an important place as dietary carbohydrate in human diet. After consumption, enzymatic digestion of starch takes place in the small intestine and main enzymes responsible of starch hydrolysis are α-amylase and α- glucosidase. α-Amylase hydrolyzes the α-1,4-linkage of starch molecules and further digestion is performed by α-glucosidase. The product of α- glucosidase activity is glucose. Glucose is highly and quickly transported into the bloodstream and causes a rapid increase in the plasma glucose level. Consumption of carbohydrates with high glycemic index is the causative factor in development of mainly type 2 diabetes, obesity and cardiovascular diseases. The presence of inhibitors of α-amylase and α- glucosidase in the dietary intake has been proposed to be effective in reducing plasma glucose level due to the retardation in the starch hydrolysis in the small intestine. In the prevention or treatment of type 2 diabetes, the enzyme inhibitors, such as acarbose used as a diabetic drug, has an important role.

However, natural enzyme inhibitors such as phenolic compounds in tea or fruits and vegetables are more favorable with no serious adverse effects (Koh et al. 2010). One of the important bioactive properties of tea is its antidiabetic effect. Tsuneki et al. (2004) showed that drinking green tea increased oral glucose tolerance. A study conducted with normal rats suggested that long term administration of green tea extract increased sensitivity and reduced insulin resistance in fructose-fed rats (Wu et al. 2004). The most active green tea catechin to prevent diabetes was claimed to be EGCG (Wu et al., 2004).

2.1.3.1.2. Antihypertensive Activity of Green Tea

In human body, the most important mechanism controlling regulation of blood pressure, fluid and electrolyte balance is renin-angiotensin system. Angiotensin-converting enzyme (ACE) involved in this mechanism converts angiotensin-I to angiotensin-II. Angiotensin is a peptide hormone responsible of vasoconstriction and increase in the blood pressure. In the metabolism, angiotensin-I is inactive and exists as the precursor of angiotensin-II, the hormone causes high blood pressure. To control the increase in the blood pressure in patients with hypertension and heart failure, ACE inhibitors are used such as captopril (Persson et al. 2006).

The effect of green tea on ACE activity was investigated in vitro and in vivo. Tea catechins showed dose dependent inhibitory effect on ACE enzyme and 30 min after oral administration of single dose of green tea a significant inhibition of ACE was observed (Persson et al. 2010). However, there are conflicting and inconsistent studies about the relation between green tea catechins and blood pressure regulation.

In a study conducted with rats, it was shown that black and green tea controlled the blood pressure increase (Negishi et al. 2004). A study investigating the hypertension risk and tea consumption in Chinese population showed that 120 mL/day green tea or oolong tea consumption reduced the hypertension risk (Yang et al. 2004). Researches claimed that green tea preserves arterial and endothelial functions, and prevents the development of atherosclerosis (Singh et al., 2005; Geleijnse et al. 1999).

2.1.3.1.3 Antiproliferative Activity of Green Tea Flavonoids

In vivo and in vitro studies suggested that tea consumption can have a protective effect on certain human cancer types, such as skin, lung, esophagus, and colon (Mantena et al. 2005; Okabe et al. 1997; Gao et al. 1994; Kumar et al. 2007). Inhibitory effect of green tea catechins on proliferation of colon carcinoma, melanoma, breast carcinoma and lung carcinoma cells, was shown (Jung et al. 2001; Valcic et al. 1996). Among the polyphenols found in green tea, (-)-epigallocatechin-3-gallate (EGCG) was suggested as the most active catechin, and has been studied extensively for its chemopreventive potential. Chen et al. (1998) suggested that EGCG inhibited the growth of colon carcinoma (Caco-2) and breast ductal carcinoma (Hs578T) cells at a proper concentration without any adversary effect on their normal counterparts. EGCG and EC at increasing doses decreased the cell viability of colon cancer cells HT29. Even though the mechanism of action of phenolics on carcinoma cells is not clear, it is known that chemical structure and differences in the signal transduction pathway modulation determine the bioactivity of phenolics such as different differentiations rates in the cancer cells (Sanchez-Tena et al. 2016). EGCG could inhibit colon cancer (HT29) tumor growth and prevented formation of new blood vessels by down-regulating vascular endothelial growth factor (VEGF) (Jung et al. 2001). On liver cancer cells (Hep G2), EGCG induced apoptosis, increased expression of p53, and blocked cell cycle progression (Kuo and Lin 2003).

2.2. Active Gel Concept

In the developing food industry, the interest in colloid-based delivery systems has been growing, especially encapsulation and release of active compounds using microcapsules and polymer films. Considering the environmental, economic and safety issues, food-grade polymers are advantageous to prepare these delivery systems. Biopolymers, such as proteins and polysaccharides are one of the most popular and promising materials to be used as building blocks. Besides the widely researched biodegradable films and their food applications, the gels prepared from biopolymers can be alternative active agent carriers for food systems (Matalanis, Jones, McClements 2011, Bower et al. 2006).

Hydrogels are polymer networks with three-dimensional structure, that are widely used in drug delivery, tissue engineering, food industry and biomedical engineering. Food gels are produced mainly using polysaccharides and proteins. Although, there exists many processed food products possessing gel properties such as jam, jelly, desserts, confectionary products etc. Mainly food hydrocolloids from natural sources can be employed particularly to produce food-like gels. In the industrial area, proteins and polysaccharides are used as stabilizer, thickening and gelling agents, to make emulsions and dispersions, inhibit or decrease syneresis and increase water retention (Sutherland 2007). More recent attention has focused on the research on development of gel materials for the delivery of active compounds. Campia et al. (2017) developed aerogel from galactomannan with the release properties of nisin, lysozyme, polymyxin B, a protease and a lipase, and suggested it as a new delivery material. Starch hydrogel crosslinked by trisodium citrate was tested for caffeine delivery, and with increasing cross-linking slower release with improved gel characteristics were obtained (Abhari et al. 2017). Moritaka and Naito (2002) compared the flavor release properties of agar and gelatin gel. An innovative study carried by Li et al. (2011) suggested a chitosan based polycationic antimicrobial poly(ethylene glycol) methacrylate and poly(ethylene glycol) diacrylate hydrogels absorbing the Gram-positive and Gram-negative bacteria from the surfaces to inhibit them. However, the developed gels in the mentioned studies were suggested for pharmaceutical or medical uses. For food preservation and food safety applications, most of the research on gel coatings did not possess any delivery function, but only barrier properties. *Aloe vera* gel was coated on grapes and nectarine cold-stored for three to seven weeks prolonged the shelf-life and preserved the quality parameters of the coated fruits (Serrano et al. 2006; Ahmed, Singh and Khan 2009).

The use of gel media as delivery systems is well known in pharmaceutical area. However, their potential in food science as antimicrobial, antioxidant and other bioactive materials needs to be researched more.

2.2.1. Use of Biopolymers in Delivery Systems

A polymer is a huge molecule made from hundreds of units called monomers that are capable of forming at least two bonds. The number of bonds that a monomer can form is called functionality. A polyfunctional unit with random branching can make cross-links

with other units to form a three-dimensional structure (Brinker and Scherer 1990). Colloids are mixtures whose particles are larger than molecules but smaller than particles and mostly found as colloidal dispersions. To design a polymeric structure, it is essential to understand the molecular characteristics of the main units. The type, number and distribution of the monomers in a polymer, determines the overall properties such as molecular weight, conformation and electrical charge (Matalanis, Jones, McClements 2011). The selection of the proteins and polysaccharides depends on their ability to assemble and form a structure, their functional requirements, the legal status, cost, ease of use and processing.

The structure of proteins depends on their amino acid sequence and environmental conditions such as pH, temperature, pressure, solvents and ionic composition. To produce protein-based systems, proteins must be able to associate with other molecules. To provide this, the denaturation or helix-coil transition temperature, isoelectric point (pI), sensitivity to ions and enzymes, and the most importantly the electrical characteristic of a protein should be known (Matalanis, Jones, McClements 2011). Also the morphology (globular, fibrous) and physical properties are important to determine the physical and functional properties during and after association (LaClair and Etzel 2010).

Figure 2.4. Simple representation of the molecular structures of proteins and polysaccharides (Source: Matalanis, Jones, McClements 2011)

Polysaccharides vary from one another by their type, sequence, bonding and number of monosaccharides within a chain, and differ in molecular weight, branching, charge, flexibility and interactions. Just like proteins, the knowledge of the helix-coil transition temperatures, electrical charge (pKa) and sensitivity to ions and enzymes is important to fabricate a polymeric structure. Electrical charge is described by the ζ-potential versus pH profile. In proteins, the charge is positive below their pI, zero at the pI and negative above pI. However, at the pI, the molecules have negative and positive regions at the surface which provides an attraction or repulsion. In polysaccharides, electrostatic characteristic depends on whether the molecule is neutral (starch, cellulose),

anionic (alginate, carrageenan) or cationic (chitosan). Anionic polysaccharides tend to be neutral below their pKa but negative above, cationic polysaccharides are neutral above pKa but positive below.

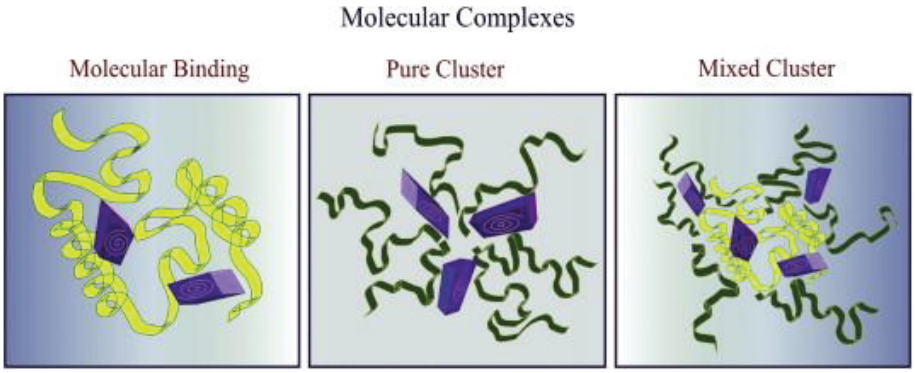

Figure 2.5. Types of molecular complexes formed between lipophilic complexes and biopolymers (Source: Matalanis, Jones, McClements 2011)

Biopolymer particles can be formed by,

i. heating above the thermal denaturation temperature; for globular proteins, the holding time, temperature, pH and ionic strength affect the size and charge.

ii. cold-set particle formation; for thermally denatured globular proteins, by altering the pH and ionic strength the self-association is promoted. Proteins attract at their pI and presence of monovalent or divalent counter-ions.

iii. changing the quality of the solvent; addition of alcohol to an aqueous biopolymer solution cause aggregation of the molecules, i.e. BSA, gelatin and caseinate nanoparticles formed after ethanol addition.

iv. Adding cross-linking agent; particle formation can be promoted by cross-linking agents such as enzymes (transglutaminase), chemicals (glutaraldehyde) and mineral ions (calcium). Chemical cross-linking consists covalent bonds, and the physical cross-linkings involve hydrogen bonds, hydrophobic interactions and ion bridging (Williams 2007).

Both proteins and polysaccharides are able to form gel, however the gelation mechanisms and properties of these two types of biopolymers are different. Proteins are gelled through controlled cross-linking or controlled aggregation. Basically, heating

above thermal denaturation temperature promotes the association, hydrophobic attraction and disulphide bond formation of globular proteins. The flexible random coil proteins such as gelatine can form gel by cooling below helix-coil transition temperature where hydrogen bonds are formed. Polysaccharides make gels by cold-setting and heat-setting methods (Morris 2007).

2.2.2. Gels

A gel is defined as a two-component system of a semisolid nature having both elastic and flow characteristics (Brinker and Scherer 1990). However, not all the gel structures are fitting in this definition such as silica gels which are rigid. So, another definition can be given as a gel is a continuous solid and fluid phases of colloidal dimensions. When polymer molecules reach macroscopic dimensions by aggregation or condensation and links to the other cluster, an extended cluster is formed called as gel structure.

One of the first definitions of gel is; “it is a semi-rigid mass of a lyophilic sol in which all the dispersion medium has been absorbed by the sol particles”. By Flory and Hermans, gels are defined as coherent colloid disperse systems containing at least two components (solid and liquid), thus they show the mechanical properties of solid and liquid component exist continuously within the system (Almdal et al. 1993). A schematic illustration of the continuity of the solid or liquid phase is given in Figure 2.6, showing interconnected particles in a continuous network is dispersed in the liquid phase.

Continuity means that both liquid and solid phases are entirely connected and can be followed without entering the other phase. Similarly, as seen in the Figure 2.6, an arrow originating from a pore can pass perpendicularly through the solid and reach to another pore. The same is valid for two different solid points, and the distance between two pores or two solid points is less than 1 μm (Brinker and Scherer 1990).

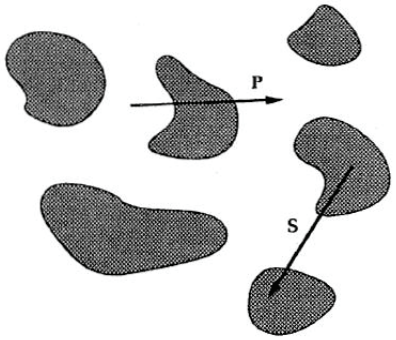

Figure 2.6. Schematic illustration of gel structure (P: from pore to pore, S: from solid to solid) (Source: Brinker and Scherer 1990).

According to the structure, four types of gels can be classified as (i) ordered lamellar structures (i.e. clays, soaps), (ii) disordered covalent polymer networks, (iii) polymer networks formed by aggregation of polymer chains via multistranded helices or crystalline domains, and (iv) particulate disordered gels (i.e. aggregated globular proteins) (Brinker and Scherer 1990).

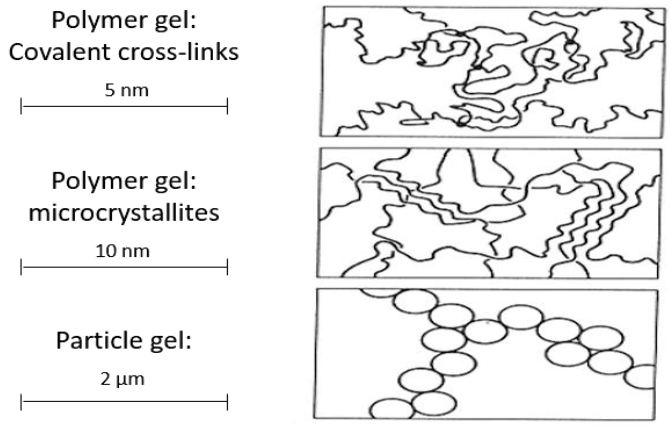

Figure 2.7. Schematic illustration of three main type of gels
(Source: Damodaran, Parkin and Fennema 2007)

2.2.3. Gelation

Gel formation is a spontaneous process occurs in a polymer dispersion or particulate suspension, and can be controlled by external conditions (Clark 1991).

2.2.3.1. The Sol

A sol is a colloidal suspension of solid particles in a liquid. A colloid is a suspension of dispersed small particles (~1-1000 nm) which are not affected by gravitational forces, and the main interactions are short-range forces such as Van der Waals attraction and surface charges. The term colloid was used to describe macromolecules that cannot pass through porous membranes (Hiemenz and Rajagopalan 1997). To define branched macromolecules, polymeric term was used, and for nonpolymeric solid particles were described as particulate. Lastly, a polymeric sol is described as the sol in which the solid phase contains no dense particles larger than 1 nm whereas particulate systems contain particles larger than 1 nm (Brinker and Scherer 1990). The relation between solubility of dense particles in solution and the radius of the particles is described by the Ostwald-Freundlich equation:

$$S = S_0 \exp\left(\frac{2\gamma_{SL}V_m}{R_g Tr}\right) \tag{2.2}$$

where S_0 is the solubility, γ_{SL} is the solid liquid interfacial energy, V_m is the molar rate of the solid phase, R_g is the ideal gas constant and T is the temperature. The size is effective on solubility mostly when the diameters of the particles are less than 5 nm. Smaller particles in this size range tend to reprecipitate on larger particles and cause particle growth. This phenomenon is known as Ostwald ripening. As a result, the average diameter of the main particles increases.

The stability of sol depends on the dipole-dipole interactions. Since every atom is a fluctuating dipole due to the electrons around nucleus, an attraction between atoms is created which is known as Van der Waals forces or dispersion energy. Three types of interaction cause Van der Waals forces; permanent dipole- permanent dipole, permanent dipole-induced dipole and transitory dipole-transitory dipole. (Brinker and Scherer 1990) Because of the distance between electrons (~30nm) the electromagnetic waves between atoms may fluctuate and this cause retardation, results as reduced attraction between atoms. For colloid systems, only unretarded forces have an important role. Attractive forces within a dispersion cause flocculation of colloids. However, in some cases the attractive forces extend and can reach the distances over and prevent aggregation by

creating barriers causing electrostatic repulsion. This is known as Steric barrier and illustrated in Figure 5.

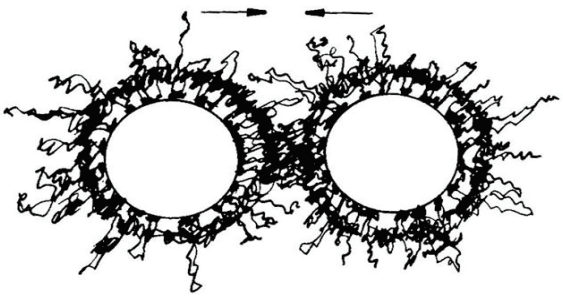

Figure 2.8. Schematic illustration of steric barrier
(Source: Brinker and Scherer 1990)

The net force between particles in suspension is the sum of attractive Van der Waals forces and electrostatic repulsion caused by the charges adsorbed on the particle surface. The repulsive barrier between two particles depends on two types of ions; charge-determining ions and counterions. Charge determining ions such as OH^- and H^+ represent the charge of the particles and the counterions separate the charges of the charge-determining ions in the solution around particle. In an electric field, charged particles move and carry the adsorbed layer and a part of counterions around them. In the slip plane around a charged particle, the fluid move with the particle is separated from the free-flowing fluid. The potential of the slip plane determines the rate of movement of the particle. This potential is called zeta potential ($\Phi\zeta$) and it is lower than surface potential. The pH at $\Phi\zeta$ is equal to zero, is the isoelectric point (pI). The pI is not the same with point of zero charge, the pH at the particle is neutrally charged. The stability of colloids depends on the $\Phi\zeta$. As the counterion concentration increases, repulsive barrier reduces and at a further level of concentration, the double-layer repulsion is reduced more to lead coagulation. (Brinker and Scherer 1990)

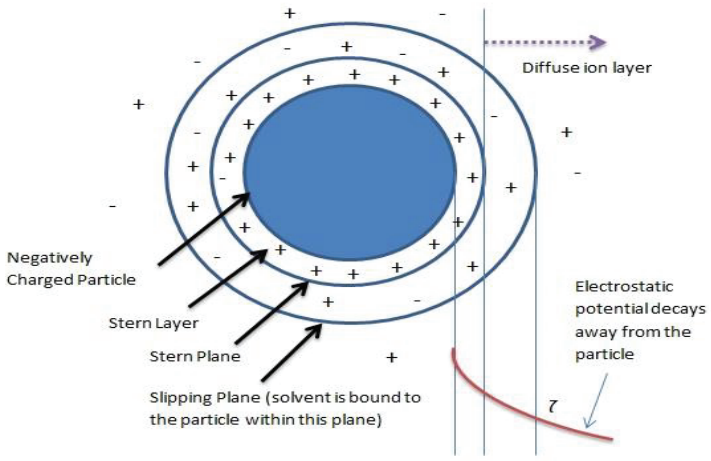

Figure 2.9. Schematic illustration of stern and diffuse double layers (surface charge is assumed to be positive) (Source: matecappliedsciences.com)

2.2.3.2. Sol to Gel Process

The process that the polymer chains are associated or crosslinked to form a three-dimensional network trapping liquid phase in it and create a rigid structure is called gellification. A monomer that can form more than two bonds can extend its size with no limit by linking. In food gel systems, this linking occurs mostly by hydrophobic interactions and intermolecular forces such as hydrogen bonds, electrostatic forces and Van der Waals interactions. The only covalent cross-linking can occur in some protein gels through disulphide bonds (Banerjee and Bhattacharya 2012).

Gel formation can be induced in two ways; physically (heat, pressure) or chemically (acid, enzymes, ionic changes) (Totosaus et al. 2002). The particulate sols can form a gel when attractive dispersion forces cause them to stick together in such a way to form a network. So, gelatin gels are formed by entanglement of chains, particulate gels are formed by van der Waals forces and polymeric gels are covalently linked. These bonds can be reversible or irreversible (Brinker and Scherer 1990). The type of the bonds and the permanent or reversible state of the gel determines its strength. However, the main factor affecting the gel to be weak or strong is the time scale. The formation of gels begins with the formation of fractal aggregates that grow until they form clusters and those clusters are linked together. This growing is described by the percolation theory.

Before reaching the gel point, the bonds are formed randomly between the aggregates or clusters which are stationary next to each other and form a network. When the sol containing many clusters is cast into a mold gelation can occur and a gel is formed

with a final shape. The gel point is the time or the degree of reaction at which the last bond is formed that completes the giant molecule. Thus, a gel is a substance that contains a continuous solid skeleton enclosing a continuous liquid phase. This continuity of the solid skeleton gives the elasticity to the gel.

2.2.3.3. The Gel

During gelation, the collapse of the repulsive double layer results as aggregation. The size distribution of these colloidal particles and the existing attractive forces form the structure of the gel. As the repulsive forces reduce gradually, the structure became more ordered. In monodisperse particles, supersaturation increases until critical concentration (C_N), where nucleation occurs very rapidly. Then, after the precipitation of particles, supersaturation reduces below C_o where no further nucleation occurs. The formed nuclei grow to an equilibrium concentration.

When the gel point is reached where any new attachment can occur, bond formation inside the network proceeds. Right after the gel is formed, still it contains sol phase entangling but not attaching, however in time, they connect to the network and result as increasing stiffness of the gel. The segments of the gel network get closer by the increasing bond forming. The final network keeps being developed by the attachment of the remaining sol phase inside the gel and causes aging of the gel. Aging process changes the structure and the properties after gelation and, if the bond formation proceeds much further contraction of the network leads the gel to shrink which is known as *syneresis*. As a result of syneresis, the liquid phase of the gel leaks through the pores and gel is deformed. In a gel system where gelation occurs by collapse of repulsive double layer, Van der Waals forces play an important role in syneresis. A further shrinkage is prevented by the remaining repulsive force, so as the electrolyte concentration increases the shrinkage occurs more. Syneresis is a reversible change except most inorganic gels that cannot swell. The bridging bonds of –O– between polymers take less space than two –OH groups and this contraction result as condensation and shrinkage as long as the gel remains flexible.

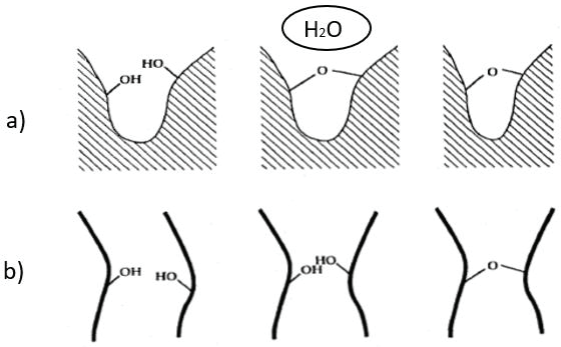

Figure 2.10. (a) Shrinkage on surface from condensation of neighboring groups. (b) Extensive shrinkage results from the flexible chain movement and new bond formation (Source: Brinker and Scherer 1990).

However, if the particles reprecipitate because of the changing solubility, and the small particles fill the small pores, the interfacial area reduces. As a result, particles cannot collapse to each other to cause shrinkage. This is known as coarsening and it depends on the factors affecting solubility of particles such as pH, temperature, concentration and type of solvent (Brinker and Scherer 1990).

2.2.4. Mixed Gels

Combining more than one hydrocolloid, mixed gels possess unique characteristics that may not be possible to obtain with single gelling agent. Combination of gelling agents can improve gel stability, reduce syneresis and improve mechanical properties and appearance. Three types of mixed gels are defined; coupled, interpenetrated and phase separated. Phase separated gels are the mostly encountered mixed gels in food systems. Phase separation and gelation compete during the process, and a micro-phase separated gels are obtained. There is limited literature about mixed gels than that are about single polymer gels and their mechanism of gelation. However, characteristics of few mixed-gels have been investigated including gellan and agar (Banerjee and Bhattacharya 2011), agar and κ-carrageenan (Meena, Prasad and Sidhanta 2009), and starch and pectin (Autio et al. 2002). Banerjee and Bhattacharya (2011), reduced syneresis significantly by mixing gellan and agar, with improved gel strength, compared to single-component gellan gel. It was claimed that, in a two-component gel where both polymers having a gelling property, the component having the lowest gelling temperature forms the continuous network, and the other component would present in a discontinuous phase. In a starch-gelatin system,

the gelation of gelatin was slower than starch, therefore amylose aggregates found discontinuously in the network weakened the gel structure (Muhrbeck and Eliasson 1991).

2.2.5. Factors Affecting Gelation and Gel

The gelation mechanism depends on the nature of the gelling agents and the gelation conditions such as temperature, the gelling time, the gelling agent concentration, the pH and the presence of ions (Banerjee and Bhattacharya 2012). These factors affect gel characteristics like the viscosity and gel strength (Osorio et al. 2007). The structure of a gel made from colloidal particles depends on the size distribution of the particles and the strength of attractive forces between them (Brinker and Scherer 1990).

- Temperature:

To obtain gels, heating is the mostly used method. During gelation, firstly the reactive sites are exposed by unfolding or dissociation of the molecules due to the energy input as a driving force. This step is generally reversible. Then in the second step, unfolded or dissociated molecules associate and aggregate to form a high molecular weight cluster. During the first and the second steps, reaction rate is depended on the temperature (Banerjee and Bhattacharya 2012). Ganji, Abdekhodaie and Ramazani (2007) showed that the gelation time of chitosan-based hydrogels decreases as the temperature of environment increases.

- Pressure:

High pressure can be used alone or combined with other processes in gelation. Under high pressure, the total volume of the system reduces, water dissociates, and the pH becomes more acidic. In a study by Zhu et al. (2014), high pressure is combined with the transglutaminase enzyme in surimi gels and greater gel strength was obtained after combination of high pressure and enzyme treatment. A smooth and glossy soy protein gels were obtained by applying 3000 kg/cm^2 pressure, and the resulting gels were softer than heat-induced gels (Okamoto, Kawamura and Hayashi 1990)

- Ionic Strength:

Monovalent or divalent ions such as sodium and calcium favour the gelation process since they can reduce or neutralize the electrostatic repulsive forces between molecules.

This method is also called as cold gelation is more applied on polysaccharide gels such as alginate, pectin, or carrageenan.

- pH:

Different pH ranges affect the gelation by altering the electrostatic attraction or repulsion forces between molecules or between molecules and solvent, also changes the solubility of salts which may affect the gel formation. The acid gel formation is explained by the fractal gel formation theory (Lucey and Singh 1997).

- Presence of Enzymes:

Enzyme induced gelation is caused by the covalent cross-linking between protein molecules. Bond formation is generally catalyzed by trans-glutaminase, peroxidise and polyphenol oxidase (Lauber et al. 2000).

- Solvent Quality:

Gelation can be affected by the type of solvent. In example, pectin can form good gel in concentrated sugar solution, since this solution allows pectin to form hydrogen bonds. In a study by Peniche et al., (2003), chitosan particles are produced using methanol/NaOH solution as aqueous media and glutaraldehyde as cross-linking agent.

- Concentration of gelling agent

Each hydrocolloid has a critical minimum concentration (C*) where gel forming cannot occur below C*. If the concentration is around C* the molar mass become effective on gelation. Higher molar mass results as faster gelation because of the higher modulus.

- Gel Strength:

For a fixed pH and concentration, as the gelatin strength increases, the melting temperature decreases (Osorio et al. 2007). However, according to Phillips and Williams (2000), there is no relationship between gel strength and the concentration.

2.2.6. Biopolymers Used in Active Gel Production

2.2.6.1. Gelatin

Gelatin, obtained from collagen by physical or chemical denaturation and partial hydrolysis, is extensively used in industry such as food, adhesive, pharmaceutical fields due to its nontoxicity, biodegradation and biocompatibility. It is an important biopolymer

with high molecular weight, low melting temperature (<35 °C), good water-solubility and thermally reversible gelling properties. Therefore, it is mostly employed as a food ingredient to increase the consistency in aqueous systems (Osorio et al. 2007; Spizziri et al. 2009).

Collagen, derived from the skin, bones and connective tissue of animals, consists of three α-chains made of repeated proline-glycine-hydroxyproline sequences which play a role in inter or intra-molecular covalent crosslinks. (Figure 2.11). The amino acid composition, especially proline and hydroxyproline, varies from species to species, so the gelling ability and properties of gelatin varies, too (Mariod and Adam 2013).

Industrially, gelatin is produced from skin and bones of cattle, pig and fish. Briefly, insoluble collagen is heated in neutral conditions to about 40-45 °C for extraction, and the non-covalent bonds are broken by chemical pre-treatment. Then, the hydrogen and covalent bonds within the triple helix are destabilized by heat, and a helix-to-coil transition resulting soluble gelatin occurs. Depending on the pre-treatment procedure, two types of gelatin are produced, type A, under acid pre-treatment, and type B, under alkaline-pre-treatment (Gomez-Guillen et al. 2011).

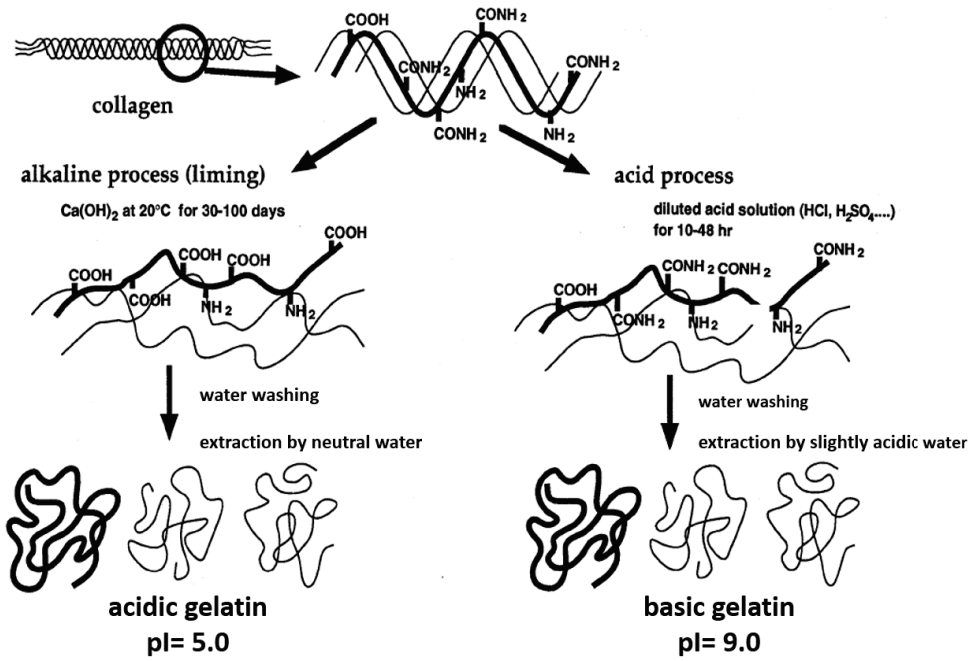

Figure 2.11. Acidic and basic gelatin production from collagen by alkaline or acid processes (Source: Tabata and Ikada 1998).

Gelatin obtained from mammalians has a wide range of molecular weight (80-400 kDa) classified as α-chain that are randomly coiled independent chains (80-125 kDa), β-

chain consisting of two linked α-chains (160-250 kDa) and γ-chain made of covalently linked three α-chains (240-400 kDa) (Ofori 1999).

Gelatin is an amphoteric protein having both positive and negative charges. Its isoelectric point is between 5 and 9 depending on the source and the process. The pI of type A gelatin is at pH 8-9 and of Type B is at pH 4.5-5.5 (Table 2.1).

Table 2.1. Typical characteristics of edible gelatin
(Source: GMIA 2012)

	Type A		**Type B**	
pH	3.8	5.5	5	7.5
Isoelectric point	7	9	4.7	5.4
Gel strength (bloom)	50	300	50	300
Viscosity (mps)	15	75	20	75
Ash	0.3	2	0.5	2

Gelatin and collagen are the different forms of same macromolecule, have different gelation mechanisms. During collagen gelation, aggregated molecules form fibrils, and microfibrillar aggregation starts to form type-1 collagen with a gelation temperature higher than 20 °C. In contrast, gelatin gelation is based on reverse coil-to-helix transition favored by cooling below 30 °C. Below coil-to-helix temperature of gelatin, disordered peptide chains transform to triple-helix structure. These triple-helix sites act as junction zones stabilized by hydrogen bonds, and gel network is formed (Figure 2.12). Similar to collagen, helices are formed but no equilibrium is reached. Both molecules are thermoreversible but, collagen forms gel as heat is raised and gelatin gel is formed upon cooling (Gomez-Guillen et al. 2011).

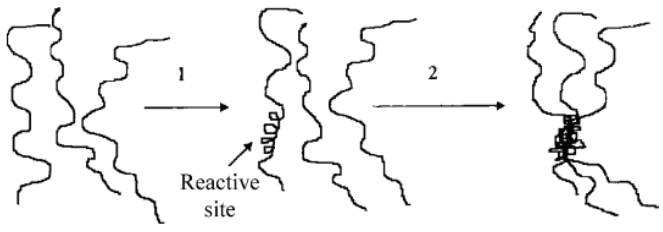

Figure 2.12. Schematic illustration of gelatin gelation
(Source: Renard et al. 2006)

Amino acid composition of gelatin varies with species and molecular weight distribution, and influence the gel strength. On gel stability, proline-hydroxyproline

content, formation of nuclear zones and thermal properties play a role (Gomez-Guillen et al. 2011).

According to a research of the effect of concentration, bloom degree and pH on T_m and T_g of gelatin gel, it has been showed that, the storage modulus increases with increasing gel concentration and the gels with different gel strength and concentration show viscoelastic behavior (Osorio et al. 2007). The melting and gelling temperature of gelatin gels increase as the concentration of gel increases and at the pH values between 3 and 6, at the isoelectric point region of gelatin. Temperature, pH, ash content, production process type, thermal history and concentration affect gelation behavior (Tsereteli and Smirnova 1991).

2.2.6.2. Soy Protein

Soy protein is a globular protein commonly used for gel, film, emulsion making, and as emulsifier (Zhao et al. 2015). Soy protein isolate can be extracted from the soy flour which is a by-product of soy oil production. Soy proteins are composed of albumins and globulins in globular structure, consisting 7S and 11S globulin fractions. The subunit fractions of globulins are associated by hydrophobic, hydrogen bonding and disulphide bonds (Shan et al. 2015, Kinsella 1979).

To form gel, globular protein structure is unfolded by denaturation and the protein aggregates (Doi 1993). During denaturation, globular proteins convert into intermediate states such as pre-molten globule and molten globule. According to Dobson (2003), hydrophobic groups buried in the surface of the native state are exposed which increases the hydrophobicity and decreases the net charge. After the network formation, a β-sheet formation is promoted. Gelation occurs by heating the soy bean flour or soy milk after salt addition such as Ca^{++} or Mg^{++}. Also, acidification induces aggregation of denatured protein molecules. Commercial soy protein isolates, which are isolated by thermal processing, have poor emulsifying properties because of their very low solubility (Damodaran, Parkin and Fennema 2007).

In the food application of soy protein, its water holding performance is as important as its gelling ability. Soy protein concentration, soy flour and soy protein isolate were used to increase water binding capacity of food systems such as meat and meat products, and dairy products such as yoghurt (Porcella et al. 2001).

2.2.6.3. Starch

Starch is found as granules in plant tubers and seed, containing two types of α-glucan; amylose and amylopectin that consist approximately 98% of the dry weight. Depending on the plant source, the ratio of these two molecules vary.

Amylose is the responsible molecule of film making. Amylose is a linear polymer of glucose linked mainly by a(α-1→4) bonds, which promote the formation of a helix structure. Amylopectin is a highly branched polymer of glucose. Glucose units are linked in a linear way with a(α-1→4) bonds. Branching takes place with a (1→6) bonds occurring every 24 to 30 glucose units (Damodaran, Parkin and Fennema 2007).

Starch granules form irreversible gels following heating above the critical gelatinization temperature in water, leaching of amylose from the granules and swelling. The leached amylose solubilizes and upon cooling if the amylose concentration is above chain overlap concentration (c*) a gel is formed. As a result, a thin layer of amylose gel around and between the granules is obtained.

Starch gelation induced by heating, and a paste formation is followed by retrogradation. After swelling of the starch granules, amylose inside leaches out and forms a three-dimensional network. Upon cooling, starch molecules partially link to form a gel or precipitate. This process is called retrogradation. The swollen starch granules are trapped in this formed matrix. This structure with swollen granules embedded in gelatinized paste can be defined as a composite material (Lii et al. 1996; Damodaran, Parkin and Fennema 2007).

2.3. Gel Characterization

In food applications, beside of the basic physicochemical properties such as solubility, transparency and color; the quality of a gel is mainly described by rheological properties. The most important commercial properties of a gel are the viscosity, gel strength and thermal stability (melting and gelling temperature) (Gomez-Guillen et al. 2011). Many factors such as concentration of gelling material, pH, electrolyte content and gelling time directly affects these parameters. In Table 2.2. the most measured parameters are given for different type of gels.

Table 2.2. The measured parameters for gel characterization

Measurement type	Instrument	Measured Parameters	Application	Reference
Compression (Fundamental)	Texture measuring system	Modulus of elasticity, Poission's ratio	Cortical bone tissue	Reilly and Burstein (1974)
Stress (Fundamental)	Texture measuring system	Residual stress, relaxation time	jello, mozzarella cheese, cheddar cheese, tofu and sausage	Singh et al., (2006)
Creep (Fundamental)	Controlled stress rheometer	Shear modulus, creep compliance	Honey	Kulmyrzaev & McClements (2000)
Oscilliation (Fundamental)	Controlled stress rheometer	Storage modulus (G'), loss modulus (G"), phase angle, complex modulus and viscosity	Gelatin gels	Osorio et al., (2007)
Puncture force (Empirical)	Texture measuring system	Puncture characteristics	Grape skin	Letaief et al., 2008
Compression (Empirical)	Texture measuring system	Peak force, firmness, compression energy	Gouda cheese	Culioli amd Sherman 1976
TPA (Imitative)	Texture measuring system	Parameters of texture profile analysis like hardness, brittleness, adhesiveness, springiness, cohesiveness	Date flesh	Rahman & Al-Farsi, 2005
Structural	DSC	Heat flow	Gellan and polyvinyl alcohol blend film	Sudhamani et al. (2003)
	Colorimeter	Color measurement	Gellan edible films	Leon et al. (2008)
	X-ray diffraction	Particle size analysis	Nano delivery system in food	Luykx et al., (2008)
Microscopic	Light microscopy	Area of the granules	Tapioca starch gel	Vittadini et al. (2006)
	SEM	Structural arrangement of components	Structural characteristics of nanoparticles	Luykx et al., (2008)
	TEM			
	AFM	Structural distribution of constituents		
		Structure of the molecules		
Molecular	NMR	Conformation changes on gelation	Infrared spectra of the components	Sudhamani et al. (2003)
	FTIR	Molecular structure		
	FT-Raman	Molecular characterization		

2.3.1. Gel Strength

Gel strength is also expressed as Bloom strength, measured by a standard method called Bloom test. The force in grams required to press a 12.5 mm diameter plunger 4 mm into 112 g of a standard 6⅔% w/v gelatin gel at 10 °C is called Bloom strength. Just as pH and temperature, gelatin strength determines the interaction with other components.

2.3.2. Rheological Methods

Rheology science studies the deformation of materials including flow. Rheological data are required in product quality evaluation, engineering calculations, and process design. Rheological properties are the mechanical properties of a material exposed a deformation and flow under the (Sahin and Sumnu 2006).

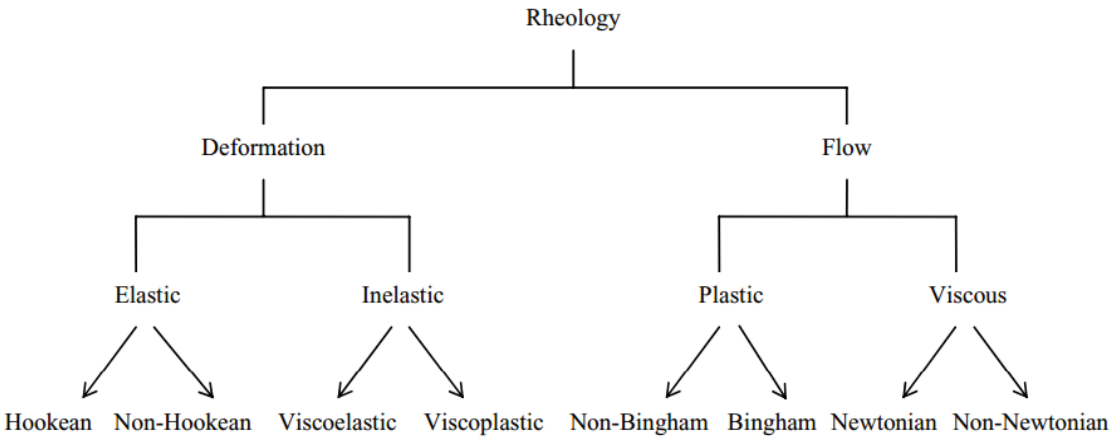

Figure 2.13. Classification of rheology
(Source: Sahin and Sumnu 2006).

From a rheological viewpoint a typical gel is a material that exhibits a yield stress, has viscoelastic properties and has a moderate modulus (Damodaran, Parkin and Fennema 2007). During the transformation from sol state to gel state, viscoelastic characteristic of the material changes and a solid or semi-solid structure are formed. Thus, to determine the viscoelastic properties of the gels, rheological measurements are the most applied methods.

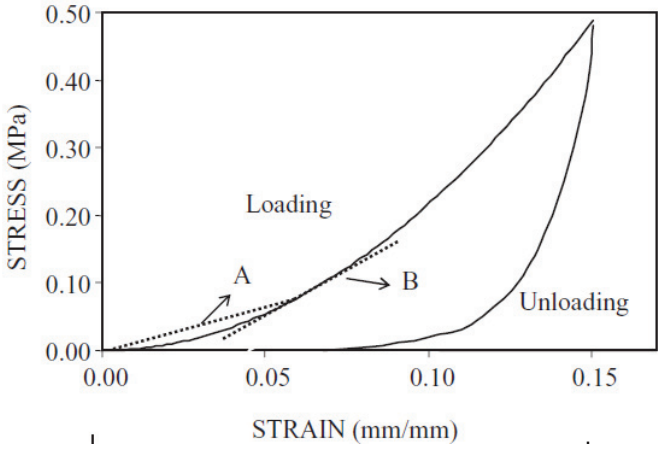

Figure 2.14. Stress-Strain curve for compression of a food material (Source: Sahin and Sumnu 2006).

In the rheological tests, two terms are important to understand; stress and strain. Stress is the applied force per unit area and has two types; normal stress and shear stress. Normal stress is the perpendicular force applied on per unit area and the shear stress is the force applied parallel to the plane per unit area. Strain is the ratio of extension to original length. Strain also has two types; normal strain and shear strain. Shear strain (γ) is the change in the angle between two planes after a deformation caused by application of stress. During unloading, the strain that is not recovered is the plastic strain and the recovered strain is elastic strain, the ratio of elastic strain to total strain is called as elasticity and the materials showing elastic behavior are called Hookean solid (Sahin and Sumnu 2006). The ratio of stress to strain is known as modulus while the ratio of strain to stress is known as compliance.

Young's modulus, known as modulus of elasticity, is defined as the ration of normal stress to normal tensile or strain. Shear modulus (G), also known as rigidity modulus, describes the relationship between shear stress and shear strain;

$$G = \tau/\gamma \tag{2.3}$$

Under a uniaxial compression in one direction, the sample may expand in the other directions. Poisson's ratio (μ) is defined as the ratio of the strain in the direction perpendicular to the applied force.

Viscous fluids start to deform when a force is applied, and the deformation is proportional with the force. When the force is removed, a viscous fluid cannot turn to its

original position, but viscoelastic fluids can. Viscous fluids generally show viscosity and solids show elasticity. However, some materials exhibit both and they are called viscoelastic materials. Most gelled products show viscoelastic behavior and examined by three different methods; stress relaxation test, creep test and dynamic test.

a) Stress relaxation test; if a gel is deformed to a certain strain, to keep this strain constant the required stress decreases in time. This decrease in stress is called relaxation and is measured as a function of time. The relaxation time is very short for viscous materials (10^{-13} s for water) but can be long for viscoelastic materials (10^{-1}-10^{6} s).

b) Creep test; when a load is applied to a material and if the deformation is continuous with time this is called creep. Creep test is performed applying an instantaneous constant stress and the strain is measured as a function of time. According to the viscous or elastic properties, the material shows small or large recovery after the stress is removed.

c) Dynamic test (Oscillatory test); in dynamic test, stress is measured at a constant strain or deformation is measured at constant stress. Concentric cylinder, cone and plate, or parallel viscometers can be used for this test. The drawback of this test is that it can be only applied when stress is proportional to strain and the breakdown of the structure may happen during the test.

Relaxation modulus is determined with oscillatory shear. From the equation 2 given below, gel point can be determined by dynamic testing with small amplitude oscillatory method.

$$\tan(\delta) = G'' / G' = \tan (n_c \pi/2) \qquad (2.4)$$

δ is the phase angle; G' and G'' are storage and loss modulus, respectively. Small amplitude oscillatory method is used to observe structural changes without destructing the gel and also to detect the gel point temperature (Arenaz and Lozano 1998). Using a viscometer with a cylinder that oscillating with a small amplitude and frequency (ω), complex shear modulus (G^*) is measured. The complex modulus consists of loss modulus G'' (viscous contribution) and storage modulus G' (elastic modulus). Also the loss modulus (G'') corresponds to the melting temperature, T_m, for heating process and the storage modulus (G') corresponds to the gelling temperature, T_g, for the cooling process.

To determine the gel point, the sudden change in rheological behavior is observed. Since the definition of gel includes the three-dimensional polymeric network that resist flow under pressure and can retain their mechanical rigidity (Banerjee and Bhattacharya 2012), the storage modulus (G′) and the loss modulus (G″) are important parameters to describe their viscoelastic behavior. G´ value is a measure of the deformation energy stored by the sample during the shear process. When the load is removed this energy becomes available and compensates partially or fully the previous deformation of the structure. The materials storing the deformation energy show reversible deformation behavior, remain unchanged. So, G´ represents elastic behavior of the materials. G´´ value is a measure of deformation energy that is lost (probably in the form of heat) during the shear. The materials that lose this energy show irreversible deformation behavior and their shapes change after the shear. G´´ represents the viscous behavior (Mezger 2006). G′ is high for elastic materials and G″ is high for viscous materials (Sahin and Sumnu 2006). Tan δ (loss factor) is the measure of the ratio of the viscous and the elastic portion of viscoelastic materials.

During the sol-gel transition, rheological behaviour of the system becomes more complicated. The viscoelastic characteristic changes depending on time, temperature and concentration. Gel point of the gel is expressed rheologically;

$$G = S_c\, t^{-n_c} \quad \text{for } t > \lambda_0 \tag{2.5}$$

Where S_c is the gel strength, n_c is the critical relaxation exponent, and λ_0 is the relaxation time. This equation can be used to determine the gel point using rheological data. In the shear stress-shear rate and viscosity-shear rate plots, the shear thinning (by increasing shear) and elastic behaviour of the gels can be observed. Towards the gel point, the elastic character increases faster than the viscosity and the Tan(δ)= G″/ G′ increases to minimum. Determination of the point when Tanδ reaching to 1 is important to analyse sol-gel transition. For the gel state tanδ<1 (G´´<G´), for the sol state tanδ>1 (G´´>G´) and at the gel point tanδ is equal to 1 (Mezger 2006). Since the gelation reactions proceed after reaching to gel point, elastic modulus and other rheological properties continue to change after gelling time.

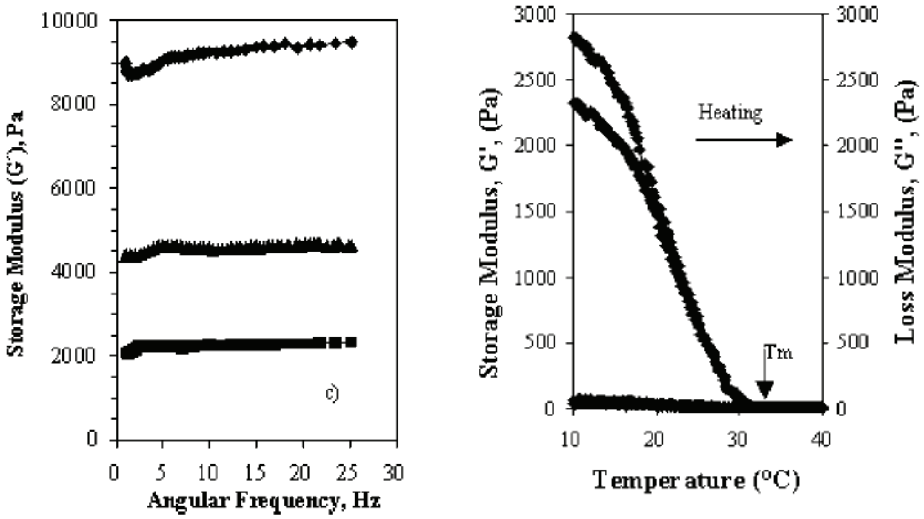

Figure 2.15. (a) Storage modulus G′ during frequency sweep of gelatin gel at 10 °C and concentrations of 5%, 7%, 10%; (b) Melting temperature determination of gels at concentrations of 5%, 7%, 10% through storage modulus and loss modulus variations during temperature sweep (Source: Osorio et al. 2007).

Rotational viscometers are the best for characterization of non-newtonian and time-dependent behaviour. For the gel systems, different types of viscometers can be used such as concentric cylinder viscometer, cone-plate viscometer and plate-plate viscometer (Zhao et al. 2015; Gudmundsson, 2002; Holm, Wendin and Hermansson 2009). Concentric cylinder viscometer consists of two annual cylinders where the sample is place in the narrow gap between them. At the cone-plate viscometer, the sample is placed on the gap between the cone and the plate, and the cone rotates at a determined angular velocity (Ω). This type of viscometer is suitable for shear thinning fluids and plastic fluids. It is well suited for testing small samples. The plate-plate viscometer consists of two parallel plates with a gap between them on which the sample is loaded. During the test, one plate rotates with an angular velocity and other stays stationary.

2.3.3. Texture Profile Analysis

The gel has the mechanical properties of a solid, it can maintain its form without any stress and under any mechanical stress it shows the phenomenon of strain (Almdal et al. 1993). Texture is an important quality characteristic in foods and the instrumental analysis of texture is an imitative, easy and short way of repeated sensory analysis.

Compression test measures the force to compress a material to a standard distance or the distance that a material is compressed under a standard force and it reflects the softness or firmness for foods.

In the texture profile analysis (TPA), the food piece is compressed twice representing the chewing action. Compression percentage varies according to the sample, but it is not recommended to apply compression rates that may cause the breakdown of the sample. By TPA, a force versus time curve is obtained and the sensory properties such as hardness, resilience, gumminess and cohesiveness are determined (Figure 2.16).

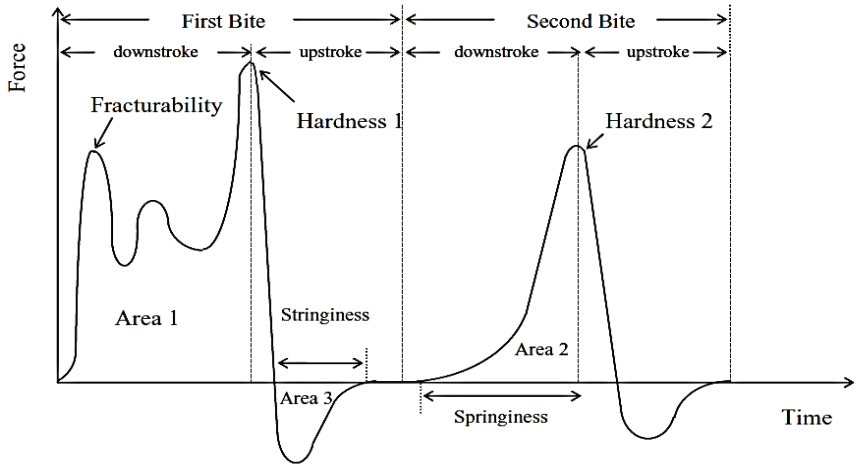

Figure 2.16. Texture profile analysis plot
(Sources: Lau, Tang and Paulson 2000; Sahin and Sumnu 2006)

From the peak forces and the areas under the curves the parameters of texture profile analysis are calculated as;

Fracturability; also the brittleness is defined as the force at the first significant break of the first positive peak. Not all the products fracture.

Hardness; is the maximum peak force of the first compression,

Cohesiveness; (Area2/Area1) is the area of work during the second compression divided by the area of work during the first compression. Cohesiveness is how well the product withstands a second deformation relative to its resistance under the first deformation.

Adhesiveness is the area of the first negative peak and represents the force required to take the plunger back.

Springiness; (Distance 2/Distance1) is the height that material recovers during the time between two compressions. Distance of the detected height during the second compression divided by the original compression distance.

Gumminess; (hardness*cohesiveness) applies only to semi-solid products. In sensory terms, it represents the energy required to disintegrate a semi-solid food and make it ready to swallow.

Chewiness; (gumminess*springiness) applies only to solid products. It is for the energy to disintegrate a solid food and make it ready to swallow.

Resilience is calculated by dividing the upstroke energy of the first compression by the downstroke energy of the first compression. It is the energy per unit volume recovered as the force is removed from the sample. The greater the resilience, the more energy will be recovered.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

The bovine skin gelatin (Type B, 225g Bloom gel strength), starch from rice, and candelilla wax to prepare gels, and lysozyme from hen egg white (Activity: ≥40000 U/mg) were obtained from Sigma (Missouri, USA). The soy protein isolate was provided from EURODUNA Food Ingredients GmbH (Barmstedt, Germany). The green tea extract used with 99% purity was provided from WILD Flavors and Specialty Ingredients (Rudolf Wild GmbH & Co. KG, Eppelheim, Germany). Bacterial strain of *Listeria innocua* (NRRL B-33314) used in antimicrobial tests was provided from United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit, Peoria, Illinois. Caco-2 cells used in cytotoxicity assay was obtained from ATCC, USA. Vacuum packed cold-smoked salmon was provided from local markets in Izmir, Turkey.

3.2. Methods

3.2.1. Preparation of Gelatin Gels and Composite Gels

The gelatin gels were prepared at the concentration of 15% (w/w) gelatin in distilled water. After the gelatin powder was dissolved in distilled water by stirring (400 rpm) until reaching 55 °C, the solution was kept at 85 °C in a water bath for 30 min to maintain denaturation of gelatin. After the denaturation step, the gel solution was cooled in molds at 4 °C for 18±2 hours. The composite gels were obtained by addition of rice starch (RS), candelilla wax (CW) and soy protein isolate (SPI) at the concentration of 7.5% (w/w) into gelatin gel solution (15% w/w) before the denaturation step in water bath. RS and SPI mixed with the gelatin solution by homogenizing at 10,000 rpm for 1 min using a homogenizer (Heidolph, Germany, rotor Φ=6.6 mm tip) before keeping in the water bath. CW was added into gelatin gel solution right before placing in the water bath and it melted during heating at 85 °C for 30 min. The melted wax phase was blended into the gel solution by homogenizing (Heidolph, Germany, rotor Φ=6.6 mm tip) at

10,000 rpm for 1 min after keeping in the water bath. Gels with no green tea extract (GTE) or lysozyme (LYS) were homogenized totally for 5 min, cooled down to room temperature in icy water, poured into the molds and cooled at 4 °C for 18±2 hours.

To obtain GTE and/or LYS containing gels, both ingredients were added after cooling the samples taken from the water bath to room temperature. Both LYS and GTE were added at 1% (w/w) of the gel solution. To disperse the active agents in the gel solution, a 4-min homogenization step took place. After pouring the gel solutions into molds and keeping in refrigerator at 4 °C for 18±2 hours, gels with 0.5 cm thickness and 6.6 cm diameter were obtained. The gels were removed gently from their molds after 18 hours and used in the analyses.

3.2.2 Determination of Lysozyme and Green Tea Extract Release Profiles of the Gels

3.2.2.1 Determination of Lysozyme and Green Tea Extract Release Profiles of the Gels in Distilled Water

To monitor the released LYS and GTE from the gels, the gels at 0.5 cm thickness and 6.6 cm diameter were placed in 100 mL distilled water in Erlen bottles and kept at 4 °C under shaking at 80 rpm. 0.1 mL samples were taken from the release media at different time intervals until reaching the equilibrium. The LYS activity was measured as described at section 3.2.2.1.1 and the total phenolic content was determined as described at section 3.2.2.1.2. Release profiles were determined from at least two replications of the release and at least three measurements at each time point.

3.2.2.1.1. Determination of Lysozyme Activity Released in Distilled Water

LYS activity was measured spectrophotometrically at 660 nm by using Shimadzu (Model 2450, Japan) spectrophotometer equipped with a constant temperature cell holder at 30 °C. Reaction mixture was prepared by mixing 0.1 mL enzyme containing solution (incubated at 30 °C for 1 min) and 2.4 mL *Micrococcus lysodeicticus* suspension (at 30 °C) prepared in pH 7.0 0.05M Na-phosphate buffer. The reaction mixture was mixed with a vortex and the decrease in absorbance was monitored for 120 s. Enzyme activity was calculated from the slope of initial portion of absorbance *vs.* time curve. One Unit was

defined as 0.001 change in absorbance within 1 min. Average of three measurements was used in calculations. Calculations were corrected by considering the total activity removed from aqueous media during sampling. Released LYS activity was expressed as the Unit released by one gram of gel (U/g gel).

3.2.2.1.2. Determination of Green Tea Extract Released in Distilled Water

The released total phenolic content was determined spectrophotometrically according to the Folin-Ciocalteu method of Singleton and Rossi (1965). Samples (0.1 mL) taken from the release media was completed to 0.25 mL by addition of distilled water. 0.25 mL sample solution was mixed with 1.25 mL of Folin-Ciocalteu (10% v/v) reagent and incubated for 3 min at room temperature. Then 1 mL of Na_2CO_3 (7.5% w/v) was added into the reaction mixture and incubated for 1 hour at room temperature. At the end of incubation, the absorbance of the samples was read at 765 nm. The calibration curve was prepared by using gallic acid (Appendix A). Average of three measurements was used in calculations. The amount of released total phenolic component was calculated as "mg gallic acid equivalent released from per gram of gel" (mg GAE/g gel).

3.2.2.2 Determination of Lysozyme and Green Tea Extract Release Profiles of the Gels on Smoked Salmon

3.2.2.2.1. Coating the Smoked Salmon Samples

To monitor the release of LYS and GTE onto smoked salmon, gels containing LYS, GTE, and LYS+GTE (1% (w/w) each) were placed on the both sides of 10 g of smoked salmon samples (freshly unpacked) cut in the same shape and diameter with the gels. Coated smoked salmon samples were wrapped with plastic wrap (PVDC) and then aluminum foil, and stored at 4 °C for 15 days. Two samples for each treatment were tested for monitoring release profiles. Released LYS activity and total phenolic content were determined according to the methods described in Section 3.2.2.2.1 and 3.2.2.2.2, respectively.

3.2.2.2.2. Determination of Lysozyme Activity Released on Smoked Salmon

The packed and coated smoked salmon samples (average meat thickness: 4.02 ± 0.4 mm) were stored at 4 °C for 15 days. At the 5th, 10th and 15th days of cold storage, the packs were opened, gel coatings were removed, and smoked salmon samples were homogenized for 1 min in 100 mL 0.05 M PBS solution (pH 6.0), using a blender (Waring, USA). After centrifuging at 10,000*g*, the supernatant was filtered to remove the fat accumulated on the surface using a filter paper. Samples (0.1 mL) were taken from the filtered supernatant, and the soluble LYS activities were measured as described in Section 3.2.2.1.1. The measurements were performed in two replicates and three parallels.

3.2.2.2.3. Determination of Green Tea Extract Released on Smoked Salmon

To determine the release profile of GTE onto smoked salmon, gels were coated on 10 g of smoked salmon discs on the both sides and the samples were wrapped with plastic film and then aluminum foil. The packed coated salmon slices were stored at 4 °C for 15 days. At the 5th, 10th and 15th days of cold storage, the packs were opened, and smoked salmon samples were homogenized for 1 min in 100 mL distilled water using a blender (Waring, USA). After centrifuging at 10000*g,* the supernatant was filtered to remove the fat accumulated on the surface using a filter paper. Samples (0.1 mL) were taken from the supernatant and the soluble total phenolic content was measured by Folin-Ciocalteu method as described in Section 3.2.2.1.2. The uncoated smoked salmon slices were also prepared in the same way and the phenolic amount coming from the salmon samples was measured and then discarded from the calculated phenolic content of gel-coated salmon samples. The measurements were performed in two replicates and three parallels.

3.2.3. Determination of Free Radical Scavenging Activity of the Gels

To determine the antioxidant activity released from the gels containing GTE, and LYS+GTE, the gels at 0.5 cm thickness and 6.6 cm diameter were placed in 100 mL distilled water in Erlen bottles and kept at 4 °C under shaking at 80 rpm. Antioxidant

activity of the released GTE were determined by taking samples from the release media according to Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) methods, as described on Sections 3.2.3.1 and 3.2.3.2, respectively. Iron chelating capacity of released GTE was determined according to the method described in Section 3.2.3.3.

3.2.3.1. Trolox Equivalent Antioxidant Capacity (TEAC) Method

The trolox equivalent antioxidant capacity (TEAC) of green tea extract released from the gels was determined spectrophotometrically (Shimadzu, Model 2450, Japan) according to Re et al. (1999). The ABTS free-radical cation solution was prepared by adding 7 mM ABTS solution into 2.45 mM aqueous solution of potassium persulfate and this final solution kept in dark overnight. ABTS solution was diluted with 75 mM phosphate buffered saline (PBS) containing 150 mM NaCl at pH 7.4. The absorbance of the diluted ABTS solution was adjusted to 0.7±0.02 units at 734 nm and preincubated at 30 °C before use. The reaction mixture was formed by mixing 2 mL diluted ABTS radical solution and the sample. The decrease in absorbance in the reaction mixture was monitored for 6 min and recorded after 0.5, 1, 3 and 6 min. The results were calculated as area under the curve (AUC) values plotting the percent inhibition vs. concentration values for the samples and trolox separately against test periods. The slopes for each test period was used to create the curve and the division of the areas of curves for each sample to that of trolox was used to calculate the AUC value. The average of triplicate measurements was used to calculate antioxidant activity based on free radical scavenging and expressed as “μmol trolox equivalents per g of gel”.

3.2.3.2. Oxygen Radical Absorbance Capacity (ORAC) Method

The ORAC method was applied by the method given by Xu and Chang (2007). Briefly, 20 μL of sample taken from the release medium was pipetted into a black 96-well plate. Then, 200 μL of 0.096 μmol/L fluorescein solution prepared in 75 mmol/L phosphate buffered saline (PBS) (contained 150 mmol/L NaCl) at pH 7.4 was added into each well and the mixture was incubated at dark for 20 min at 37 °C. The reaction was started by adding 20 μL of 100 mmol/L AAPH (prepared in 75 mol/L phosphate buffer

saline with 150 mmol/L NaCl). The fluorescence of reaction mixture was monitored periodically for 40 min at the excitation wavelength of 485 nm and emission wavelength of 520 nm by using a microplate reader spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, MA, USA). The average of triplicate measurements was used to calculate antioxidant activity based on free radical scavenging and expressed as "μmol trolox equivalents per g of gel".

3.2.3.3. Determination of Iron Chelating Capacity

The iron chelating capacity (ICC) of green tea extract released from the gels was determined according to the spectrophotometric method given by Arcan and Yemenicioğlu (2007). Briefly, 2 mL of sample taken from the release medium was mixed with 0.1 mL of 1 mmol/L $FeCl_2.4H_2O$ solution and incubated for 30 min at room temperature. Then, 0.1 mL of 0.5 mmol/L ferrozine was added into the solution and incubated for 10 min. After incubation, the absorbance of solution was read at 562 nm. The percent ICC of released green tea extract was determined by using deionized water in place of sample. The ICC of released green tea extract was calculated by dividing the slope of the initial linear portion of their ICC (%) vs. concentration (μg/reaction mixture) curves by that slope of the same curve of the chelating agent EDTA. The average of three replicates was used for calculations and the results were expressed as "μmol EDTA per g of gel".

3.2.4. Determination of Bioactive Properties of the Gels

3.2.4.1. Antihypertensive Activity of the Gels

The antihypertensive activity of green tea extract released from the gels was determined by measuring their inhibitory effects on angiotensin-converting enzyme (ACE) according to the method described by Shalaby, Zakora, and Otte (2006) with minor modifications. 5 μL of 0.25 Units/mL ACE prepared in 0.01 mol/L phosphate buffer saline (PBS) (NaCl concentration: 0.5 mol/L) at pH 7.0 was mixed with 5 μL of green tea extract solutions taken from the release medium. The enzyme-sample mixture was incubated for 15 min at 37 °C and the enzymatic reaction was initiated by adding 150 μL

1.75 mmol/L FAPGG substrate solution (at 37 °C) prepared in PBS into this mixture. The assay was performed in 96-well microtiter plates (UV flat bottom, 8404, Thermo Fisher Scientific, Waltham, MA, USA). The absorbance of the reaction mixture was monitored at 340 nm for 30 min at 37 °C using microplate reader spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, MA, USA) and the ACE activity was determined from the slope of the initial linear portion of absorbance-time curve. The ACE inhibition (%) was calculated according to the following formula of % ACE inhibition

$$\% \text{ Inhibition} = [1-(\text{activity of sample}/\text{activity of control})] \times 100 \quad (3.1)$$

Activity of control was measured using 5 µL PBS instead of sample. Results were expressed as "µmol captopril per g of gel".

3.2.4.2. Antidiabetic Activity of the Gels

3.2.4.2.1. Human Salivary α-amylase Inhibition Assay

The antidiabetic activity against human salivary α-amylase (HSA) enzyme was determined using the procedure given by Koh et al. (2010). In this assay 5 U/mL HSA were used. Briefly, 25 mg/mL rice starch solution in 50 mM phosphate buffer saline (NaCl: 6.85 mM, pH: 6.9) were prepared by heating at 100 °C for 1 min. After cooling rice starch solution was diluted to the concentration of 2 mg/mL. To prepare the reaction mixture, 82 µL samples taken from the release media were mixed in an Eppendorf tube with 8 µL of rice starch solution (2 mg/mL). To start the digestion 10 µL HSA (5 U/ml) was added into each tube and the mixture were incubated at 37 °C for 30 min. For the termination of reaction, 100 µL 3,5-dinitrosalycilic acid (DNSA) were added into each tube. DNSA reagent (1% w/v) was prepared by dissolving 0.1 g of DNSA in 8 mL deionized water containing 2 mL 2 N NaOH and 3 g Na-K-tartarate. For color development, the tubes were immersed in 100 °C water bath for 5 min. After addition of 500 µL deionized water into the tubes, 200 µL aliquots were pipetted in the wells of a transparent microplate (flat bottom). Absorbance at 540 nm was measured using a microplate reader spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, MA, USA). Control group was measured using phosphate buffer solution instead of samples

with green tea extract. The whole experiment was performed using denatured HSA treated at 100 °C for 10 min to obtain absorbance values of blank for control and samples. HSA inhibition was calculated according to the formula given in Eq.3.2. Results were expressed as "µmol acarbose equivalent per g of gel".

$$\%Inhibition=([(A_{control}-A_{controlblank})-(A_{sample}-A_{sampleblank})])/(A_{control}-A_{controlblank})*100 \quad (3.2)$$

$A_{control}$, $A_{controlblank}$, A_{sample}, $A_{sampleblank}$ are the absorbances measured for the reaction mixtures prepared with buffer using active enzyme, buffer using inactive enzyme, sample using active enzyme, and sample using inactive enzyme, respectively.

3.2.4.2.2. α-Glucosidase Inhibition Assay

The antidiabetic activity based on α-glucosidase (AGH) inhibition of green tea extract released from the gels was determined according to the method of Koh et al. (2010). Mammalian α-glucosidase (AGH) mixture were obtained from rat intestine aceton powder. Firstly 0.033 g rat intestine acetone powder were dispersed in 2 mL PBS (50 mM, pH 6.9, containing 6.8 mM NaCl) by vortexing for 5 min. After centrifugation at 4 °C, 8760 *g* for 30 min the supernatant was taken and used as AGH mixture in the assay. Buffer was used as control of the sample. 340 µL of sample in a test vial was mixed with 20 µL of AGH and incubated at 37 °C for 10 min. Then 40 µL of PNPG solution was added to initiate the digestion to all vials. After 15 min, 200 µL of 1 M Na_2CO_3 was added for reaction termination. Absorbance at 400 nm was measured using a microplate reader spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, MA, USA). The entire experiment was repeated using denatured AGH treated at 100 °C for 10 min. AGH inhibition was calculated according to the formula given in Eq.3.3, and all measurements were done as three times. α-glucosidase inhibition was expressed as "µmol acarbose equivalents per g of gel".

$$\%Inhibition=([(A_{control}-A_{controlblank})-(A_{sample}-A_{sampleblank})])/(A_{control}-A_{controlblank})*100 \quad (3.3)$$

$A_{control}$, $A_{controlblank}$, A_{sample}, $A_{sampleblank}$ are the absorbances measured for the reaction mixtures prepared with buffer using active enzyme, buffer using inactive enzyme, sample using active enzyme, and sample using inactive enzyme, respectively.

3.2.4.3. Antiproliferative Activity of the Gels

3.2.4.3.1. Cell Culture

Human colon cancer cells, Caco-2 cell line (ATCC HTB-37), obtained from American Type Culture Cell (Rockville, MD) was used in the cytotoxicity experiments. The cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 at 37 °C. Cells were grown for 24 h before use in cytotoxicity assay.

3.2.4.3.2. Cytotoxicity Assay

The cytotoxicity of GTE against Caco-2 cells were evaluated by cell counting method using a hemocytometer. Briefly, cells were harvested during the logarithmic growth phase, seeded at a density of 5000 cells/well onto a 96-well plate and incubated at 37 °C under 5% CO_2. After plating for 48 h, series of GTE solution (5-10-15-20-40-60-80-120 μg/mL) were added into the wells and the cells were then incubated at 37 °C for 48 h more. At the end of 48 h, the growth media were removed, and the cells were washed with PBS twice. Cells were detached from the wells using 20-30 μL trypsin-EDTA solution. The number of the living cells were determined by cell counting method using a cell counting chamber (hemocytometer) after the cells were dyed with 0.4% trypan blue dye. The cells dyed in blue were considered as death cells. The viable cell numbers were plotted against GTE concentration.

The effect of released GTE from the gels were tested against Caco-2 cells using spectrophotometric CCK-8 assay. This method simply gives the viability of the cells after any treatment. To be able to treat the cells equally with the released GTE from the gels, release media obtained after the 72 h-release tests were freeze-dried using a lyophilizer (Labconco, USA). 15 mg/mL solutions were prepared from the dry material obtained by

lyophilization of the release media. Pure GTE powder and dry material obtained from control gels with no GTE were also tested preparing 15 mg/mL GTE solution. From the stock solution (15 mg/mL) equal volumes from each group were given to Caco-2 cells for toxicity assay. For toxicity assay by CCK-8 kit, three replicates of Caco-2 cells were plated and tested. Briefly, 100 µL cell suspension (5000 cells/well) was dispensed in 96-well plate, and the cells were incubated at 37 °C, 5% CO_2 for 24 h in an incubator. After 24 h, 0-3.5-10.3-20.6-41.2 µL of release media solution mixed with cell growth medium and completed to 0.2 mL (Total concentrations of the dry material in the total media in each well were 0-21-62-123.6-247.2 µg/0.2 mL). Caco-2 cells were incubated for 48 h after adding GTE-growth media mixture. At the end of 48 h, firstly the media were removed, and the cells were washed with physiologic PBS. In each well 100 µL fresh media mixed with 10% CCK-8 solution (Sigma-Aldrich, USA) were added. Cells were incubated for 1 h at 37 °C, 5% CO_2 for color development, and absorbance was measured at 450 nm, using a varioscan plate reader (Multiscan Go, Thermo, USA). Control group was prepared similarly without any GTE treatment and its absorbance measured as described above. The absorbance measured for control Caco-2 cells was taken as 100% viable and the viability of GTE treated cells were calculated according to control group. Results were given by plotting viability (%) vs concentration of lyophilized release media in cell growth volume (µg/0.2 mL).

3.2.5. Antimicrobial Activity of the Gels

3.2.5.1. In vitro Antimicrobial Activity of the Gels against *Listeria innocua*

To determine the antimicrobial activity of the gels containing GTE, LYS, and GTE+LYS, *Listeria innocua,* a gram-positive strain sensitive to LYS, was selected as test microorganism representing *L. monocytogenes*. The gels were prepared the day before the test. *L. innocua* was activated by transferring one loop of frozen culture (10 µL) to 9 mL nutrient broth and incubated at 37 °C for 24 h. One-mL aliquot from the active culture was transferred to another 9-mL nutrient broth in tube and incubated at 4 °C for 24 h more to maintain the adaptation of growth at 4 °C. The initial number (~10^7 cfu/mL) of microorganism was diluted 4 times and the final dilution was made by mixing 10 mL of culture and 90 mL nutrient broth. The gels with 0.5 cm thickness and 6.6 cm diameter

were placed in 100 ml diluted culture media in Erlen flasks under aseptic conditions. The flasks sealed with cotton and aluminum foil were placed in a refrigerator shaker set to 80 rpm shaking rate, and 4 °C temperature. The number of microorganism were determined as cfu/mL at days 0, 7, 11 and 15. Enumeration of the colonies were performed by spread-plate method, using Oxford listeria selective supplement (Merck, Darmstad, Germany). Spread plates were incubated at 37 °C for 48 h for enumeration, and black colonies with a bright halo around them were counted as *L. innocua*. Microbiological counts were expressed as colony-forming unit per mL (CFU mL^{-1}), and the means and standard errors were calculated.

3.2.5.2. Antimicrobial Activity of the Gels against *Listeria innocua* inoculated on Cold-Smoked Salmon

To determine the antimicrobial activity of LYS containing gels when coated on cold-smoked salmon, *Listeria innocua,* a gram-positive strain sensitive to LYS was selected as test microorganism. The gels were prepared the day before the test. *L. innocua* was activated by transferring one loop of frozen culture (-80 °C) to 9 mL nutrient broth and incubated at 37 °C for 24 h. One-mL aliquot from the active culture was transferred to another 9-mL nutrient broth in tube and incubated at 4 °C for 24 h more to maintain the adaptation of growth at 4 °C. After dilution with 0.1% pepton water two times (Merck, Darmstad, Germany) an inoculum of $1x10^6$ CFU/mL was obtained. Smoked salmon pieces (6.6 cm diameter, each 10 g, freshly unpacked) were inoculated with 0.150 mL of inoculum (10^6 CFU/mL) on both surfaces and the inoculum was spread with a sterile plastic rod. The inoculated slices were kept under sterile conditions in safety cabinet for 10 min (for each side) for absorption of inoculum. Each piece of inoculated salmon was covered on the both sides with the gels (0.5 cm thickness, 6.6 cm diameter, 8-9 gram each). The gel coated samples were wrapped with plastic wrap (PVDC) and then with aluminum foil. The packaged smoked salmon samples were prepared in duplicate and stored at 4 °C for 15 days.

The samples were analyzed following the packaging and at the 5^{th}, 10^{th} and 15^{th} days of cold-storage *L. innocua* count was determined. A portion (10 g) of each sample was placed into a stomacher bag with 90 mL sterile 0.1% peptone water and immediately homogenized using a stomacher (BagMixer ® 400, Interscience, France) for 60 s. The serial decimal dilutions were prepared from this homogenate and appropriate dilutions

(0.1 mL) were spread plated, in triplicate, onto Oxford Listeria Selective Agar (Merck, Darmstad, Germany) with Oxford Listeria Selective Supplement (Merck, Darmstad, Germany). The plates were incubated at 37 °C for 48 h and small black colonies with halos around them were enumerated. The counts were performed in triplicate plates. Microbiological counts were expressed as colony-forming unit per gram (CFU g^{-1}) and the means and standard errors were calculated.

3.2.5.3. Antimicrobial Activity of Gel/SPI Gel

The antimicrobial activity of the gel materials without release conditions were determined for absorbent gels. Gel, Gel/SPI, Gel+LYS and Gel/SPI+LYS were tested for the antimicrobial activity of incorporated LYS into the gels. In this purpose, 10 g of gels, prepared the day before the test, were inoculated by 5 mL of *L. innocua* inoculum (~10^5 cfu/mL). The inoculated gels were placed in plastic sterile petri dishes, wrapped with plastic film and then aluminum foil, and stored at 4 °C for 7 days. At day 0 and day 7, bacterial load was determined. 10 g of gels were homogenized in 90 mL peptone water (0.1%) for 120 s, using a stomacher (BagMixer ® 400, Interscience, France). The serial decimal dilutions were prepared from this homogenate and appropriate dilutions (0.1 mL) were spread plated, in triplicate, onto Oxford Listeria Selective Agar (Merck, Darmstad, Germany) with Oxford Listeria Selective Supplement (Merck, Darmstad, Germany). The plates were incubated at 37 °C for 48 h and small black colonies with halos around them were enumerated. Microbiological counts were expressed as colony-forming unit per gram (CFU g^{-1}) and the means and standard errors were calculated.

3.2.6. Characterization of the Gels

3.2.6.1. Texture Profile Analysis

Gels were poured into plastic molds with 24 mm diameter and cooled at 4 °C for 18±2 h. The texture profile of the gels was determined as described by Fiszman and Damasio (1999), using a texture analyzer (TAXTplus, Stable Micro Systems, Godalming, UK). The cylindrical samples with the height of 13 mm and the diameter of 24 mm were compressed under 75 mm diameter plunger at a test speed of 0.5 mm/sec using 50 kg load cell. The compression level was 50% to avoid large deformation, recommended by Pons

and Fiszman (1996). The samples were kept at 4 °C until measurement and were tested in 3 replicates in each batch. The recorded textural parameters were hardness (N), cohesiveness, and gumminess, and resilience.

3.2.6.2. Rheological Measurements

Dynamic oscillatory measurements of Gel, Gel/RS, Gel/CW and Gel/SPI gels were carried out after keeping them in the refrigerator for 18 h at 4 °C, using a controlled stress AR-2000 rheometer (TA Instruments, New Castle, DE, USA) with 25 mm diameter parallel plates. The gel discs with 25 mm diameter and 3 mm thickness were loaded in the gap between the two plates. To determine the linear viscoelastic range of the samples, stress sweep test was performed under 10 Pa stress. Frequency sweep test was also run from 0.1 to 25 Hz, and 1 Hz frequency was selected for the test. Before each measurement 1 s^{-1} pre-shear and 1 min equilibration at 25 °C were applied.

To determine the melting and gelling temperatures, after the equilibration step samples were heated from 25 °C to 75 °C at 5 °C min^{-1} heating rate. At 75 °C the loss and storage moduli were measured during 10 min incubation, followed by cooling back to 25 °C at 5 °C min^{-1}. The elastic modulus (G′), which represents the energy stored due to elastic deformation, was used to indicate the viscoelastic nature of the gels. The point where elastic modulus (G′) and loss modulus (G′′) crossover during heating and cooling stages indicates the melting (T_m) and gelling (T_g) temperatures, respectively.

3.2.6.3. Water Absorption Capacity of the Gels

The absorbed water by the gels was calculated as the difference between initial and final weights after soaking the gels into water at 4°C. Gels were weighed after annealing for 16 h at 4 °C and initial weights were recorded. Each gel was soaked in 50 mL deionized water shaking at 80 rpm at 4 °C. At the days 0.25, 1, 2, 5, 7, and 8 the gels were removed from the water, excessive water gently dried by paper towel, and weighed. The increase in the weight of each sample as percentage (%) was plotted against time. Total absorbed water (A_t) was determined by Eq.3.4;

$$A_t = (W_w - W_D)/W_D * 100 \quad (3.4)$$

3.2.6.4. Solubility of the Gels in Water

To estimate the soluble gelatin concentration in water, Bradford assay was followed as a rapid method for protein quantitation. The assay reagent was made by dissolving 100 mg of Coomassie blue G250 in 50 mL of 95% ethanol. Then the solution was mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Whatman No. 1 filter paper and then stored at room temperature. The gel samples were placed in shaking (80 rpm) beakers each containing 50 mL distilled water, and the soluble gelatin gel concentrations were monitored at two different temperatures, 4 °C and 20 °C. 100 μL samples were taken into tubes at every 24 h. 1 mL of protein reagent was added into tubes and mixed by vortexing. After 1 h incubation in room temperature, the absorbance was measured spectrophotometrically at 595 nm according to the method by Kruger (1994). The calibration curves were prepared using series of bovine serum albumin. Average of three measurements was used in the calculations.

3.2.6.5. Released Water from the Gel Network

Water-holding properties of the gels were measured using centrifugation method according to the method of Banerjee and Bhattacharyan (2011). Gels with 13 mm height and 23 mm diameter were placed in centrifuge tubes after storage at 4 °C, and were centrifuged at 3000*g* for 15 min. Before and after centrifugation, the gels were weighed, and the released water amount was calculated from the difference between pre- and after centrifugation. Test was performed in two replicates. The released amount of water measured at 16 and 120 h after gel preparation.

3.2.6.6. Microstructure of the Gels

The microstructural examination of the gels was performed by using ESEM (environmental scanning electron microscopy) detector of scanning electron microscopy (FEI Quanta 250 FEG, Oregon, USA) under 300 Pa. The micrographs of each sample were taken at 1000x, 2000x and 2500x magnification. All samples were examined directly the day after preparation at room temperature without any pre-treatment.

3.2.7. Statistical Analysis

Statistical analysis was performed by using MINITAB® release 17 (Minitab Inc., State College, Pa., U.S.A.). The mean values obtained from the analyses were analyzed by one-way analysis of variance (ANOVA). Significance threshold was $P < 0.05$. Results were given as "mean ± standard error".

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Characterization of the Gels

4.1.1. Mechanical Properties of the Gels

The mechanical properties of the gels were tested by performing texture profile analysis (TPA) using a texture analyzer. The gelatin concentration of the gels was selected after testing a series of gelatin gel with increasing concentrations. As the protein concentration increased, firmer and harder gelatin gels were obtained. The hardness values of gelatin gel series are presented in Figure 4.1.

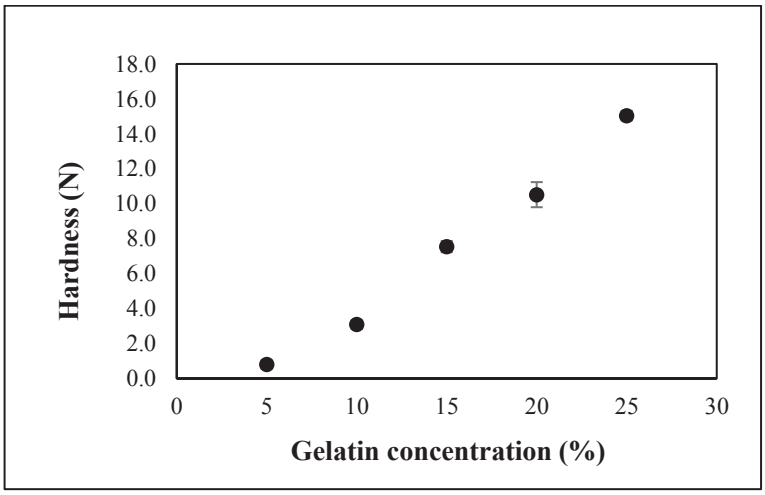

Figure 4.1. Hardness (N) of the gelatin gel series with gelatin concentrations between 5-25% (w/w) (Analysis was performed using 5 kg cell load).

For composite gels, 15% gelatin concentration was selected. Gelatin gel with an average hardness was chosen to prepare composite gels, considering the possible significant increase in hardness introduced by other polymers. It was aimed to keep the gelatin concentration as low as possible, and to increase the hardness by mixing with a second polymer. From the TPA of the composite gels, hardness, cohesiveness, resilience, springiness, and gumminess parameters were obtained. Since 5 kg cell load was insufficient to compress some hard gels, 50 kg cell load was used in the TPA of composite gels. Results are presented in Table 4.1.

Hardness gives information about the gel strength of the gels under compression. The hardness values measured during the first compression varied according to the gel composition. No brittleness (fracturability) was observed during the compression cycles. Mixing gelatin with 7.5% SPI increased the hardness of gelatin gel dramatically due to the reinforcing effect of SPI in the gel structure. When LYS and GTE were incorporated into Gel/SPI, the interactions of SPI with LYS and GTE might have resulted as harder Gel/SPI gels. SPI-LYS interaction due to the charge attractions may be responsible of the increase in the gel hardness. LYS, positively charged at pH of Gel/SPI solution (6.2±0.1), might have had an electrostatic attraction with the SPI at this pH which was close to or slightly above its isoelectric point (5.0-5.6). Another known phenomenon is the phenolic-protein interactions, occurred between GTE and SPI, and may have influenced the increase in hardness. Mixing gelatin gel with RS and CW has increased hardness of gelatin gel, too. Both RS and CW mixed into Gel solution contained 1.5-fold more dry matter than that had Gel within the solution. Higher polymer concentration resulted as increased hardness, as reported by other studies (Lau, Tang and Paulson 2000; de Jong and van de Velde 2007; Hurler et al. 2011). Gel/SPI and Gel/CW had very similar hardness values. However, in Gel/CW and in Gel/RS, since no interaction of CW and RS with LYS and GTE was expected, incorporation of these active agents slightly reduced this parameter. LYS and GTE caused no significant difference in Gel. There might be an attractive interaction between gelatin network and active agents, too. However, according to the hardness results, this effect was more dramatic in Gel/SPI. The remarkable increase in the hardness of Gel/SPI incorporating LYS and GTE could be due to the increased protein concentration within the same volume of gel. Additionally, in Gel/SPI, SPI may be found partially in the free form, which could create more affinity with the active agents. Therefore, higher non-covalent interactions formed between these active agents and the SPI, present both in gelled and free form.

The most significant reduction in hardness and gumminess of Gel/RS and Gel/CW was occurred when GTE and LYS were incorporated together into the gels. In a research carried by Gimenez et al. (2013), GTE incorporated in agar films reduced the tensile strength of the films, due to the loss of intermolecular interaction among agar molecules. Lau et al. (2000) suggested that presence of Ca ions in gellan/gelatin mixed gel structure weakened the gel by binding to anionic sites of gellan molecules and reducing the formation of linkages between aggregated helices. In gelatin and mixture gels, when GTE and LYS were incorporated together, more pronounced reduction in hardness was

observed. Possible interactions occurred between GTE and LYS may have disrupt the gel network partially and reduced hardness for the gels with GTE+LYS.

Gumminess was also higher in Gel/SPI, especially in the ones incorporated with GTE and LYS, than that of Gel, Gel/CW and Gel/RS. There is a proportional relationship between hardness and gumminess (Shafiur Rahman and Al-Mahrouqi 2009). Here, as the hardness of the gels increased, higher gumminess values were measured. Gumminess represents the energy required to break down the material. Gumminess values of Gel/SPI +GTE and Gel/SPI+LYS were higher than other gels, parallel to their hardness. Gel/CW also showed higher gumminess. Candelilla wax particles and SPI phase mixed with the gelatin network had an increasing effect in hardness and therefore in gumminess.

Table 4.1. Texture profile analysis (TPA) parameters of the gels.

Type of gel	Hardness (N)	Cohesiveness (%)	Resilience (%)	Springiness (%)	Gumminess
Gel	23.16 ± 6.9^{f}	77 ± 8^{abc}	104 ± 2^{ab}	84 ± 4^{cdef}	18.05 ± 7.2^{fgh}
Gel+GTE	22.84 ± 4.5^{f}	75 ± 2^{abcd}	98 ± 7^{bc}	98 ± 2^{ab}	17.2 ± 3.1^{gh}
Gel+LYS	25.62 ± 0.05^{def}	74 ± 1^{bcd}	89 ± 4^{cd}	97 ± 3^{ab}	18.87 ± 0.4^{efgh}
Gel+GTE+LYS	20.92 ± 2.6^{f}	85 ± 2^{a}	115 ± 5^{a}	90 ± 6^{cdef}	17.74 ± 2.6^{gh}
Gel/RS	29.79 ± 2.1^{cde}	73 ± 1^{bcd}	77 ± 1^{ef}	82 ± 5^{def}	21.69 ± 1.9^{defg}
Gel/RS+GTE	23.74 ± 1.5^{f}	76 ± 1^{abc}	93 ± 5^{bcd}	87 ± 10^{cde}	18.02 ± 1.3^{gh}
Gel/RS+LYS	21.56 ± 3.0^{f}	72 ± 1^{bcd}	89 ± 7^{cde}	89 ± 7^{bcd}	15.51 ± 1.9^{h}
Gel/RS+GTE+LYS	19.08 ± 1.7^{f}	78 ± 1^{abc}	97 ± 2^{bc}	92 ± 6^{abc}	14.88 ± 1.4^{h}
Gel/CW	36.07 ± 0.7^{bc}	76 ± 1^{abc}	74 ± 1^{f}	78 ± 1^{ef}	27.65 ± 0.06^{cd}
Gel/CW+GTE	30.81 ± 1.7^{cde}	77 ± 1^{abc}	74 ± 2^{f}	85 ± 2^{cdef}	23.87 ± 1.58^{def}
Gel/CW+LYS	32.41 ± 0.2^{bcd}	78 ± 0.5^{abc}	76 ± 0.6^{ef}	82 ± 0.8^{cdef}	25.26 ± 0.19^{de}
Gel/CW+GTE+LYS	25.01 ± 1.6^{ef}	78 ± 1^{abc}	75 ± 1^{f}	82 ± 4^{cdef}	19.29 ± 1.0^{efgh}
Gel/SPI	37.16 ± 2.04^{b}	60 ± 10^{bcd}	84 ± 9^{def}	99 ± 1^{ab}	22.32 ± 4.3^{defg}
Gel/SPI+GTE	51.95 ± 3.55^{a}	79 ± 1^{ab}	76 ± 1^{f}	81 ± 2^{ef}	41.03 ± 2.06^{a}
Gel/SPI+LYS	47.69 ± 0.93^{a}	67 ± 5^{de}	76 ± 1^{f}	82 ± 0.6^{ef}	31.94 ± 2.0^{bc}
Gel/SPI+GTE+LYS	47.09 ± 1.7^{a}	71 ± 5^{cd}	83 ± 3^{def}	77 ± 3^{f}	25.74 ± 1.8^{cd}

a-h: Values within each parameter followed by the same letter are not significantly different ($P > 0.05$)

Cohesiveness describes how well a material withstands a second deformation after the resistance shown during the first deformation. It reflects the degree of difficulty to break down the gel structure (Lau et al. 2000). A product is more cohesive as it adheres to itself better under a certain compression. According to Glibowski and Kowalska (2012), a 0% cohesive material cannot have rebuilt its structure after compression, where a 100% cohesive material can totally recover between two cycles of compressions. Gel+GTE+LYS showed the highest cohesiveness, which was attributed to its higher resistance to several compressions than other gels. GTE+LYS incorporation made

positive effect on the cohesiveness of the gels. Increased polymer concentration in composite gels did not affect the cohesiveness. All the gels with similar cohesiveness values could maintain their original shape without breaking down into pieces after compression cycles. Highly cohesive products are more tolerated to production, handling, and packaging stresses exposed during manufacturing.

Resilience represents the energy recovered when the force is removed after the first compression. In composite gels, relatively lower resilience values were measured. It was reported that, as solid or gelling agent concentration increased, resilience reduced (Shafiur Rahman and Al-Mahrouqi 2009). Incorporation of GTE+LYS increased the resilience values of the gels. All the gels showed good shape recovery behavior after compression cycles. Resilience of harder gels such as Gel/CW and Gel/SPI was slightly lower than softer gels, namely Gel and Gel/RS.

Springiness, also called as elasticity is the measure of breaking down in the gel structure, and represents the rubberiness of a food product in the mouth. Low springiness in gel materials means that they break into many small pieces easier under compression than elastic gels such as gelatin gel. Softer gels had relatively higher springiness values. Springiness closer to 1 means that the gel shows high elasticity. Within each gel, as hardness reduced by LYS and GTE incorporation, springiness increased slightly.

It is desirable to obtain gels with high strength and flexibility, with reversible deformation character. Mixing gelatin with SPI and CW improved elasticity and resistance under high strain. Commonly used approaches to develop mechanical features of the gels are using cross-linking agents, mixing two gelling networks or incorporation of nanoparticles into gelatin gels (Ge et al. 2018) The interactions between gelatin and LYS, GTE and gelling polymers had an effect on the mechanical properties, could be mostly coming from the hydrogen bonding and electrostatic interactions formed.

4.1.2. Rheology of the Gels

Rheologic characteristics of Gel, Gel/RS, Gel/CW, and Gel/SPI were determined using a parallel-plate rheometer. To determine if the stress and frequency applied are in the linear viscoelastic region, frequency sweep test and stress sweep test were performed. 1 Hz (6.28 rad/s) frequency and 10 Pa stress parameters selected for the test were within the linear viscoelastic region (Figure 4.2).

The change in storage modulus (G′) and loss modulus (G′′) during temperature ramp test of Gel, Gel/RS, Gel/CW and Gel/SPI are illustrated in Figure 4.3. G′, G′′, Tan δ and melting temperatures estimated from temperature ramp test are given in Table 4.2.

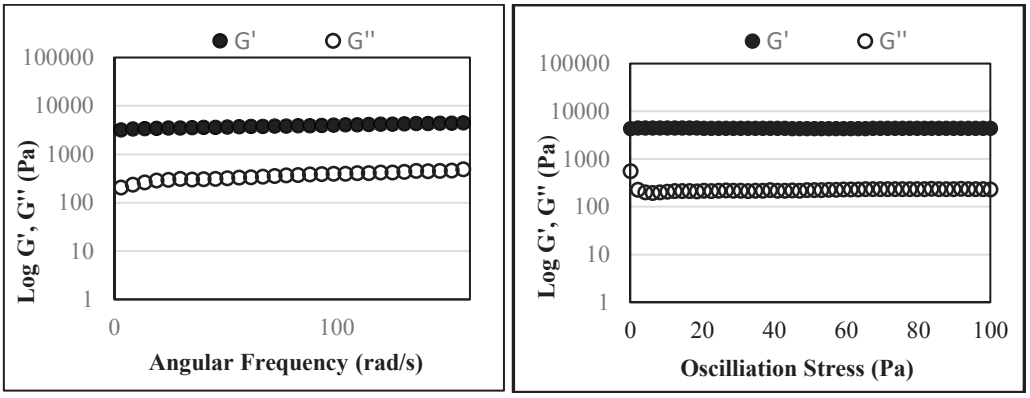

Figure 4.2. Frequency sweep (0.6- 157 rad/s) and stress sweep (0.1-100 Pa) tests applied on gelatin gel (Gel) sample.

All the samples showed the characteristics of viscoelastic materials according to their G′ being higher than G′′ (Tanδ<1). This behavior is typical for gel-like substances. G′ and G′′ were measured at 27 ºC for all samples, and at least 6 measurement points were taken for two different samples. Storage modulus (G′), loss modulus (G′′) and Tanδ of Gel/SPI were higher than other gels, therefore it had a higher elastic behavior. This difference may be originating from the polymer characteristics of soy protein isolate and its nature as gel. The highest G′ and G′′ after Gel/SPI were measured for Gel/RS and Gel/CW, respectively. Increasing cross-links and hydrogen bonding formations within the gel structure cause increase in the measured G′ and G′′, so in the solid-like behavior of the gels (Apichartsrangkoon 2003). Hardness of Gel/RS measured during TPA was slightly higher than Gel however under shear stress, Gel/RS showed greater G′ than that of Gel. In Gel/RS, two different gelling polymer introduced higher number of linkage to the gel network. It was reported that the gelation of starch occurs faster than gelatin (Muhrbeck and Eliasson 1991). In that case gelled amylose aggregates constitutes the discontinuous phase. Here, the retrogradation of amylose network introduced more rigidity and worked as distributed filler particles resulting as a higher G′ in Gel/RS gel. This result showed that, the improved viscoelastic behavior may not be reflected by hardness parameter.

The rheological measurement clearly implied that mixing gelatin gel network with gelling SPI and RS or with CW particles improved elastic behavior of the gels. The

increased gelling agent concentration and the filling effect of the second polymers played a role to increase viscoelastic behavior. It was reported that as the solid concentration increases, elastic modulus increases proportionally (Bot et al. 1996; Carvalho, Onwulata and Tomasula 2007). Also, depending on the interactions between the gelling components, and the nature of the second polymers mixed into gelatin gel determines the change in modulus. Since the added polymer into the gelatin gel had stiffer characteristics, they increased G′. If there were no interactions between the mixed polymers, G′ would reduce since the gel networks would be weakened. For the composite gels, it was not expected that the mixed polymers would interact with gelatin gel by electrostatic interactions however, the gelled aggregates of SPI and RS filled the network and homogeneously dispersed CW particles that may have created hard emulsion droplets within the network have influenced the rigidity and therefore the viscoelastic behavior of the gels.

Table 4.2. Comparison of storage moduli (G′), loss moduli (G′′), Tan δ read at 27 °C and melting temperatures (T_m) of the gels.

Gel Type	**G′**	**G′′**	**Tan δ**	**T_m (°C)**
Gel	1037 ± 17^{c}	120 ± 16^{b}	0.116 ± 0.01^{a}	34.2
Gel/RS	4607 ± 549^{b}	620 ± 174^{a}	0.129 ± 0.03^{a}	34.2
Gel/CW	3544 ± 254^{b}	417 ± 117^{ab}	0.116 ± 0.03^{a}	33
Gel/SPI	5817 ± 416^{a}	508 ± 37^{a}	0.087 ± 0.001^{a}	34.4

a-c Values within each parameter followed by the same letter are not significantly different ($P > 0.05$)

Tanδ (G′′/G′) reflects the relativity of viscous and elastic components. It is a parameter used to determine the gelling point during sol-gel transition. When Tanδ is more than 1 (G′′/G′ >1) the gel is in sol phase, when it is below 1 (G′′/G′<1) the gel is in solid or gel phase. The point where Tanδ = 1 is the gelling or melting point that sol phase switches to gel phase or vice versa. Mixing gelatin gel with SPI, RS and CW did not cause a significant change in the melting temperature of the gels. These values were obtained from the crossing point of G′ and G′′ measured during temperature ramp tests. This data was also validated by calculating Tanδ, and the first temperature point where it corresponds to 1 was taken as the point that the gels started to transfer into sol phase. It was observed that increased number of linkages and gelling agent concentration did not affect the melting point of the composite gels.

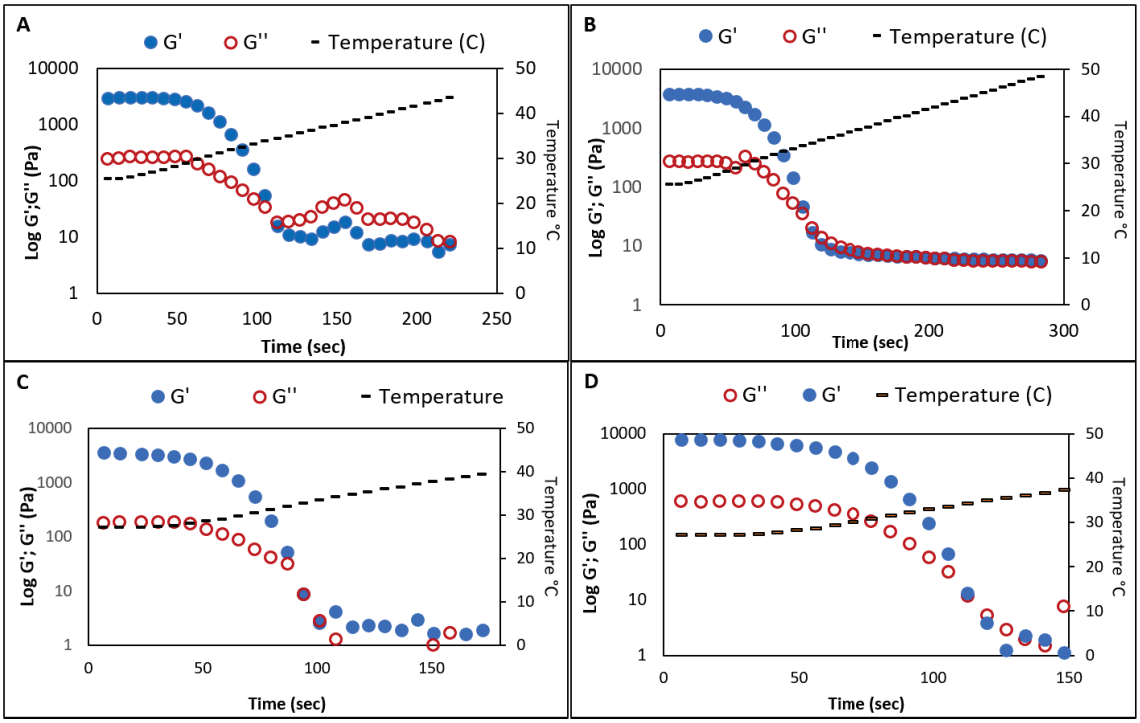

Figure 4.3. G′ and G′′ of Gel (A), Gel/RS (B), Gel/CW (C) and Gel/SPI (D) during heating from 25 ºC to 75 ºC (the graphics display only the initial heating part until 40-60 ºC where G′ and G′′ show regular decrease and the crossing point could be determined).

Produced gels showed viscoelastic behavior and mixing gelatin with RS, CW and SPI increased the stiffness of the network, evidenced by higher storage modulus (G′) than that of pure gelatin gel. In recent studies, gel stiffness and elasticity were modified mixing gelatin with different polymers and nanomaterials. Higher G′ and G′′ were obtained when chitosan based nanoparticles were incorporated into gelatin hydrogel (Ge et al., 2018). In a two-component starch-bovine serum albumin (BSA) system, amylopectin aggregates diffused from the swelled starch granules formed a discontinuous phase within a continuous BSA network, and improved the gel strength and G′ by acting as a filler (Muhrbeck and Eliasson 1991). Calcium caseinate-soy protein isolate mixed gels showed the highest G′ compared to the mixtures of calcium caseinate with fish protein isolate, whey protein isolate, egg albumin and wheat gluten. The increased G′ was attributed to the increased aggregated entanglements occurred after denaturation (Onwulata, Tunick and Mukhopadhyay 2014).

4.1.3. Solubility of the Gels in Water

The solubility of the gels in water during the release tests were determined by Bradford protein solubility method at 4 ºC and 20 ºC, and the results are given in Figure 4.4. Gel/SPI gave the highest solubility in all time points due to the soluble soy protein within the gel matrix in all temperatures and sampling times. This result supported the idea of free SPI present in the Gel/SPI network. Even though, the Gel/SPI solution was heated enough to cause protein denaturation, significant amount of soy proteins may be still found apart from the gelled soy protein network when the incorporated SPI concentration was 7.5% (w/w).

At 4 ºC, the solubility of the gels was low and remained constant for 48 hours of storage. At 72nd h of storage in water, the soluble gelatin concentration from all the gels increased. The lowest and highest solubilized protein concentration at 4 ºC corresponded to 0.04-0.08% of the gelatin found in the gels, except Gel/SPI. At cold storage temperature, gels could maintain their integrity even under continuous shaking in aqueous media. The released total protein concentrations from the gels were not dramatically different from each other.

At 20 ºC, the soluble protein concentration increased gradually as the storage time increases. However, the solubility of Gel/RS and Gel/CW was lower than Gel and Gel/SPI, and did not increase dramatically during the storage for 72 h. Compared to the solubility of the gels stored at 4 ºC, solubility of Gel increased 2.5-4 folds at 20 ºC (0.11-0.26% of protein). However, solubility of Gel/RS and Gel/CW increased 1-1.5-fold. It can be proposed that mixing gelatin gel with rice starch and candelilla wax may be helpful to provide the integrity of the gels at higher temperatures than 4 ºC. As mentioned in section 4.1.2., RS and homogeneously dispersed wax particles have increased the integrity and elasticity of gelatin gel. Gel/RS and Gel/CW with higher melting temperature had less dissolved protein from their gel network. This modification in the physical structure, may have reduced the solubilized gelatin from the gels, especially at 20 °C. However, the total maximum solubilized protein concentration from the gels kept in aqueous media were quite low. This concentration would be even lower when the gels would be coated on food surfaces where the penetration of free water would be less. This is a positive characteristic of the gels since the migration from the biodegradable gels onto food surface would be insignificant.

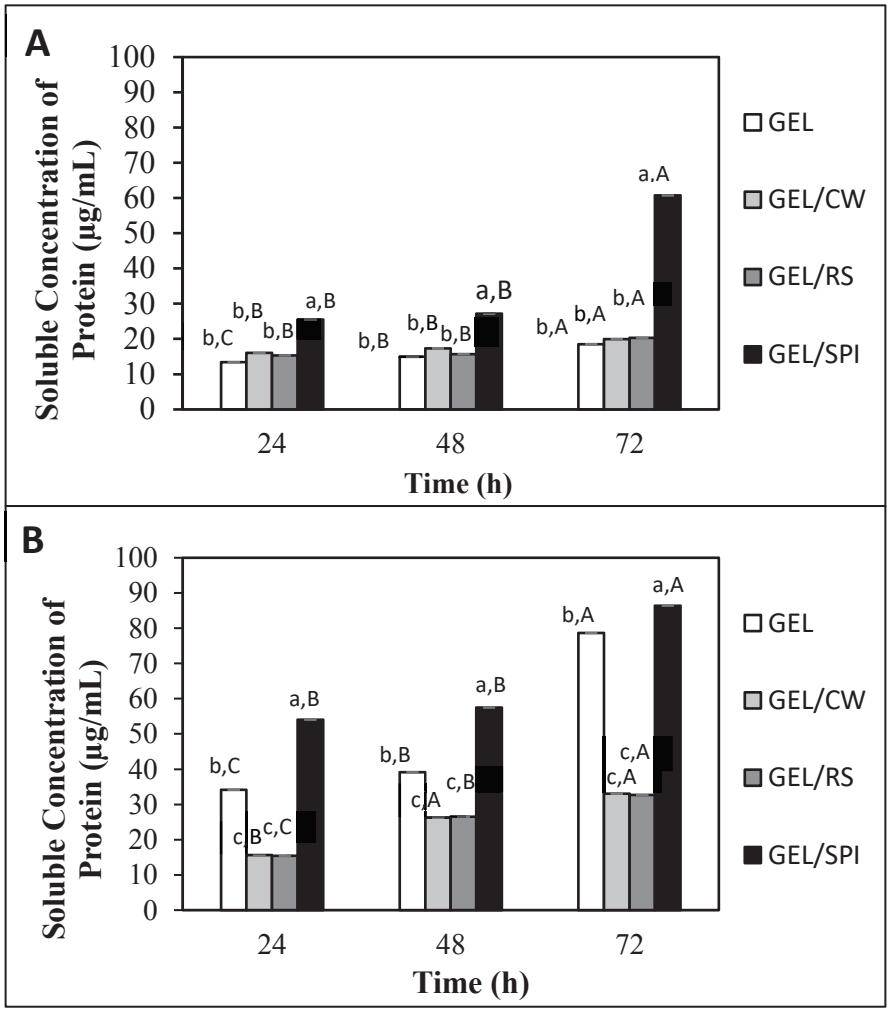

Figure 4.4. Soluble protein concentrations (µg/mL) of the gels kept in water for 72 h at 4 ºC (A) and 20 ºC (B) (a-c: Values within each storage time followed by the same letter are not significantly different at P > 0.05, A-C: Values within treatment followed by the same letter are not significantly different at P > 0.05)

4.1.4. Stability of the Gels

The amount of water released from the gel structure is related to syneresis during the annealing of the gels. The removed water was determined from the differences between the initial gel weights and the weight after removal of leaked water by centrifugation. After pouring the gel solutions in the molds, at 16th and 120th h of storage at 4 ºC, the amount of water leaked from the gel structure was given in Figure 4.5.

The amount of leaked water from the gels were very low (0.5-2%) in 5 days of storage at 4 °C. 16 h after pouring the gels, highest water release was measured from

Gel/SPI (0.86%). However, the water loss was reduced by 0.3% at 120th h from this gel. Data obtained for 16th h were statistically similar for all gels. At 120th h, the greatest water loss was occurred from Gel (2%). Released water from Gel after 16 h was 0.5% of its weight, and this loss increased to 2% after 120 h. The increases in released water from Gel/RS and Gel/CW were 0.17% and 0.3%, respectively. At 120th hour, water loss from Gel/RS and Gel/CW were similar. Least syneresis was occurred in Gel/SPI gel.

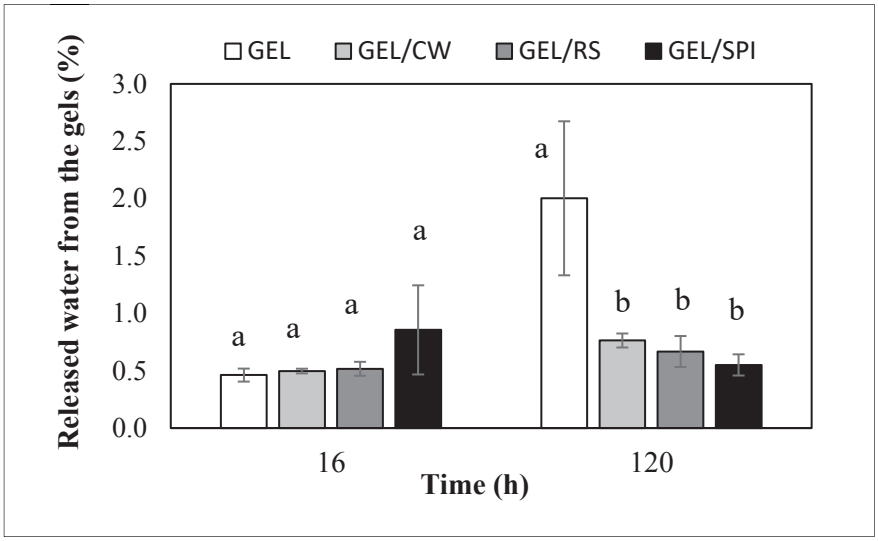

Figure 4.5. Released amount of water from the gels after 16h and 120h storage at 4 ºC (a-b: Values within each storage time followed by the same letter are not significantly different at P > 0.05)

Comparing the mean values, Gel/SPI was the most successful to withhold the water during 5 days of storage. Soy protein is known for its high water-holding capacity and used as binder to prevent syneresis and drip loss in food formulations such as meat, sausages, dairy and cakes (Kinsella 1979). Also, it was reported that as the concentration of gelling agent increases, syneresis reduces (Banerjee and Bhattacharya 2011). In gellan-$CaCl_2$ gel mixed with "particle gellan-$CaCl_2$ gel", syneresis was lower than pure gellan gel mixed with "particle gellan-$CaCl_2$ gel" during 24 h storage at 20 °C (Moritaka, Kimura and Fukuba 2003). The higher syneresis was attributed to the interaction between $CaCl_2$ of particle gel and pure gellan during the ageing process. It can be concluded that the syneresis is related to the density and aggregation ratio in the gel network.

The polymer concentration in the composite gels were higher than Gel, and higher concentration contributed in reducing syneresis. These results clearly showed that gelatin gel network is less capable of holding the water, and mixing additive polymers with gelatin gel resulted as less syneresis. Due to the higher entanglements and stiffer structure

the shrinkage of gelatin must have been reduced, and resulted as less syneresis upon 5 days of ageing. Nevertheless, the released free water from all the gels was still very low (0.5-2%) which is good for the food applications, since the gels would not release much free water onto food surface that can favor the microbial growth.

4.1.5. Water Absorption of the Gels

Gel, Gel/RS, Gel/CW and Gel/SPI were soaked into the deionized water and the weight increase were monitored for 8 days. Swelling, and so the increase in the gel weights were measured to calculate water absorption of the gels. The results are shown in Figure 4.6. No significant increase was measured in the weight of gels after day 7. Gel/SPI gel absorbed significantly higher water than other gels and its weight increased 156% of its initial weight within 7 days. At the end of 7 days of storage in the water, the increase in the weight of the gels compared to their initial weights are 58.5% for Gel, 156.8% for Gel/SPI, 74.6% for Gel/RS, and 57.3% for Gel/CW. Candelilla wax did not affect the water absorption, where rice starch contributed to the water absorption of gelatin gel. RS with its natural gelling and water absorption properties increased the overall water intake, but it was only half of the Gel/SPI gel did. Soy protein in concentration, isolate of flour form, has been used in doughs, comminuted meats, dairy foods and custards due to improve water binding of the system (Porcella et al. 2011). Water absorption capacity of different commercial soy protein isolates were reported as 6.0-11.3 mL water /g protein (Zayas 1997). Hydration capacity of soy protein was reported as 0.33 g water/g protein (Damodaran 2008; Chou and Morr 1979). Gelatin gel increased its weight by 24.5% within 24 h. A similar result was reported by Kunitz (1928) where 14% gelatin gel blocks soaked in acetate buffer (pH 4.7) at 5 °C gained 17% of its initial weight. The swelling and water retaining capacity of the gels are related to the intermolecular forces and hydrogen bonding between the polymer matrix and water. Even though there are different views on the physical state of water in gel, several suggestions stated that water exists as polarized multilayers on protein surface with decreasing rotational motion as the distance from the surface is increased (Chou and Morr 1979). In Gel/SPI denatured globular SPI and gelatin molecules as joint random coils favored protein-water interaction, so that the swelling of Gel/SPI gel. Water absorption and holding without dissolving is an important function of proteins and therefore the gels.

Water content, defined by the weight percentage of water in a swollen gel, is also a measure of crosslinking density (Young et al., 2005). It was reported that swelling can be less as the cross-linking increases and the network becomes stiffer (Ge et al., 2018). Even though Gel/SPI had the highest stiffness, an important part of SPI may be found within the gel matrix in free form, so it may have caused dramatically high swelling. Also, Gel/RS and Gel/SPI must have shown the properties of phase-separated mixed gels. Therefore, two different gelled networks found in the same body as a mixture but not crosslinked to each other. So, each phase must have contributed independently to the swelling.

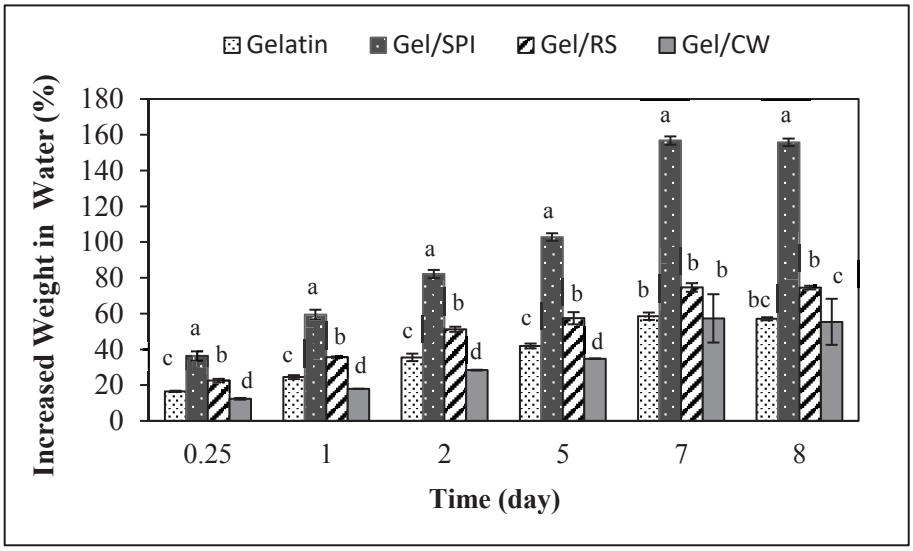

Figure 4.6. Water absorption of the gels monitored for 8 days at 4 °C (a-d: Values within each storage time followed by the same letter are not significantly different at $P > 0.05$)

High water absorption property of the gels may be useful in the case that they are used as absorbent pads inside the packaging placed under the poultry, seafood or meat products to eliminate the accumulation of free water caused by drip loss. Thus, with Gel/SPI the growth of pathogens and spoilage microorganisms could be controlled. To prevent the accumulation of exuded juices inside the packaging of food products using absorbent pads is important for prevention of microbial growth and for improvement of the appearance. However, regular absorbent pads may still create a problem since the immobilized juice within the pad can promote the spoilage, pathogen growth or off-odor production. Therefore, the produced antimicrobial biodegradable absorbent pads can be a potential to develop the regular absorbent pad for use in retail food packaging.

4.2. Release Profiles of the Gels

4.2.1. Lysozyme Release Profile of the Gels in Water

The release rate of the antimicrobials incorporated into the gels should be known to select a suitable food system to apply the gels as coatings. Released LYS profiles of Gel, Gel/RS, Gel/CW and Gel/SPI are presented in Figure 4.7. The release tests conducted in distilled water at 4 °C clearly showed the significant release of LYS from the gels during six days of storage. In all gels, released LYS activity reached at its maximum at 120th hour and then reduced slightly. Throughout the release in water, all the gels kept their integrity and no burst release was observed due to the dissolution of gels.

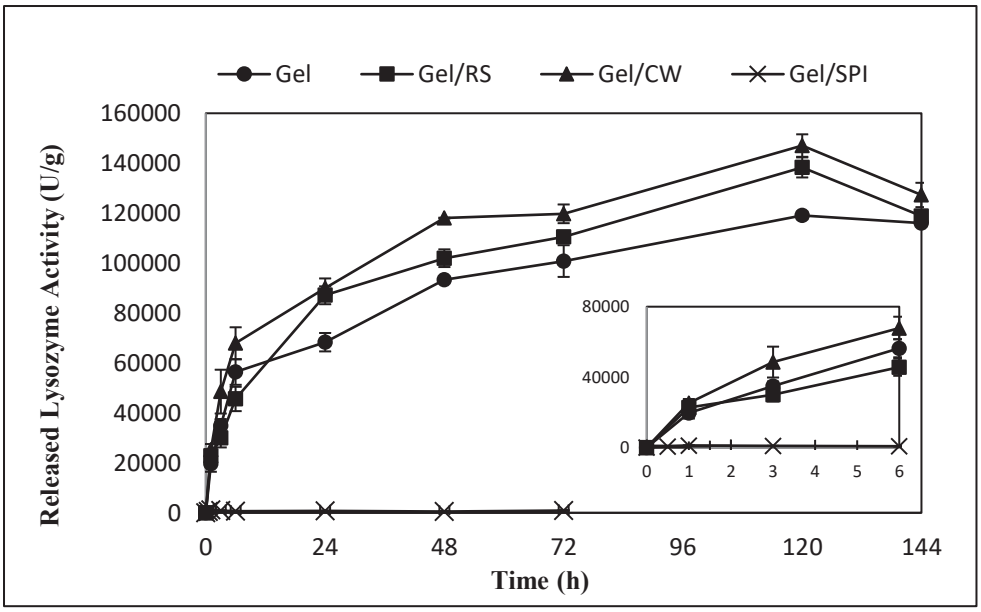

Figure 4.7. LYS release profile of Gel, Gel/RS, Gel/CW and Gel/SPI gels containing LYS (1%) (Release in the first 6 hours of storage is shown separately).

After the 1st hour of the release, released LYS activity of the gels differentiated from each other. No significant LYS activity was released from Gel/SPI for 72 h of release. After this time point, release of LYS from Gel/SPI was not monitored. The reason of not releasing of LYS from this gel must have been the electrostatic interactions between LYS and SPI. At the pH of gel solution (6.2±0.1), LYS was positively charged and SPI, with pI≈5.0-5.5, mainly possessed negative net charge. Therefore, charge-charge attractions did not allow any LYS release from the gels under neutral conditions.

Addition of CW and RS had an increasing effect in the release of LYS from the gel matrix. Gel/CW had the highest release rate and reached 147069 U/g of gel in 120 h. Gel/RS followed a similar release profile to Gel/CW and reached 138314 U/g of gel. At 120^{th} h of the storage, the maximum released LYS activity by Gel was 119071 U/g gel. The maximum released activities by Gel/RS and Gel/CW were 16% and 23.5% higher than Gel, respectively.

This increase in maximum released LYS activity may be favored by the altered structure of gelatin gel matrix by rice starch granules and wax particles. Incorporation of wax particles into edible films was employed to change the physical properties and release characteristics of the materials (Boyaci et al. 2016; Arcan and Yemenicioglu 2013). The numerous microparticles of wax distributed homogeneously in the gel matrix has changed the release profile of gelatin gel. Same effect was observed for rice starch incorporation as well. In Section 4.1.5, it was reported that the Gel/RS gels absorbed higher amount of water compared to Gel, so higher swelling of the gel matrix may have resulted as higher diffusion of LYS into the aqueous media. In the same water absorption test, it was shown that Gel/CW absorbed less water than Gel. However, in the aqueous medium, while the gelatin gel continued to swell, hydrophobic wax particles kept their volume stable. Therefore, the tight integrity of the wax particles with the gelatin gel loosened, and micro gaps occurred between the wax particles and swollen gel matrix to allow higher diffusion of LYS through the gel. The micrographs obtained by ESEM showed that the composite gels have more porous structure with more cavities (Figure 4.8). As seen in Figure 4.8-E, candelilla wax is dispersed through the whole matrix in small particles, where Gel has smooth structure (Figure 4.8-A). It was reported that the release rate of biomolecules from gelatin carriers is affected by the crosslinking density of the gel (Young et al. 2005). The extensively dispersed wax particles, also the rice starch granules may have reduced the crosslinking among gelatin molecules and allowed a higher release of LYS.

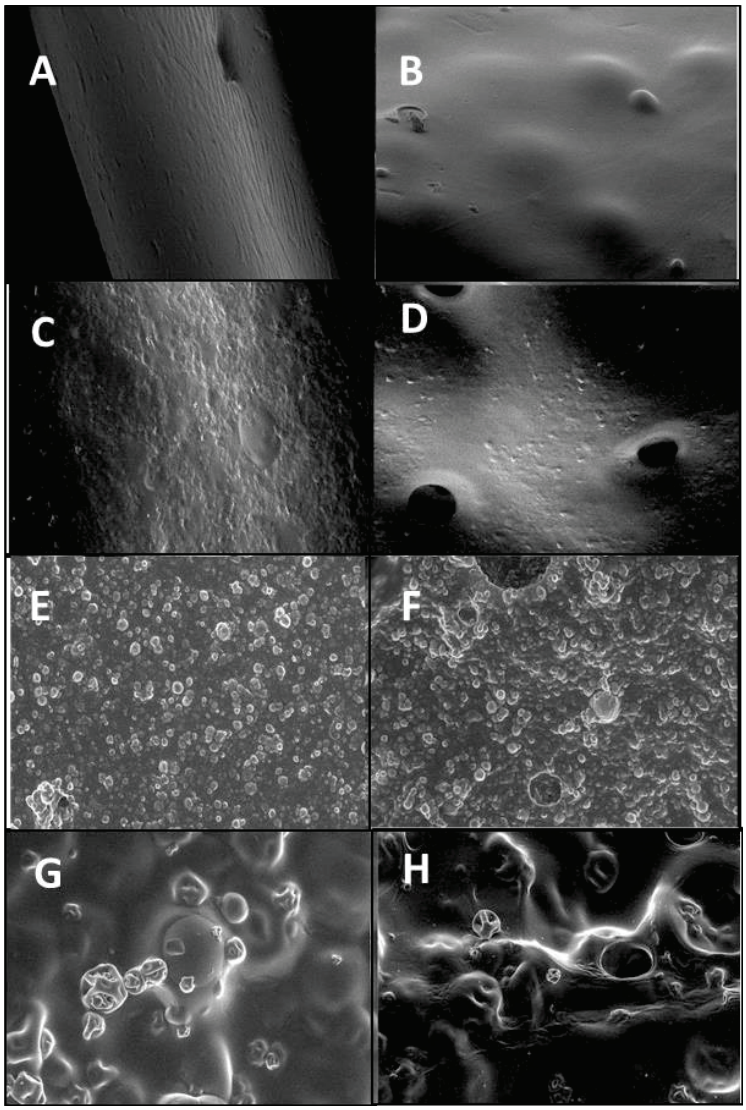

Figure 4.8. ESEM photographs of Gel (A), Gel+GTE+LYS (B), Gel/RS (C), Gel/RS+GTE+LYS (D), Gel/CW (E), Gel/CW+GTE+LYS (F), Gel/SPI (G), Gel/SPI+GTE+LYS (H) (Magnitude 1000x).

In the release tests conducted with Gel, Gel/RS and Gel/CW containing 1% GTE, the highest LYS activity was reached at 120th hour of the storage (See Figure 4.9). After the 120th h, LYS activities were reduced or remained stable. In the first six hours of the release, Gel/CW showed the highest release rate similar to the gels without GTE, followed by Gel and Gel/RS. However, all the gels reached to a similar LYS activity at 48th hour of storage (76333-84213 U/g gel). Until the end of release period, the gels with GTE did not show a dramatic difference from each other regarding to their release profiles. The maximum activity was measured at 120th hour for Gel/RS and Gel/CW and at 144th for Gel. The maximum released activities of LYS released from the gels are given in Table 4.3. The released LYS from Gel/CW was 7% and 12% higher than Gel/RS and Gel, in

the presence of GTE. Gel/RS released 5% higher activity compared to Gel. Statistically, no significant difference was observed between the maximum released LYS activity from the gels incorporated with GTE and LYS together (Table 4.3).

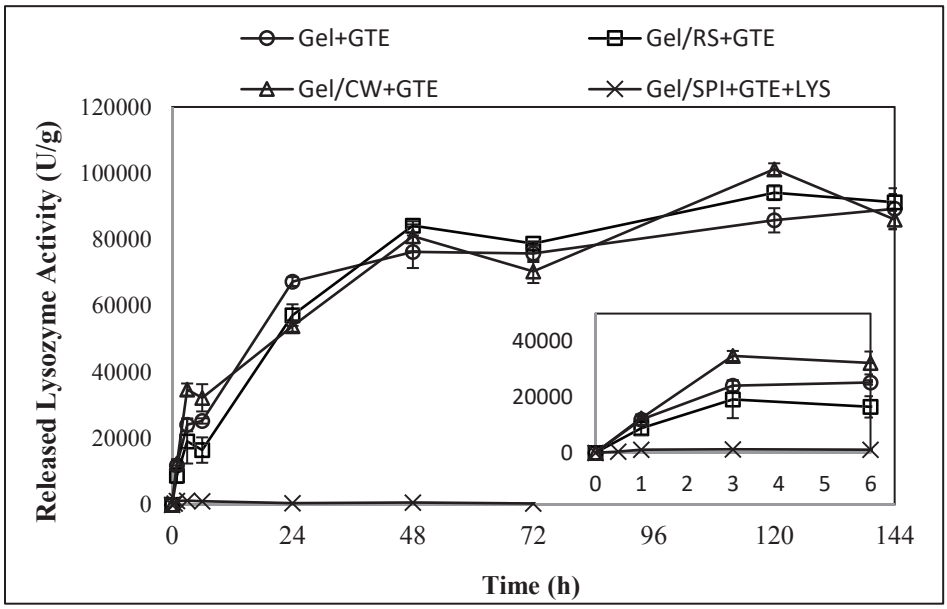

Figure 4.9. LYS release profile of Gel, Gel/RS, Gel/CW and Gel/SPI gels containing LYS (1%) and GTE (1%) gels (Release in the first 6 hours of storage is shown separately).

For all the gels, incorporation of GTE together with LYS resulted in lower final LYS activity and release rates, and this reduction was higher for Gel/RS and Gel/CW than Gel. Incorporation of GTE together with LYS into the gels caused 25%, 32% and 31% reduction in the maximum released LYS activities from Gel, Gel/RS and Gel/CW, respectively.

Presence of GTE had slowed down the release rate of LYS and the maximum released activities. It was reported by Ghosh, Sahoo and Dasgupta (2008) that tryptophan (Trp) residues of LYS binds the EGCG found in green tea extract through Van der Waals interactions and hydrogen bonding. Additionally, GTE catechins and phenolic acids could interact with gelatin molecules and modified the gel structure slightly. These interactions may have caused lower release of LYS from the gels.

Table 4.3. Maximum LYS activities released from the gels in water.

Gel type	Maximum released activity (U/g gel)	
Gel	119071 ± 1561[bc]	(120)*
Gel+GTE+LYS	89283 ± 6204[d]	(144)
Gel/RS	138314 ± 4016[ab]	(120)
Gel/RS+GTE+LYS	94130 ± 2222[d]	(120)
Gel/CW	147070 ± 4500[a]	(120)
Gel/CW+GTE+LYS	101241 ± 1766[cd]	(120)
Gel/SPI	1099 ± 162[e]	(1)
Gel/SPI+GTE+LYS	1246 ± 41[e]	(3)

[a-e] Values by the same letter are not significantly different (P > 0.05)
Data is given as mean ± standard error.
* Time (h) at which the maximum LYS activity was measured.

Type B bovine gelatin, used in the gel production in this study, has isoelectric point (pI) between 4.7 and 5.2, and its 1.5% solution´s pH is reported as 5.0-7.5 (Product information sheet, Sigma-Aldrich, USA). Gelatin is negatively charged in the gel making solutions since the pH values are slightly above its pI. In the same conditions, LYS is positively charged (pI≈11.4) and majority of LYS is bound to gelatin by electrostatic interactions. In the release tests performed in distilled water, positively charged LYS and negatively charged gelatin made a polyion complexation, and only free LYS that was not bound to gelatin molecules could release. Nevertheless, to increase the released LYS activity, gels can be prepared by adjusting the pH of gel making solution to slightly below the pI of gelatin or by selecting an acid-treated gelatin with a higher pI (≈9.0). Moreover, at the pH values below the isoelectric point of gelatin (≈4.7-5.2) the LYS release would be increased due to the charge-charge repulsion. Despite the electrostatic interaction between LYS and gelatin, significant activities were released from the gels containing 1% LYS (w/w). The results obtained from in vitro release model are generally comparable to those of the in-situ release studies and allows prediction of in-situ release profiles. The antimicrobial effect of the released LYS was evaluated further in antimicrobial analyses.

4.2.2. Total Phenolic Release Profile of the Gels in Water

The release profiles of GTE from the gels are presented in Figure 4.10, and the maximum released total phenolic contents from the gels as “mg gallic acid equivalent per

g of gel" are given in Table 4.4. No significant phenolic compounds were released from the control gels. Gel+GTE and Gel/RS+GTE released highest amount of phenolics, followed by Gel/RS+GTE+LYS, Gel/CW+GTE and Gel/CW+GTE+LYS. Gel and Gel/RS showed higher swelling capacity than that Gel/CW did. Higher water amount that penetrated in the gel network may have caused higher solubility of GTE into the aqueous media. Even though, Gel/SPI absorbed much higher water, released phenolic content was the lowest among the gels containing GTE. This may be resulting from the SPI-phenolic interactions.

The released amount reached an equilibrium after 48th hour of storage. It can be said that water soluble GTE was not affected by the modified gel structure. The gels containing LYS and GTE together released slightly lower phenolic content than the gels containing only GTE. This slight reduction may be caused by GTE-LYS interaction, mentioned in Section 4.2.1. However, the decrease in the total phenolic content caused by LYS incorporation was not as significant as the LYS activity drop caused by GTE incorporation, and the difference between released total phenolic content from gels with GTE and gels with GTE+LYS was maximum 0.08 mg GA/g gel.

Table 4.4. Maximum released total phenolic contents from the gels in water at 4 °C.

Gel Type	**Total Phenolic Content (mg GAE/g gel)**
Gelatin Gel	0.055 ± 0.003^{g}
Gel+GTE	1.055 ± 0.03^{a}
Gel+GTE+LYS	0.967 ± 0.018^{c}
Gel/RS	0.066 ± 0.004^{g}
Gel/RS+GTE	1.077 ± 0.01^{a}
Gel/RS+GTE+LYS	1.015 ± 0.015^{b}
Gel/CW	0.051 ± 0.002^{g}
Gel/CW+GTE	1.00 ± 0.025^{bc}
Gel/CW+GTE+LYS	0.977 ± 0.011^{bc}
Gel/SPI	0.179 ± 0.008^{f}
Gel/SPI+GTE	0.844 ± 0.006^{d}
Gel/SPI+GTE+LYS	0.804 ± 0.001^{e}

$^{a-g}$ Values within each storage time followed by the same letter are not significantly different ($P > 0.05$)

The released phenolic content from 1 g of the gels were between 0.80 and 1.05 mg GAE/g gel. This amount corresponded approximately 10% of the incorporated GTE into the gels. In release kinetics of the active agents, the molecular interactions between the agent and the carrier material play an important role. It is known that green tea

catechins create reversible bonds with proteins (Ozdal, Capanoglu and Altay 2013; Wu and Bird 2010). Hydroxyl groups of phenolic compounds form hydrogen bonds with polar groups of gelatin. Zhang et al., (2010) suggested that the reactive sites of oxidized phenolic compounds interact with amino groups of gelatin, forming C-N covalent bonds as cross-link linkages, however here no oxidation conditions were provided during gel preparation. The interactions of phenolic compounds with proteins decrease the total antioxidant activity measured by different methods and total phenolic content in various studies (Ozdal, Capanoglu and Altay 2013). Liang et al. (2013) reported an interaction between lysozyme and procyanidins creates insoluble aggregates, and gum arabic could inhibit this interaction due to its heteropolysaccharide structure and acidic character. A similar interaction may occur in the gels containing LYS and GTE together.

It is reported that there is a strong relationship between antioxidant capacity and polyphenol concentration of green tea (Turkmen et al. 2006). Antioxidant activity of GTE released from the gels were measured by ORAC and TEAC methods, and the results are given in details in Section 4.4.1. Antioxidant activities obtained from ORAC method showed no important difference between the gels incorporating GTE and GTE+LYS. It can be said that parallel results were obtained from total phenolic analyses and antioxidant activity analyses of the gels. The incorporation of antioxidant GTE into antimicrobial gelatin gels could be beneficial to improve oxidative stability of oxygen-sensitive packed foods such as meat and meat products (Hong, Lim and Song 2009; Kang et al. 2007; Siripatrawan and Noipha 2012). Moreover, GTE, when released into food products lacking phenolics, could contribute to human health by increasing the phenolic compound consumption in western diet (Crespy and Williamson 2004; Wolfram 2007).

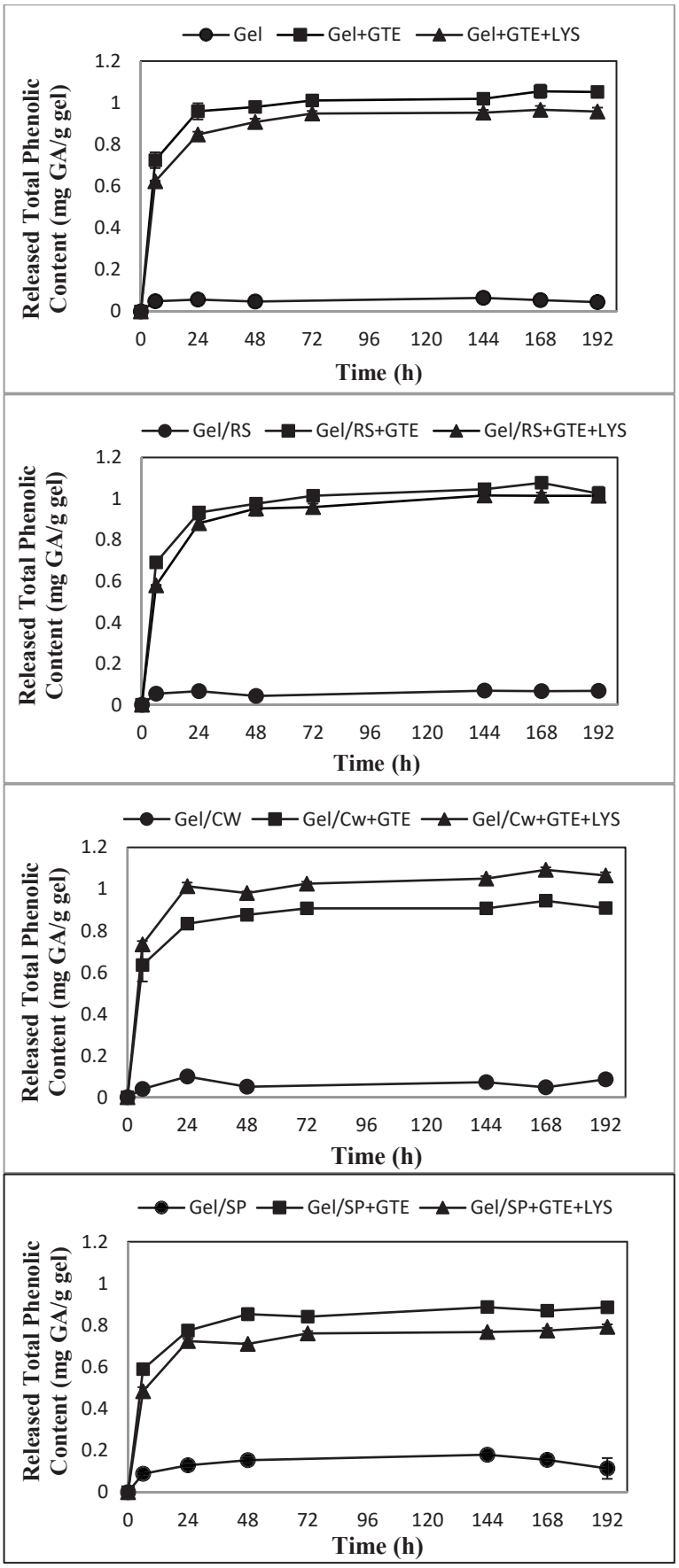

Figure 4.10. Released total phenolic compounds from (a) Gel, (b) Gel/RS, (c) Gel/CW and (d) Gel/SPI gels containing 1% GTE and 1% GTE+1% LYS

4.3. Antimicrobial Activity of the Gels

4.3.1. Antimicrobial Activity of the Gels Based on Their LYS Delivery in Aqueous Growth Media

Gel, Gel/RS and Gel/CW gels with 1% LYS and 1% LYS+1% GTE were tested against *Listeria innocua* in an aqueous growth media for 15 days and with an initial inoculation level around 2.7 log cfu/mL. Since Gel/SPI released no LYS, its antimicrobial activity was evaluated as an absorbent pad in Section 4.3.2. Antimicrobial activity results of LYS delivering gels are shown in Figure 4.11 and Table 4.5. In the antimicrobial tests, the gels without LYS or GTE suppressed the growth of microorganisms compared to control culture. While the increase in the number of control group was 5.2 log CFU/ml, the increase in the growth in the presence of Gel, Gel/RS, and Gel/CW was 2.4, 4.1 and 1.9 log CFU/m, respectively. This result indicated that gelatin showed a significant antimicrobial activity even if there were no antimicrobial agent incorporated in it. This may be resulted from the small peptides released from the gel, originated from the antimicrobial fractions of bovine gelatin. It was reported that small peptides derived from gelatin showed antimicrobial activity against *P. aeruginosa* (Gopal et al. 2012). Some peptide fractions of tuna and squid gelatin showed antimicrobial activity against *L.innocua* (Gomez-Guillen et al. 2010). The exact profile of the released compounds from the control gelatin gel is unknown, however depending on the production methods and type, gelatin may have released some small bioactive peptides into the release media. The gels incorporated by only GTE showed no reduction in the *L. innocua* count, but suppressed the growth compared to the control group and control gel. Increase in the *L. innocua* number was 1.54, 2.6, and 1.65 log CFU/mL for Gel+GTE, Gel/RS+GTE, and Gel/CW+GTE gels, respectively. In several studies, the inhibitory effect of green tea extract was shown against *L. monocytogenes*. Theivendran, Hettiarachchy and Johnson (2006) inhibited *L. monocytogenes* by ~2.5 log CFU/mL in culture media with 1% green tea extract. Inhibitory effect of green tea extract against bacterial pathogen is individually dependent on the phenolic compounds in the extract. Listerial growth increase was higher for Gel/RS+GTE at the end of the storage. Presence of the soluble rice starch dissolved in the aqueous culture could have been provide a source of nutrition for the culture and affected the growth in a positive way compared to Gel and Gel/CW. It is known that

starch is one of the ingredients used in selective *Listeria* growth medium in 1 g/L concentration (product information sheet).

Table 4.5. Antimicrobial activity of the gels against *Listeria innocua* during 15 days of storage at 4 °C.

	Log cfu/mL			
Gel Type	**Day 0**	**Day 7**	**Day 11**	**Day 15**
Control	2.69 ± 0.08^{a}	4.63 ± 0.15^{a}	6.57 ± 0.05^{a}	7.88 ± 0.04^{a}
Gel	2.69 ± 0.11^{a}	3.62 ± 0.04^{c}	4.31 ± 0.05^{c}	5.1 ± 0.03^{c}
Gel+LYS	2.76 ± 0.05^{a}	1.63 ± 0.15^{f}	$<1^{*}$	<1
Gel+GTE	2.75 ± 0.09^{a}	3.44 ± 0.03^{c}	4.00 ± 0.16^{d}	4.29 ± 0.11^{d}
Gel+LYS +GTE	2.56 ± 0.05^{a}	2.29 ± 0.14^{e}	1.72 ± 0.12^{fg}	<1
Gel/RS	2.76 ± 0.04^{a}	4.28 ± 0.03^{b}	5.35 ± 0.02^{b}	6.88 ± 0.02^{b}
Gel/RS+LYS	2.77 ± 0.11^{a}	1.39 ± 0.06^{f}	<1	<1
Gel/RS+GTE	2.72 ± 0.11^{a}	3.49 ± 0.06^{c}	4.39 ± 0.03^{c}	5.32 ± 0.03^{c}
Gel/RS+LYS +GTE	2.83 ± 0.05^{a}	1.53 ± 0.25^{f}	<1	<1
Gel/CW	2.65 ± 0.07^{a}	3.28 ± 0.09^{c}	4.01 ± 0.05^{d}	4.52 ± 0.10^{d}
Gel/CW+LYS	2.74 ± 0.07^{a}	2.66 ± 0.40^{d}	1.89 ± 0.11^{e}	1.15 ± 0.08^{e}
Gel/CW+GTE	2.60 ± 0.20^{a}	2.87 ± 0.10^{d}	3.02 ± 0.02^{f}	4.25 ± 0.03^{d}
Gel/CW+LYS+GTE	2.72 ± 0.08^{a}	2.07 ± 0.12^{e}	1.58 ± 0.11^{g}	<1

$^{a-g}$ Values within each storage time followed by the same letter are not significantly different ($P > 0.05$)

* <1 indicates that the number in the samples were below the detection limit.

The gels incorporated by LYS and LYS+GTE showed inhibitory effect against *L. innocua*. At the end of day 15, the total inhibitions caused by Gel+LYS, Gel/RS+LYS and Gel/CW+LYS were 1.76, 1.77, and 1.6 log CFU/mL, respectively. The number reduced below the level of detection (< 1 log CFU/mL) by Gel+LYS and Gel/RS+LYS at day 11. The release profiles of the gels monitored for six days were similar to each other as described in Section 4.2.1, therefore the antimicrobial effects of released LYS from the gels were no significantly different than each other. When the release profiles were considered, the Gel/CW showed the highest released LYS activity in water, however the inhibitory effect of Gel/CW was slightly lower than Gel and Gel/RS. As seen in Figure 4.7, the released LYS activities reduced after day 5, and reached to a similar level for all the gels. In the microbiological analysis, microbial load was counted at 7th day after the first inoculation, where the difference between released LYS activities would become insignificant, so all the gels showed similar antimicrobial activity in the aqueous growth medium.

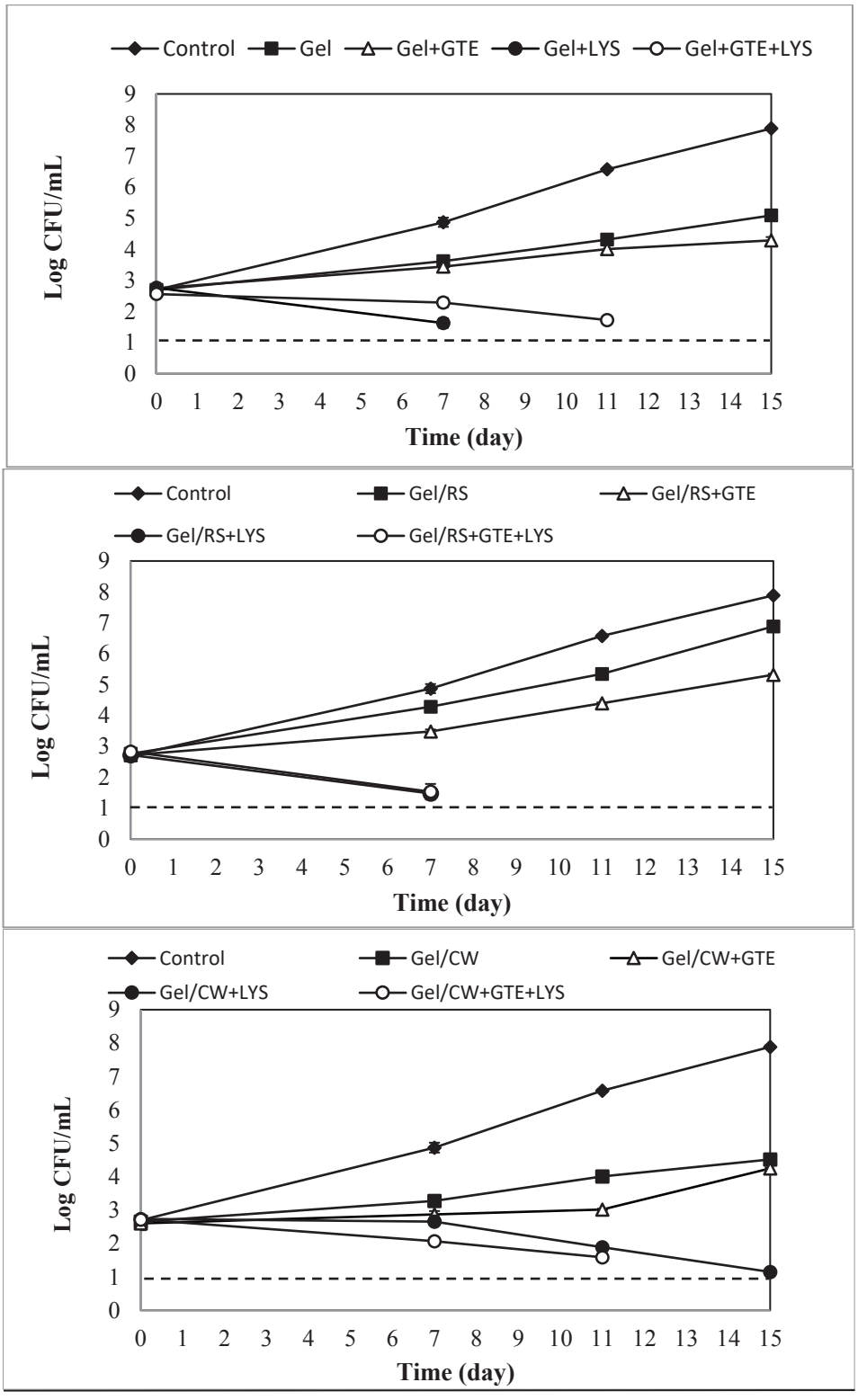

Figure 4.11. Antimicrobial activity Gelatin, Gelatin/RS and Gelatin/CW gels containing 1% LYS and 1% GTE against *L.innocua* for 15 days of storage at 4 °C (----: limit of detection).

Addition of GTE together with LYS did not cause any difference in antimicrobial activity for Gel/RS. Gel/CW incorporated with GTE+LYS showed high inhibitory effect, and at the end of the storage the reduction in the *L.innocua* counts were 1.72 log CFU/mL. GTE slightly lowered the antimicrobial activity of Gel+LYS+GTE, and the number of bacteria dropped below the limit of detection at day 15, while this reduction occurred at day 11 for Gel+LYS. Even though the *Listeria* count may have lowered below the limit of detection earlier than 15 days in the presence of Gel+LYS+GTE, the exact day of this reduction was unknown since there was no sampling for enumeration between 11^{th} and 15^{th} days. While GTE has reduced the LYS release from the gels, in the antimicrobial tests, presence of GTE did not lower the inhibitory effect of LYS dramatically. In the evaluation of the antimicrobial potential of the gels, not only the released LYS activity but also the suppressive effect of gelatin and GTE played a role.

It was reported that LYS showed bacteriostatic effect against *Listeria monocytogenes* for 2-3 weeks when inoculated in fresh pork sausage however did not inhibited significant number of cells. In camembert cheese, LYS alone showed inhibitory effect for around 35 days of storage. (Hughey et al. 1989). Depending on the food type and spoilage level, when applied alone LYS may show bacteriostatic or inhibitory effect against *Listeria* species. To understand this behavior the gels incorporated with LYS were applied to inoculated food samples and the results were given in Section 4.3.2.

For listeria-risk animal derived foods, when 15 days of cold storage time were considered, the most effective gels against *Listeria innocua* were Gel/RS+LYS+GTE Gel/RS+LYS and Gel+LYS gels. These gels were followed by Gel/CW+LYS+GTE and Gel+LYS+GTE gels. Since the addition of GTE is intended to prevent oxidative deterioration and for delivery of phenolic compounds to improve human health, Gel/RS with LYS and GTE is a good candidate to possess a high potential of antimicrobial activity against *L.monocytogenes.* As a result, addition of 1% LYS together with 1% GTE into gelatin gel and gelatin based mixed gels with rice starch or candelilla wax showed significant antilisterial activity against *L. innocua* for 15 days storage at 4 °C.

4.3.2. Antimicrobial Activity of the Gels Based on their Water Absorption Capacity

Gel/SPI did not release any significant LYS activity in the aqueous media, due to the charge-charge interactions. However, this gel showed remarkably high water-

absorption capacity, which could be effective in preventing microbial growth resulting from the accumulated exuded juice inside the package of meat, poultry and seafood products. Hence, Gel/SPI gel could be applied on food systems as edible antimicrobial absorbent tray pads.

Antimicrobial activity of Gel/SPI were tested against *L. innocua* inoculated on the gel materials. The initial number of inoculated microorganisms on the gels and the number after 7 days of storage at 4 °C were presented in Table 4.6. The initial *L. innocua* number was at the same level for all the gels and initial number was around 4.1-4.4 log cfu/g. It was clearly understood that LYS entrapped within the gel network via electrostatic attractions could show antimicrobial effect against *L. innocua*. The number of *L. innocua* reduced after 7 days in all the samples. However, this reduction was more significant for Gel/SPI+LYS, and 1.15 log cfu/g reduction in the initial number was obtained. The reduction caused by Gel+LYS was almost half of the inhibition caused by Gel/SPI+LYS (0.62 log cfu/g), and lower than the activity of Gel/SPI (0.77 log cfu/g reduction).

Table 4.6. Antimicrobial activity of Gel and Gel/SPI incorporated with LYS against *Listeria innocua* inoculated on the gels stored at 4 °C.

Gel Type	Bacterial Count (Log cfu/g)		Total inhibition (Log cfu/g)
	Day 0	Day 7	
Gel	$4.23 \pm 0.02^{a,A}$	$3.71 \pm 0.08^{a,B}$	-0.52
Gel+LYS	$4.07 \pm 0.10^{a,A}$	$3.45 \pm 0.12^{b,B}$	-0.62
Gel/SPI	$4.44 \pm 0.04^{a,A}$	$3.67 \pm 0.13^{a,B}$	-0.77
Gel/SPI+LYS	$4.27 \pm 0.11^{a,A}$	$3.12 \pm 0.22^{c,B}$	-1.15

[a-c] Values by the same letter within each storage day are not significantly different ($P > 0.05$)
A-B Values by the same letter within each treatment are not significantly different ($P > 0.05$)

When Gel+LYS and Gel/SPI+LYS was compared, Gel/SPI+LYS showed two folds higher activity than Gel+LYS. Since, the absorption capacity of Gel/SPI was higher than Gel, higher volume of inoculum must be absorbed by Gel/SPI and therefore higher inhibition in the *L. innocua* number was obtained. Gels without LYS showed significant antimicrobial activity, too. In the literature, the studies on biodegradable absorbent pads developed for food packaging are lacking. Natrajan and Sheldon (2000) tested a commercial absorbent tray pad treated with nisin, EDTA, citric acid and Tween 20, and tested the pad against *Salmonella* Typhimurium and mesophilic bacteria on commercial chicken stored at 4 °C. At the end of 168 h storage, 6.3 and 3.1 log CFU/g

reductions were obtained for mesophilic bacteria and *Salmonella* Typhimurium, respectively. Similarly, Oral et al. (2009) sprayed cellulose based commercial absorbent pad with oregano essential oil and tested it on chicken drumsticks stored at 4 °C. They observed the antimicrobial pads suppressed the total viable count growth. However, in both studies commercial pads coated were used after treating with the antimicrobial agents. Cellulose-silver nanoparticles hybrid absorbent pad was developed and tested on fresh-cut melons, and yeast growth was suppressed remarkably (Fernández, Picouet and Lloret 2010).

4.4. Bioactive Properties of the Gels

4.4.1 Antioxidant Activity of the Gels

Antioxidant capacity of GTE released from the gels were determined using TEAC and ORAC methods and the results were given in Table 4.7 as "μmol Trolox per gram of gel". TEAC method, also known as ABTS radical cation decolorization assay, is a method to screen antioxidant activity of both lipophilic and hydrophilic antioxidants. The radical cation $ABTS^{\bullet+}$ is formed in a stable form prior to the reaction and reduced to ABTS in the presence of antioxidants. The percentage inhibition of $ABTS^{\bullet+}$ is determined spectrophotometrically from the degree of decolorization at 734 nm wavelength. The antioxidant activity is calculated relative to the reactivity of Trolox as a standard. ORAC method is based on hydrogen atom transfer, wherein a competitive reaction between antioxidant and fluorescein for a peroxy radical (generated by AAPH) takes place. By electron transfer from antioxidant to peroxy radical, the decay in fluorescence caused by radicals is eliminated. There are various studies comparing electron transfer based methods (TEAC) and hydrogen atom transfer based methods (ORAC) in determination of antioxidant capacity of the foods (Roy et al. 2010; Zulueta et al. 2009).

According to TEAC method, Gel+GTE+LYS, Gel/CW+GTE and Gel/SPI+GTE released the highest antioxidant activity (10065-10412 μmol Trolox/g of gel). The gels without GTE did not show any significant antioxidant activity. Gel+GTE+LYS showed higher antioxidant activity than Gel+GTE, however presence of LYS did not show the same effect in Gel/CW+GTE and Gel/SPI+GTE. The lowest antioxidant activities were measured for Gel/RS+GTE and Gel/RS+GTE+LYS. It is known that there is a strong relationship between antioxidant capacity and polyphenol concentration of green tea.

Considering the released phenolic compound profiles of the gels, the antioxidant activities measured by TEAC method did not gave parallel results with the phenolic release results. During total phenolic release in water, Gel+GTE+LYS and Gel/SPI+GTE released the lowest amount while they released highest antioxidant activities. Similarly, Gel/RS+GTE released the highest total phenolic content in water, but the antioxidant activity released from Gel/RS+GTE was the lowest according to TEAC method. The difference between the released total phenolic contents and the released antioxidant activities could be resulting from the differences in the released phenolic compound profiles. The highest release of total phenolics may not give the highest antioxidant activity in vitro. Antioxidant activities of different catechins and phenolic acids may be different (Roy et al., 2010; Prior and Cao 1999; Henning et al. 2003). According to Roy et al. (2010), antioxidant capacity of tea catechins measured by ORAC method showed great variety (43823-6987 μmol TE/g). Antioxidant capacity of the catechins from the highest to lowest followed the order; EC>ECG>EGC>EGCG>Gallic acid. In the same study 2mg/mL green tea extract solutions gave antioxidant capacity between 4513-8077 μmol TE/g.

In this study, according to ORAC method, control gels gave relatively high antioxidant activity than that in TEAC method. These results were different than TEAC method, since the mechanism of actions of two methods are different. It seemed like ORAC method was less sensitive to determine the differences between the released antioxidant activities from the gels. Except Gel/RS+GTE+LYS, all the gels containing GTE released similar antioxidant activity. Control gels showed almost the half of the antioxidant activity that was shown by GTE containing gels. Only Gel/SPI showed as high activity as the gels with GTE. This could be resulting from the antioxidant activity shown by dissolved SPI. However, Gel/SPI gels with GTE did not show significantly higher activity that could be resulted from the additive effect introduced by SPI. It is known that green tea catechins create reversible bonds with proteins (Ozdal, Capanoglu and Altay 2013; Wu and Bird 2010). It was shown that the interactions of phenolic compounds with proteins decrease the total antioxidant activity measured by different methods and total phenolic content (Ozdal, Capanoglu and Altay 2013).

Table 4.7. Antioxidant capacity and iron chelating capacity of GTE released from the gels

Gel Type	ORAC (µmol Trolox/g of gel)	ABTS (µmol Trolox/g of gel)	Iron Chelating Capacity (µmol EDTA/g gel)
Gel	4414 ± 418^{c}	311± 21^{e}	4.02 ±0.2^{d}
Gel+GTE	7483 ± 413^{a}	8300 ± 1359^{bc}	15.16 ± 1.6^{b}
Gel+GTE+LYS	8139 ± 377 ^{a}	10065 ± 449^{a}	-*
Gel/RS	4914 ± 740 ^{bc}	239 ± 16^{e}	3.16 ± 0.5^{d}
Gel/RS+GTE	7465 ± 248 ^{a}	7307 ± 418^{d}	16.15 ± 2.2^{b}
Gel/RS+GTE+LYS	6840 ± 947 ^{ab}	7624 ± 439^{cd}	-*
Gel/CW	4480± 386 ^{bc}	298 ± 16^{e}	3.28 ± 0.1^{d}
Gel/CW+GTE	7863 ± 458 ^{a}	10286 ± 735^{a}	8.32 ± 0.3^{c}
Gel/CW+GTE+LYS	8034 ± 282 ^{a}	8361 ± 225^{b}	-*
Gel/SPI	8743 ± 576^{a}	889 ± 50^{e}	34.66 ± 0.3^{a}
Gel/SPI+GTE	7471 ± 248^{a}	10419 ± 320^{a}	35.39 ±0.02^{a}
Gel/SPI+GTE+LYS	8230 ± 153^{a}	7833 ± 135^{bcd}	35.25 ± 0.2^{a}

$^{a-e}$ Values within each storage time followed by the same letter are not significantly different ($P > 0.05$)

* Measurements were not possible due to the turbidity resulted from the released lysozyme

Iron chelating capacity (ICC) of GTE released from the gels was given in Table 4.7 as µmol EDTA per g of gel. ICC of gels contain both GTE and LYS could not be measured properly due to the turbidity introduced by LYS in the reaction mixture. The ICC was similar for Gel+GTE and Gel/RS+GTE gels, measured as 15.2 and 16.2 µmol EDTA/g gel, respectively. Although the released total phenolic content and the antioxidant activity were high from Gel/CW, its ICC was the lowest. The highest ICC values were measured for Gel/SPI gels. Since no significant LYS was released from Gel/SPI, no interference was observed for ICC of Gel/SPI+GTE+LYS. The ICC of all the Gel/SPI gels were similar and seemed like soluble SPI mainly determined the results. The iron chelating capacity of polyphenols is dependent on the presence of o-dihydroxy polyphenols such as polyphenols having catechol and galloyl groups. The incorporated GTE contains various flavonoids and phenolic acids that are not specifically known. Although the levels of released total phenolic content of the gels were similar, the exact content of the released GTE solution in unknown and can be different for each gel depending on the interactions with the gel material. This variation can be responsible for the variable ICC of GTE released from the gels. Metal chelation mechanism was considered as minor mechanism and its contribution to the free radical scavenging activity is not fully specified (Sugihara et al. 2001).

4.4.2 Antidiabetic and Antihypertensive Properties of the Gels

Antidiabetic activities of the gels were tested by measuring their inhibitory effects against human salivary α-amylase (HSA) and α-glucosidase (AGH) enzyme solutions. According to the results given in Table 4.8, the highest inhibitory activities against HSA were obtained by Gel/RS+GTE+LYS (49.75 μmol acarbose/g gel). Other gels released similar activity against HSA changing between 37.6 and 44.3 μmol acarbose/g gel. Gel/SPI showed significant inhibitory activity as the gels with GTE did, resulting from the soluble SPI. The plant-sourced proteins were investigated before for their digestive enzyme inhibitory effects and antidiabetic activities (Kwon et al. 2010: Kobrehel, Yee and Buchanan 1991; Garcia-Olmedo et al. 1987). The antidiabetic effect of soy protein and genistein, an isoflavone, was shown in diabetic rats by different studies (Lee 2006; Mezei et al. 2003, Ademiluyi and Oboh 2013).

Table 4.8. Antidiabetic and antihypertensive activity of GTE released from the gels

Gel Type	Antidiabetic Activity (μmol acarbose/g gel)		Antihypertensive Activity (μmol captopril/g gel)
	Against HSA	Against AGH	
Gel	22.75 ± 3.5^{cd}	-	50.68 ± 23.4^{b}
Gel+GTE	43.89 ± 2.8^{ab}	79.85±7.2^{ab}	141.02 ± 6.23^{a}
Gel+GTE+LYS	40.37 ± 3.7^{ab}	34.27±2.8^{cd}	112.38 ± 29.6^{ab}
Gel/RS	9.46± 2.8^{d}	-	94.44 ± 13.4^{ab}
Gel/RS+GTE	35.37 ± 0.5^{abc}	13.97±4.5^{d}	106.24 ± 18.4^{ab}
Gel/RS+GTE+LYS	49.75 ± 2.1^{a}	40.99±0.3^{cd}	151.10 ± 10.01^{a}
Gel/CW	16.49 ± 9.9^{d}	-	92.89 ± 3.4^{ab}
Gel/CW+GTE	33.70 ± 7.8^{bc}	92.2±11.2^{a}	136.62 ± 15.6^{a}
Gel/CW+GTE+LYS	37.61 ± 0.4^{ab}	100.09±13.2^{a}	165.3 ± 4.7^{a}
Gel/SPI	38.38 ± 1.9^{ab}	-	126.83 ± 40.7^{ab}
Gel/SPI+GTE	40.98 ± 3.9^{ab}	54.86±16.4^{bc}	136.05 ± 1.63^{a}
Gel/SPI+GTE+LYS	44.30 ± 6.8^{ab}	81.20±18.4^{ab}	103.77 ± 8.15^{ab}

$^{a-d}$ Values within each storage time followed by the same letter are not significantly different (P > 0.05)

Antidiabetic activity of the gels against AGH were given in Table 4.8 as "μmol acarbose/g gel" representing the acarbose equivalent of GTE released from 1 g of gel. Highest activity released from Gel/CW+GTE and Gel/CW+GTE+LYS. When inhibition was expressed as μmol acarbose/g gel, the inhibitory effects of GTE released from the gels seemed different from each other. Based on their percentage inhibition, 60-77% inhibition range by the gels was obtained. The inhibitory effects of phenolics released

from different gels against alpha-amylase enzyme were not parallel to inhibitory effects against alpha-glucosidase enzyme. Against alpha-amylase, Gel/RS+GTE showed the highest activity, while against alpha-glucosidase it showed the lowest activity. It can be suggested that the released phenolic acid and catechin content and composition may varied from each gel and showed different bioactive properties.

Antihypertensive activity measurement is a method based on the inhibition of angiotensine converting enzyme (ACE) of GTE released from the gels. In Table 4.8, it was shown that the highest ACE inhibitory effects were shown by Gel+GTE, Gel/RS+GTE+LYS, Gel/CW+GTE, Gel/CW+GTE+LYS and Gel/SPI+GTE. LYS incorporation together with GTE did not affect antihypertensive activity of Gel/RS and Gel/CW, however reduced the activities of Gel and Gel/SPI. Control gels showed lower but significant antihypertensive activity. Dissolved small peptides during release in water might have had ACE inhibitory effect. ACE inhibitory effect was generally screened for bioactive peptides (Shalaby et al., 2006; Vermeirssen, Camp and Verstraete 2002). Gelatin peptides, especially when hydrolyzed are known for good antihypertensive activity (Kim et al. 2001; Aleman et al. 2011).

These results indicated the potential antidiabetic and antihypertensive activity of green tea extract which could promote human health. In vitro bioactive effects of GTE were proved by other studies (Persson et al. 2006, Actis-Goretta, Ottaviani and Fraga 2006, Matsui et al. 2007). GTE, incorporated into the gels, showed potential health supporting biofunctional properties after release from the gels.

4.4.3. Cytotoxicity of the Gels against Caco-2 Cells

The release media taken at the end of the release tests of the gels were tested against Caco-2 human colon carcinoma cell line. The cytotoxic effect of GTE released from the gels were compared to the cytotoxic effect of GTE solution. Firstly, the toxicity of GTE against Caco-2 cells were tested by treating the cell culture grown for 48 h by GTE at increasing concentrations. The number of the cells counted using hemocytometer under microscope after 48 h treatment are given in Figure 4.12. The photographs of the Caco-2 cells at the end of treatment are presented in Figure 4.13. Concentrations lower than 20 µg GAE/mL given to the cells did not cause any inhibition, but also triggered the growth. Low levels of phenolics may cause promoted growth since the antioxidant

activity can reduce the stress factors without damaging the cells. This may have resulted as higher cell numbers compared to control group. Human prostatic adenocarcinoma cells (LNCaP) treated with increasing levels of tea catechins for 6 days showed better cell growth after treated by low levels of EC and EGC (1-25 µM) (Paschka, Butler and Young 1998).

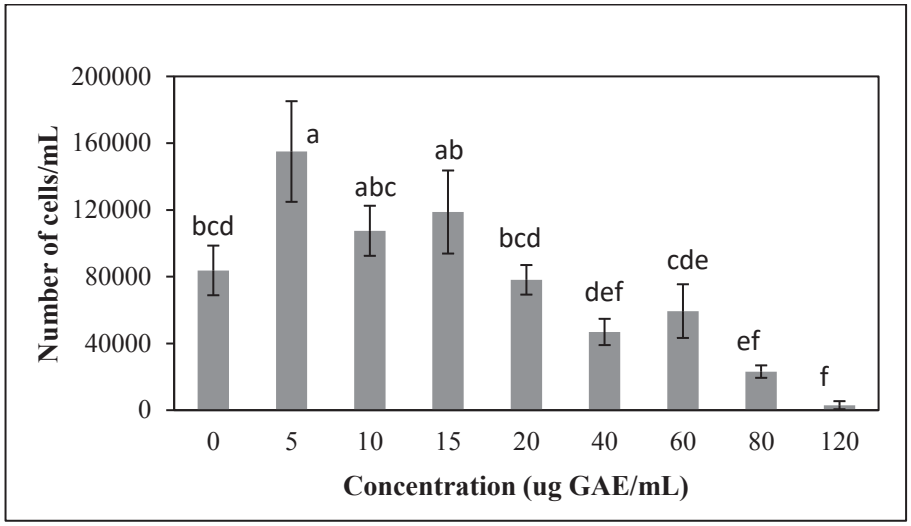

Figure 4.12. Number of Caco-2 cells after 48h treatment of GTE solution (15 mg/mL) at increasing concentrations (µg GAE/mL).

Similarly, EGCG increased proliferation at lower concentrations but showed inhibition in proliferation at higher concentrations (>80µM) on pancreatic and bone cancer cells (Lai et al. 2017). The GTE concentrations above 40 µg GAE/mL in the cell growth media caused gradual reduction in the Caco-2 cell number, and almost a total inhibition was obtained at 120 µg GAE/mL. Based on the concentrations that caused inhibition in the Caco-2 cell number, IC_{50} value of the GTE used in the gel production was calculated as 62.85 µg GAE/mL, according to the cell counting method (Appendix B).

The cytotoxicity of pure GTE solution was compared to the GTE released from the gels. The released GTE from the gels was treated to the cells after lyophilization of the release media. 15 mg/mL solutions were prepared from GTE powder and from the lyophilizate of the release media of the gels. From these solutions, same volumes were taken and completed to 500 µL with cell growth medium. Each well of Caco-2 cell was treated with 0.2 mL of the final solution. The phenolic content of 15 mg/mL of GTE, and release media of Gel+GTE, Gel/RS+GTE and Gel/CW+GTE were measured as 2907±43, 1099±9, 1134±15, and 1099±11 µg GAE/mL, respectively. Therefore, even the given

volumes of the released media and GTE solution were the same, each media contained different total phenolic content. The phenolic content of 1 ml of GTE solution (prepared from GTE powder) was around 2.6 folds higher than that in 1 mL of released media solutions of the gels (prepared from lyophilized media content).

Viability of the Caco-2 cells were measured spectrophotometrically using CCK-8 kit that gives yellow color depending on the dehydrogenase activity of the cells. The effect of GTE and the GTE released from the gels against Caco-2 cell growth are presented in Figure 4.14. The absorbance of control group was taken as "100% viability", and the reduction or increase in the viability were calculated according to the control group. As seen in Figure 4.14, pure GTE solution caused the highest inhibition in the viability of Caco-2 cells due to its higher phenolic content. When the cells were treated with 123.6 µg/0.2 mL (contains ~120 µg GAE/mL) and 247.2 µg/0.2 mL (contains ~240 µg GAE/mL) caused 29.6% and 55.8% reduction in the cell viability, respectively. According to the cell counting method using hemocytometer under microscope (Figure 4.12), 120 µg GAE/mL caused almost a total inhibition, however in the cell counting kit (CCK-8) method, same concentration gave less than 50% inhibition in the viability. In the counting method using microscope, with trypan blue dying, the cells were detached by trypsin after removal of the media by suction. During suction using a vacuum pump, the inhibited cells by GTE after 48h treatment could be already detached and be removed from the wells. The remaining cells, that were assumed to be viable, were detached by trypsin. After homogenizing the viable cell suspension, randomly taken droplets were evaluated by hemocytometer counting method. This method may allow to overestimate the inhibitory effect of GTE depending on the suction, trypsin detachment and randomly sampling practices. However, CCK-8 method is based on the dehydrogenase activity of the cells. It utilizes water soluble tetrazolium salt found in the kit and produces formazan dye, giving peak at 450 nm. In this method, Caco-2 cells were not detached from the wells by trypsin. The cells that could be in the different phases of their life cycle could contribute to the absorbance read spectrophotometrically, and the effect of the detachment of the cells from the wells had less role in the viability measurements by CCK-8 than that in hemocytometer counting method.

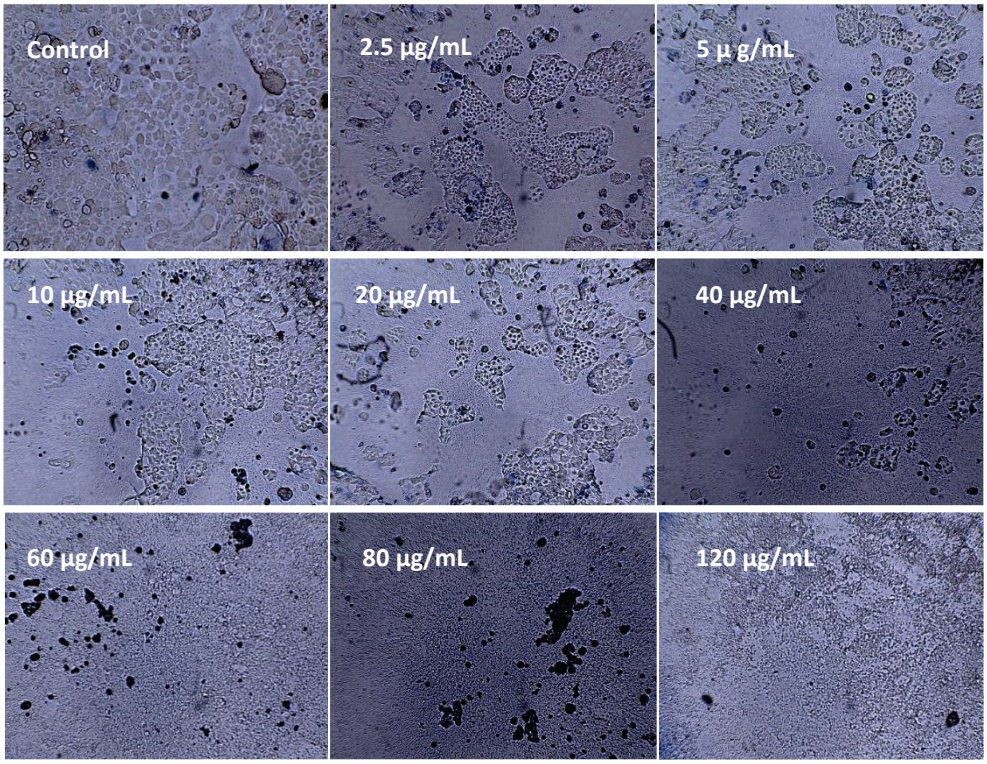

Figure 4.13. The photographs of Caco-2 cells dyed with trypan blue, treated by GTE solution at different concentrations for 48 h.

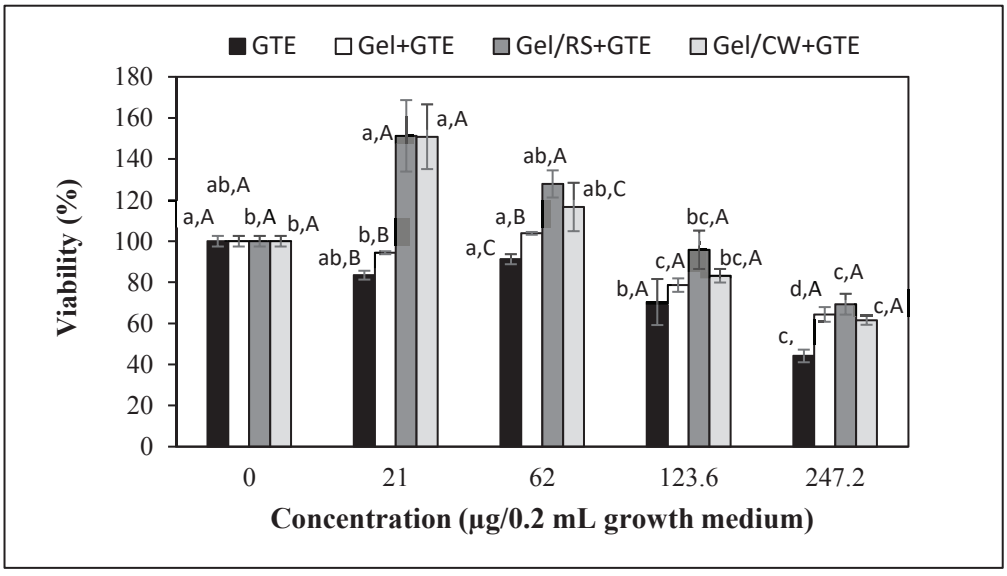

Figure 4.14. Viability of Caco-2 cells after 48 h treatment of GTE solution (15 mg/mL) and released GTE from Gel+GTE, Gel/RS+GTE and Gel/CW+GTE (a-c: Values within each treatment followed by the same letter are not significantly different at $P > 0.05$, A-C: Values within each volume followed by the same letter are not significantly different at $P > 0.05$)

21 and 62 µg/0.2 mL of release medium of Gel+GTE caused no difference compared to the control group. As the given concentration has increased to 123.6 and 247.2 µg/0.2 mL, 31.4 and 35.6% reductions in the viability were observed. Release media of Gel/RS+GTE and Gel/CW+GTE gels when given at 21 and 62 µg, a significant increase (16-51%) in the cell viability was observed compared to control group. This result could be resulted from the antioxidant activity of GTE at the low levels that could reduce the cell stress and promoted the growth of Caco-2 cells. Release media of Gel/RS+GTE and Gel/CW+GTE treated at 123.6 µg caused 4.2% and 16.8% inhibition, respectively. When the treatment concentration was increased to 247.2 µg, 30.7 and 38.5% inhibition was obtained by Gel/RS+GTE and Gel/CW+GTE, respectively. When the highest concentration treated to the cells was considered, Gel+GTE, Gel/RS+GTE, and Gel/CW+GTE showed no difference in their inhibitory effect. Pure GTE at the highest level showed 1.5-1.8 folds higher toxicity against Caco-2 cells. However, the total phenolic content of 15 mg/mL solutions of GTE was 2.6 folds higher than the solutions of lyophilized release media of the gels at the same concentration. It can be said that; the phenolic content and cell cytotoxicity were not directly proportional.

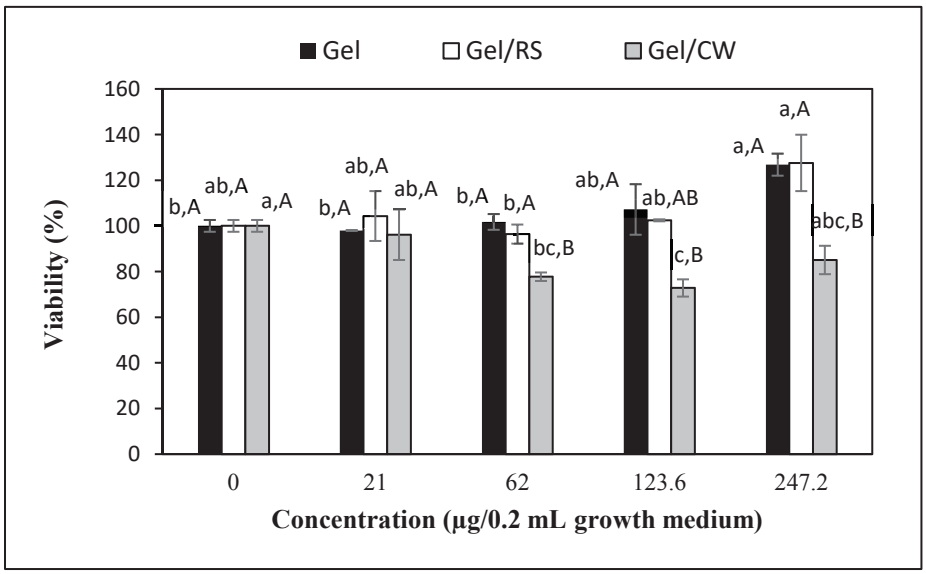

Figure 4.15. Viability of Caco-2 cells after 48 h treatment of control Gel, Gel/RS, and Gel/CW gels (a-c: Values within each treatment followed by the same letter are not significantly different at $P > 0.05$, A-C: Values within each volume followed by the same letter are not significantly different at $P > 0.05$)

To see the effect of the compounds coming from the gels into the release media, such as small peptides and soluble starch, the release media of control gels with no GTE

were also lyophilized and treated to the Caco-2 cells. As seen in Figure 4.15, lyophilizates of Gel and Gel/RS at all volumes did not cause any difference compared to control, except the highest concentration caused 26% increase in viability. However, lower viability values were measured (15% to 27% lower) for Gel/CW at the concentrations between 62-247.2 μg/0.2mL.

These results suggested that, the gelatin gel and the composite gels containing GTE had the potential to create a functional food product, when the foods would be coated by the developed gels. Increased phenolic content of the bioactive gel-coated foods that are poor in phenolics such as meat, poultry and sea food could have the potential to support phenolic intake by consumers. However, determination of the dose of the delivered amount and the effectivity of the phenolics after the digestion route require further studies.

4.5. Food Applications of the Antimicrobial, Antioxidant and Bioactive Gels

The developed gels were examined for their physical nature, release characteristics, antimicrobial effects, and bioactive properties. However, the tested activities, especially the release profiles of the active agents and their effect after being delivered into the real food system could be different than that of the in vitro assays. The tests performed in a real food system are generally defined as in situ tests. For a model food system, cold-smoked salmon (CSS) was selected. CSS is a ready-to-eat listeria-risky food, consumed without any treatment. It is generally stored at 4 °C and vacuum packed in retail market.

4.5.1. Lysozyme Release Profile of the Gels on Smoked Salmon

The LYS release profiles of the gels coated on smoked salmon slices were monitored to compare the release profile with the release in water. Release profile obtained from food applications can be different than in vitro release profiles since the food material is not subject to flow conditions. Released LYS activity on smoked salmon slices from the gels within 15 days of storage at 4 °C are presented in Figure 4.16. Measured enzyme activities are represented as Unit per gram of gel. As seen in Figure 4.16, release of LYS increased gradually until the end of storage. The smoked salmon samples were not stored any further since the indications of microbiological spoilage has

been observed after 15 days under the given storage conditions. At day 5, released activities from all the gels were similar to each other, and varied between 24900 and 33600 U/g gel. Between the day 5 and day 10, the highest increase in the LYS activity was measured for Gel, and this increase was constant until day 15. The activity increase for Gel/RS and Gel/CW was lower than Gel between days 5 and 10, but release from Gel/RS reached the same level as Gel at 15th day (54450-56447 U/g). At day 10, Gel released 1.3-1.4 folds higher activity than Gel/CW and Gel/RS. At the end of the storage, Gel/CW released 1.4 folds lower activity than Gel and Gel/RS. It can be said that in the first 5 days of storage, free LYS released independently form the structure and swelling rate of the gels. After that timepoint, it can be said that Gel showed a constant increase in released activity, while composite gels gave release profiles affected by incorporated RS and CW. Gel/RS showed more controlled release than Gel while reaching the same activity at the end of the storage. The reason of lower LYS release at 15th day from Gel/CW could be its limited swelling due to the higher hydrophobicity introduced by wax particles. This limitation was not observed in water where the gels were more available to fluid diffusion into the gel matrix. However, on food sample, diffusion occurs only through the contact area in a single direction. Therefore, wax particles could show more significant role in the swelling of the gel. Compared to the maximum released activities in aqueous media, Gel, Gel/RS and Gel/CW released 2.2, 2.5 and 3.8 folds lower LYS activities on smoked salmon, respectively. It is expected that the diffusion would be much slower in the food system compared to the water, since the mobility of LYS and swelling of the gels would be much limited. However, this characteristic can be advantageous since the lower diffusion rates for antimicrobial agents are desirable for a longer period of preservation on the food surface (Appendini and Hotchkiss 2002).

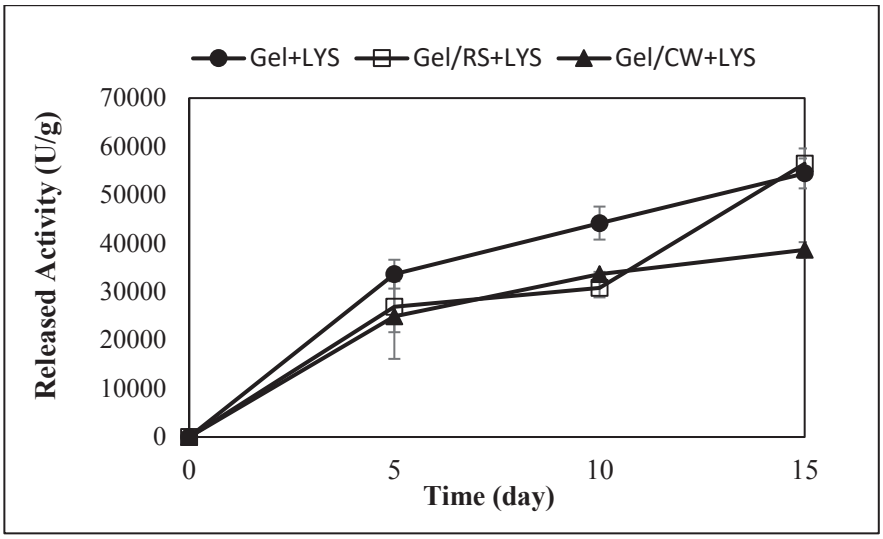

Figure 4.16. LYS release profile of Gel, Gel/RS and Gel/CW coated on both sides of smoked salmon slices

Table 4.9. Released LYS activities from the gels coated on smoked salmon stored at 4 °C for 15 days.

Gel Type	Released LYS Activity (U/g)		
	Day 5	Day 10	Day 15
Gel	$33637 \pm 2978^{a,C}$	$44186 \pm 3410^{a,B}$	$54450 \pm 3091^{a,A}$
Gel+GTE+LYS	$22091 \pm 2687^{bc,C}$	$36918 \pm 3544^{b,B}$	$43052 \pm 1915^{b,A}$
Gel/RS	$26913 \pm 5264^{ab,B}$	$30777 \pm 1940^{e,B}$	$56447 \pm 3157^{a,A}$
Gel/RS+GTE+LYS	$14777 \pm 1511^{c,C}$	$25582 \pm 752^{de,B}$	$42007 \pm 4310^{b,A}$
Gel/CW	$24900 \pm 8760^{b,A}$	$33652 \pm 4757^{bc,A}$	$38651 \pm 1597^{bc,A}$
Gel/CW+GTE+LYS	$23301 \pm 2409^{bc,B}$	$31489 \pm 1799^{cd,A}$	$35423 \pm 2287^{c,A}$

$^{a-e}$ Different letters within each storage time show significant differences at $p < 0.05$.
$^{A-C}$ Different letters within each treatment show significant differences at $p < 0.05$.

LYS release profiles of the gels incorporated with LYS and GTE together were shown in Figure 4.17. Similar to the profiles obtained for the gels containing only LYS, Gel and Gel/RS released the highest activities at 15th day, which were 1.2 folds higher than the maximum released activity from Gel/CW. At the 5th and 10th days Gel and Gel/CW released similar activities. Gel/RS showed more controlled release profile compared to Gel and Gel/CW. Release rate of Gel/CW reduced gradually throughout the storage period. Similar to the release profiles for gels with LYS and GTE obtained in aqueous media, incorporation of GTE resulted as relatively lower LYS release. The reason of this reduction could be the interaction of GTE with gelatin and LYS, as described in Section 4.2.1.

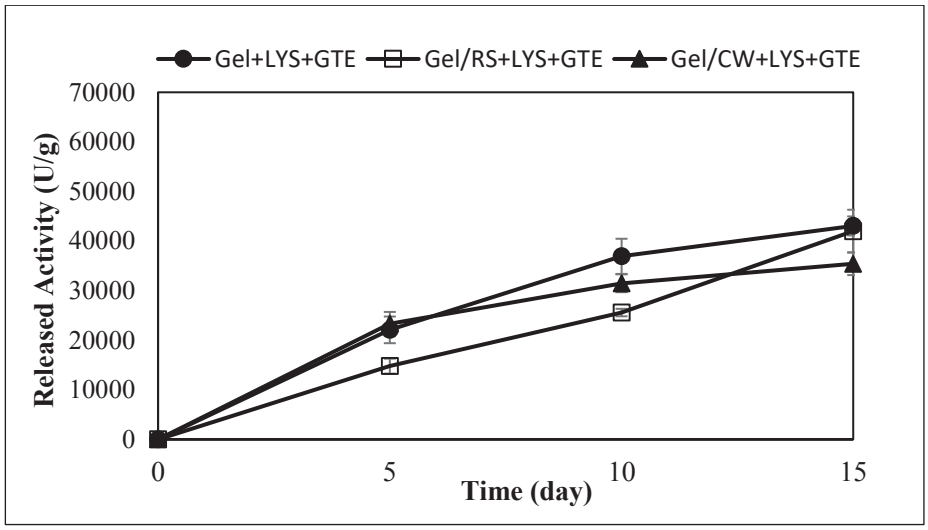

Figure 4.17. LYS release profile of Gel, Gel/RS and Gel/CW with GTE (1%) coated on both sides of smoked salmon slices

These results indicated that the gels would show a significant antimicrobial activity when coated on smoked salmon slices. Since the release rate of LYS from the gels would vary significantly from liquid media to salmon surface, the antimicrobial effect of the gels would be different in in-vitro and in-situ antimicrobial tests. The results obtained from antimicrobial challenge tests are given in Section 5.3.

4.5.2. Total Phenolic Release Profile of the Gels on Smoked Salmon

The released amount of GTE from the gels coated on smoked salmon were determined and are presented in Figure 4.18 and Fig 4.19. The release of GTE followed an increasing pattern until the end of the storage for all gels. Further measurement was not performed since it was not considered as suitable to store smoked salmon slices due to the spoilage indications appearing in the storage time longer than 15 days at 4 °C, under laboratory storage conditions. At the end of 15th day, the highest phenolic release was obtained from Gel/CW+GTE (0.786 mg GAE/g gel) and the lowest from Gel/RS+GTE (0.453 mg GAE/g gel). Maximum released levels were between 0.45 and 0.78 mg GAE/g gel, and these levels were lower than the released total phenolic amount from the gels in water. However, due to the limited diffusion on smoked salmon, lower released amounts were expected. The different gel compositions played more significant role in the release of phenolics from the gels when placed on smoked salmon slices, compared to their release in the water. In aqueous media, the maximum released phenolic content from the gels were close to each other (0.96-1.07 mg GAE/g gel). In water, Gel/RS released the

highest phenolics, however on smoked salmon, Gel/RS gels released the lowest amounts. This could be related to the limited swelling of starch granules on food samples, and resulted as lower diffusion of phenolics than that in liquid media. However, Gel/CW gels were not affected by the swelling mechanism, and the modified gel structure by wax particles played the main role in the diffusion of phenolics onto smoked salmon. On the food system the affinity between Gel/CW due to the increased hydrophobicity introduced by the wax content and lipid-rich smoked salmon could be higher.

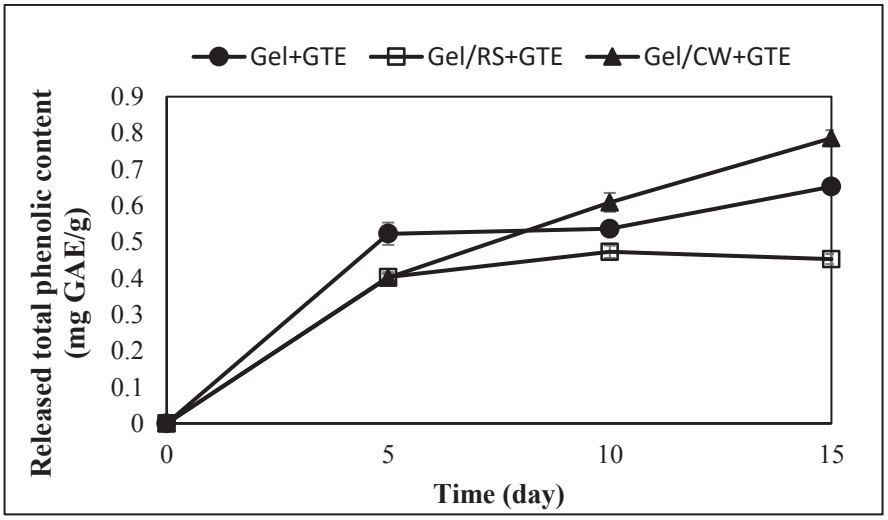

Figure 4.18. Released total phenolic content from Gel, Gel/RS and Gel/CW with GTE (1%) coated on smoked salmon slices at 4 °C.

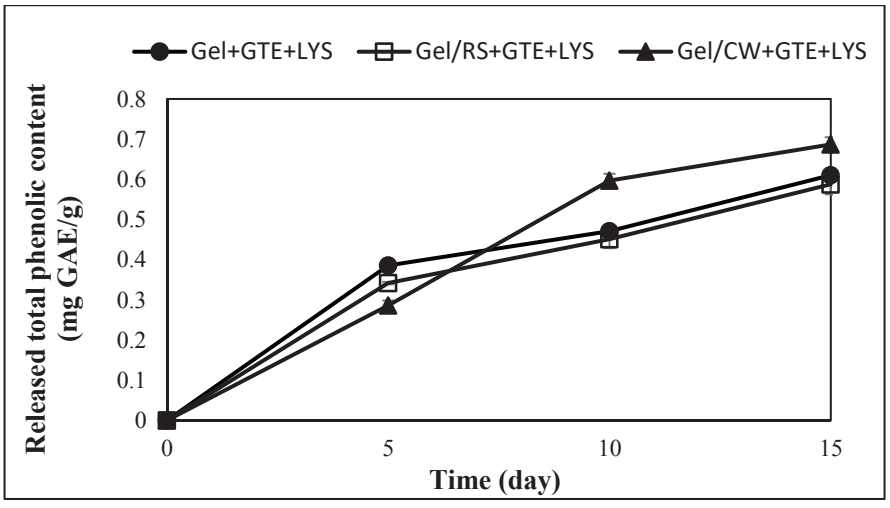

Figure 4.19. Released total phenolic content from Gel, Gel/RS and Gel/CW with GTE (1%) and LYS (1%) coated on smoked salmon slices at 4 °C.

The release of GTE onto food samples could be beneficial to control lipid oxidation in oxygen-sensitive foods such as meat and meat products. Hong, Lim and Song (2009) showed that the degree lipid oxidation of pork loin coated with *Gelidium corneum*-gelatin film containing green tea extract could be controlled during 10 days of storage. According to Kang et al., (2007) green tea powder added (0.5%) pectin films coated on irradiated pork patties reduced lipid oxidation both in aerobic and vacuum conditions. Similarly, pork sausages coated with chitosan films containing green tea extract (20% of film forming solution) reduced the lipid oxidation compared to uncoated sausages stored at 4 °C for 20 days. It can be said that the GTE incorporated in the gels could have a protective effect against oxidation in oxygen sensitive foods. In this study, the developed gels were applied on smoked salmon slices stored at 4 °C. Smoked salmon was not sensitive to oxidation as uncured and unsmoked fish does. Therefore, oxidation preventive effect of the gels was not determined since the determination of degree of lipid oxidation by TBARS method gave no significant result within 15 days of storage at 4 °C.

4.5.3. Antimicrobial Activity of the Gels on Smoked Salmon

To perform an effective antimicrobial activity on a real food system by the developed gels is challenging but crucial. The indigenous flora and the food substances may affect the antimicrobial activity of LYS and the growth of inoculated target microorganism. Therefore, the results may not be the same as in the antimicrobial tests performed in an inoculated growth medium. For the food challenge test, cold-smoked salmon was selected as a model food. Cold-smoked salmon is a listeria-risky food since the elimination of *Listeria monocytogenes* from fish processing plant is very challenging (Peiris et al., 2009; Dass, Cummins and Abu-Ghannam 2011). Hence, as a ready-to-eat minimally processed food, cold-smoked salmon is considered as highly susceptible to *L. monocytogenes* contamination during processing steps such as skin removal, slicing and packaging. Especially in the case of the tools and knives used for skin removal and fillet slicing are contaminated, the bacteria would be cross-contaminated on the surfaces of thin cold-smoked salmon slices before vacuum packaging step (Uyttendaele et al. 2004). *L. monocytogenes* can survive at low temperatures (0-0.3 °C), and various atmosphere conditions, salt concentrations (4-6%), and pH values (4.1-7.0) (Cole, Jones and Holyoak 1990; Barakat and Harris 1999).

To see the antimicrobial effect of the developed gels on cold-smoked salmon, the gels containing LYS and LYS+GTE were tested on *L. innocua* inoculated cold-smoked salmon stored at 4 °C. Enumeration of the bacteria was performed at the 0^{th}, 5^{th} and 10^{th} days of cold storage by plate counting method. The initial inoculated listerial numbers were the same in all groups. Gels were coated on both sides of the samples after inoculation, and then sampling was performed immediately, so that there may not have been sufficient time for LYS to diffuse onto salmon surfaces to cause any inhibition. No listerial cells were detected in uncoated uninoculated samples. Since GTE showed no inhibitory effect against L. *innocua* in the tests performed in growth media, the gels with only GTE were not tested in the food challenge test. Antimicrobial effect of the gels incorporating only LYS were shown in Table 4.10 and Figure 4.20.

Table 4.10. Antimicrobial activity of the gels incorporated with LYS against *Listeria innocua* inoculated on smoked salmon for 15 days of storage at 4 °C

	Log cfu/g			
Gel Type	**Day 0**	**Day 5**	**Day 10**	**Day 15**
Control	$3.19 \pm 0.12^{a,C}$	$3.74 \pm 0.09^{a,B}$	$4.06 \pm 0.09^{a,B}$	$4.61 \pm 0.06^{a,A}$
Gel	$3.08 \pm 0.08^{a,B}$	$3.13 \pm 0.06^{b,B}$	$3.24 \pm 0.12^{b,B}$	$4.61 \pm 0.01^{a,A}$
Gel+LYS	$3.15 \pm 0.09^{a,A}$	$2.93 \pm 0.08^{b,AB}$	$2.33 \pm 0.15^{d,C}$	$2.69 \pm 0.09^{c,B}$
Gel/RS+LYS	$3.27 \pm 0.10^{a,A}$	$2.94 \pm 0.16^{b,A}$	$3.00 \pm 0.16^{bc,A}$	$2.91 \pm 0.07^{bc,A}$
Gel/CW+LYS	$3.19 \pm 0.12^{a,A}$	$2.96 \pm 0.11^{b,AB}$	$2.82 \pm 0.05^{c,B}$	$2.98 \pm 0.09^{b,AB}$

[a-d] Values by the same letter within each storage day are not significantly different (P > 0.05)
[A-C] Values by the same letter within each treatment are not significantly different (P > 0.05)

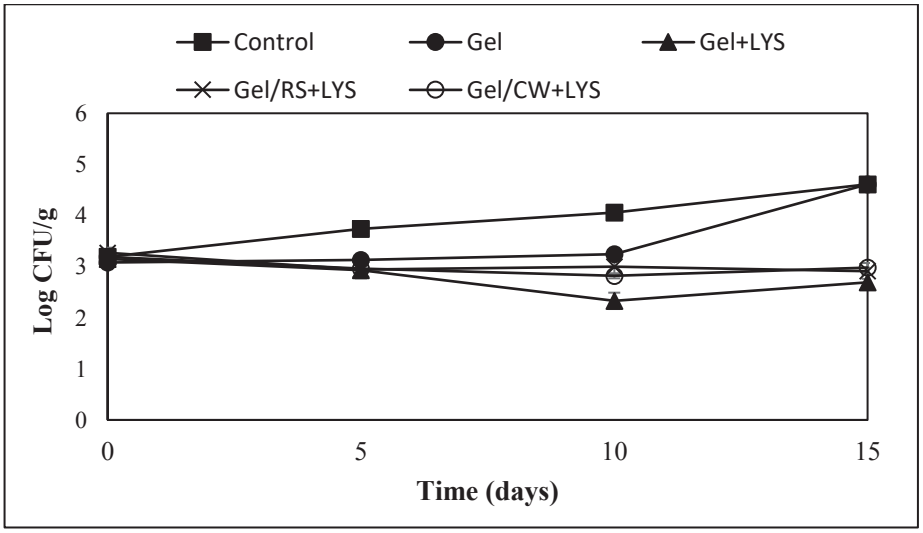

Figure 4.20. Growth of *L. innocua* inoculated on smoked salmon coated with Gel, Gel+LYS, Gel/RS+LYS and Gel/CW+LYS stored at 4 °C.

In control group, without any gel coating, the number of bacteria increased steadily until the 15th day and reached to 4.61 log CFU/g. The bacterial number in Gel coated group remained stable for 10 days, but then increased by 1.4 log CFU/g between the 10th day and the 15th day. Control group and Gel coated group reached to the same level at day 15, however Gel suppressed the growth of *L. innocua* compared to control during the first 10 days. Gel+LYS caused 0.22 and 0.6 log CFU/g inhibition in the number at the 5th and 10th days, respectively. However, 0.36 log CFU/g increase was observed after the 10th day. According to the release profile of Gel+LYS onto smoked salmon samples, it could be seen that LYS release constantly increased for 15 days. Considering the rate of *L. innocua* growth in the control group, the released LYS from Gel may have not been sufficient to cause further inhibition in the bacterial count. Nevertheless, the overall reduction in the bacterial number caused by Gel+LYS was 0.46 log CFU/g at the end of the storage time. The most significant reduction in the bacterial count was observed in the first 5 days for Gel/RS+LYS and Gel/CW+LYS. However, statistically the number of *L. innocua* has been kept stable by Gel/RS+LYS and Gel/CW+LYS during the 15 days of storage. When the LYS release profiles of Gel/RS and Gel/CW was considered, the both gels released similar activities onto smoked salmon samples. Between day 10 and day 15, Gel/RS released increasing activity on cold/smoked salmon. However, since the bacterial count increased dramatically in the same period, Gel/RS could only have suppressed the growth. The overall inhibition caused by Gel, Gel/RS and Gel/CW incorporated by LYS were 0.46, 0.36 and 0.21 log CFU/g, and the antimicrobial effects shown by the gels on smoked salmon were parallel to their effectiveness shown in aqueous media. As expected, the decimal reductions in *L. innocua* number were much lower in smoked salmon than in aqueous growth media. This result was related to the diffusion of LYS in solid media, which was slower than in agitated liquid media.

The antimicrobial activities of the gels incorporated by GTE+LYS were shown in Table 4.11 and Figure 4.20. As seen in the results, gels with GTE and LYS together showed bacteriostatic effect than inhibition. While the L. innocua number in uncoated control group increased around 1.4 log CFU/g, the gels with GTE and LYS prevented the bacterial growth in smoked salmon samples stored at 4 °C for 15 days. Gel with no active agent also showed bacteriostatic effect, however the number increased after day 10 as observed in the antimicrobial test performed with the gels containing only LYS. During this dramatic increase period, Gel+LYS+GTE, Gel/RS+LYS+GTE and Gel/CW+LYS+GTE inhibited or suppressed the growth. Compared to the gels with only

LYS, the gels incorporated by GTE+LYS showed lower inhibitory effect. This result was parallel to the release profiles of LYS into water and onto smoked salmon. GTE had a slightly reducing effect in the LYS activity. This effect could be lessened by reducing the GTE concentration incorporated into the gels. Nevertheless, in the presence of GTE together with LYS, significant antimicrobial effect was observed.

Table 4.11. Antimicrobial activity of Gel, Gel/RS and Gel/CW incorporated with LYS+GTE against *Listeria innocua* inoculated on smoked salmon for 15 days of storage at 4 °C

	Log cfu/g			
Gel Type	**Day 0**	**Day 5**	**Day 10**	**Day 15**
Control	4.20 ± 0.03^{a}	5.54 ± 0.03^{a}	5.36 ± 0.12^{a}	5.58 ± 0.07^{a}
Gel	4.19 ± 0.01^{a}	4.66 ± 0.10^{b}	4.29 ± 0.03^{b}	4.80 ± 0.08^{b}
Gel+LYS+GTE	4.04 ± 0.04^{a}	4.44 ± 0.04^{c}	4.11 ± 0.04^{c}	4.22 ± 0.05^{c}
Gel/RS+LYS+GTE	4.10 ± 0.10^{a}	4.17 ± 0.06^{d}	4.10 ± 0.02^{c}	3.96 ± 0.04^{d}
Gel/CW+LYS+GTE	4.07 ± 0.07^{a}	4.22 ± 0.06^{d}	3.93 ± 0.03^{d}	4.05 ± 0.04^{cd}

[a-d] Values by the same letter within each storage day are not significantly different (P > 0.05)

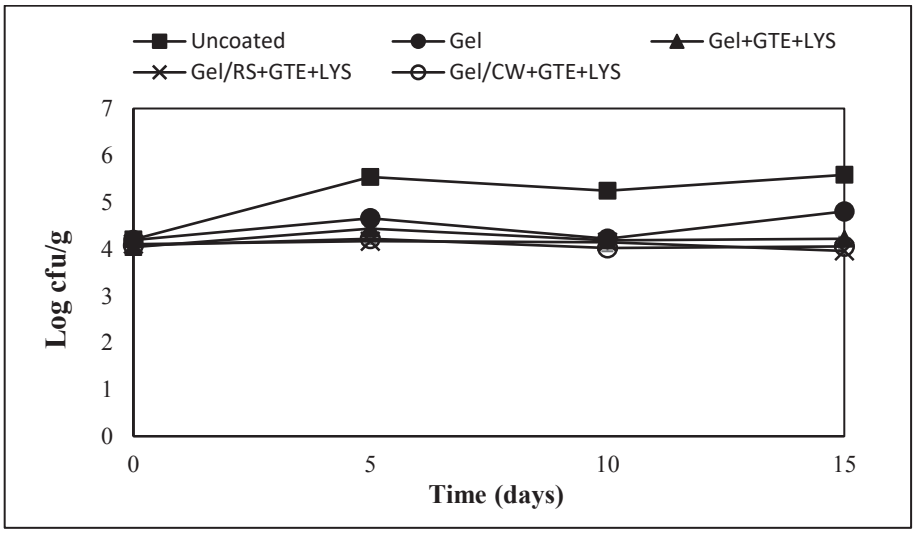

Figure 4.21. Growth of *L. innocua* inoculated on smoked salmon coated with Gel, Gel+GTE+LYS, Gel/RS+GTE+LYS and Gel/CW+GTE+LYS stored at 4 °C.

The inhibition caused by the antimicrobial gels is related to the initial inoculation level. It was reported by the researches that antimicrobial systems showed higher antimicrobial activity when the initial spoilage number was lower (Francis and O´Beirne 1997; Janes, Kooshesh and Johnson 2002). To observe a more effective inhibition by the produced gels, a challenge test with lower initial count of *L.innocua* can be performed,

however according to the preliminary studies, when 3 log cfu/g inoculation was selected as the initial number very slow growth of *L.innocua* in the uncoated control group was observed in 10 days of storage at 4 °C. Consequently, the antimicrobial tests performed in real food systems are challenging and several trials are required to find the conditions to demonstrate the antimicrobial performances of the developed systems in the best way.

Additionally, in the antimicrobial tests performed in liquid media or in food systems, area/volume ratio of the antimicrobial material plays a role in the activity of the antimicrobial agents. It was demonstrated that as the area/volume ratio increased the antimicrobial effect was increased too (Appendini and Hotchkiss 2002). In the further studies, it would be beneficial to determine the optimal thickness providing a sufficient activity and avoid thicker gels for more practical applications and to save the material.

CHAPTER 5

CONCLUSION

This thesis that aimed development of active gelatin and gelatin based composite gels to deliver natural antimicrobial and bioactive compounds onto food have reached the following major conclusions:

- Composites of GEL with SPI, RS and CW showed different textural properties than classical GEL gels. The major effect of composite gel making is the increased hardness. Moreover, a slight increase in gel elasticity occurred in gels except for those containing SPI by addition of active components, green tea extract and lysozyme.
- The composite gel making with different components also helped modification of physical properties of gelatin. The most dramatic and useful change occurred in water absorption of gels by use of SPI in composite gel making.
- GEL, GEL/RS and GEL/CW contain comparable amounts of soluble LYS and GTE. Thus, these gels are quite suitable to deliver the indicated active agents on food surface.
- GEL/SPI gel contains a soluble fraction of GTE, but these gels bind all of the incorporated LYS with ionic interactions. Thus, with their considerably high water-binding capacity and totally bind fraction of LYS these gels could serve a perfect active pad material to control drip-loss in packed food.
- The different LYS and GTE release profiles of GEL, GEL/RS and GEL/CW gels on smoked salmon used as a model food clearly showed the possible benefits of composite gel making in controlled release of active agents. GEL gels showed the fastest LYS release rates on food while GEL/RS and GEL/CW showed sustained LYS release properties. On the other hand, GEL/CW gels performed as the best reservoir for delivery of GTE onto smoked-salmon.
- LYS containing GEL, GEL/RS and GEL/CW with or without GTE showed significant antimicrobial activity against *Listeria innocua* in aqueous culture media. In contrast, indicated gels applied on smoked salmon showed mainly a growth inhibitory effect rather than inactivation.

- The released GTE from GEL, GEL/RS and GEL/CW showed significant TEAC and ORAC based antioxidant capacity, antidiabetic activity based on inhibitory effect against human saliva alpha-amylase (HSA) and alpha-glucosidase, and antihypertensive activity based on inhibition of angiotensin converting enzyme (ACE). Moreover, GTE released form the specified gels also showed cytotoxic effect against human colon carcinoma cell line (Caco-2). These results suggested that the delivery of GTE from gels onto food could cause potential health benefits in consumers.

The overall results of this thesis clearly showed that the gels applied as a coating material onto food surface or as a pad at the bottom of food could act as tools for delivery of both natural antimicrobial and bioactive agents onto food surface. With their high water-binding capacity active gels could also act as absorbent pads to prevent drip-loss from food. Moreover, embedding food into gels could turn these active tools a part of the food with pleasant textural properties. This thesis showed the potential of gels to play significant roles in food preservation and in development of functional foods with multiple health benefits.

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APPENDIX A

STANDARD CURVES USED IN THE ANALYSES

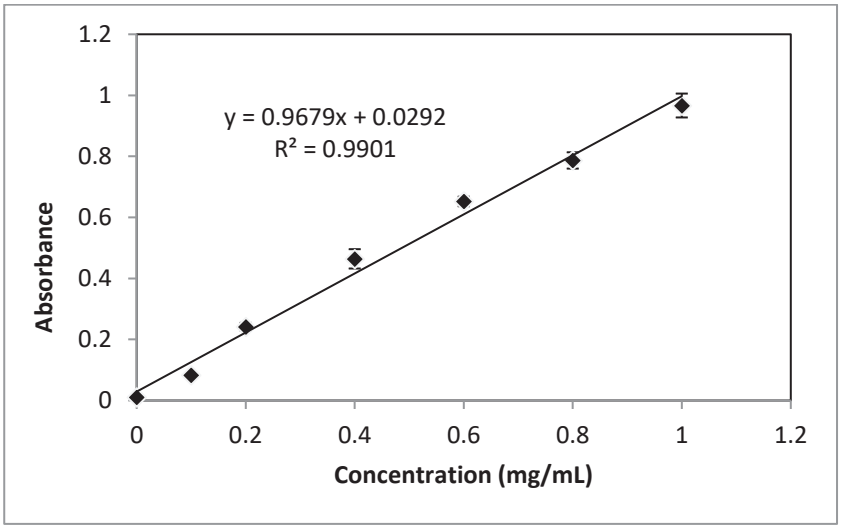

Figure A.1. Bovine serum albumin (BSA) standard curve for determination of soluble protein concentration

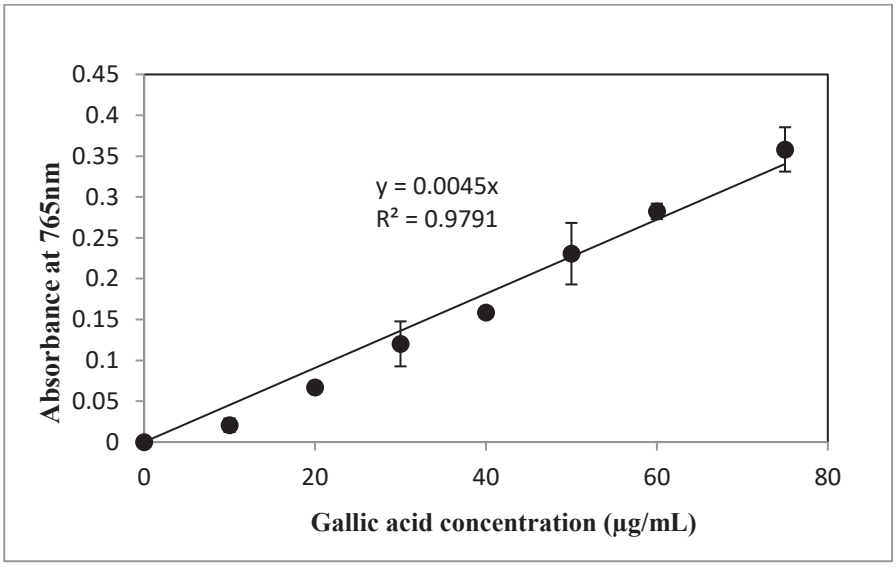

Figure A.2. Gallic acid standard curve for determination of total phenolic content

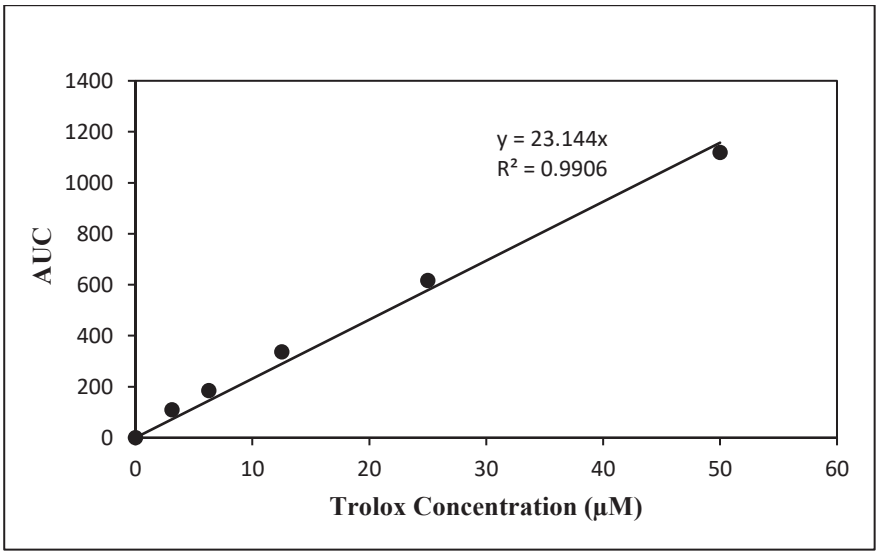

Figure A.3. Trolox standard curve for determination of antioxidant activity based on ORAC

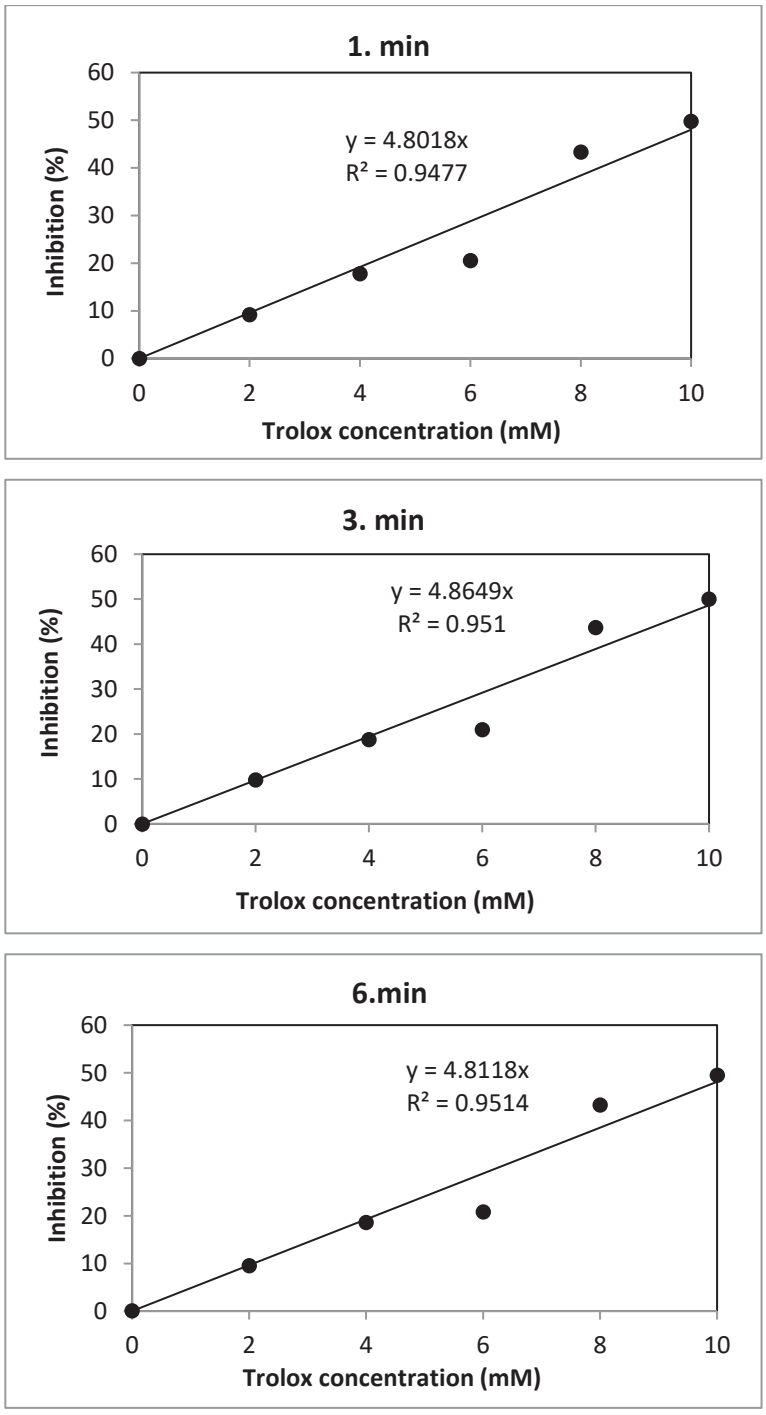

Figure A.4. Trolox standard for determination of antioxidant activity based on TEAC

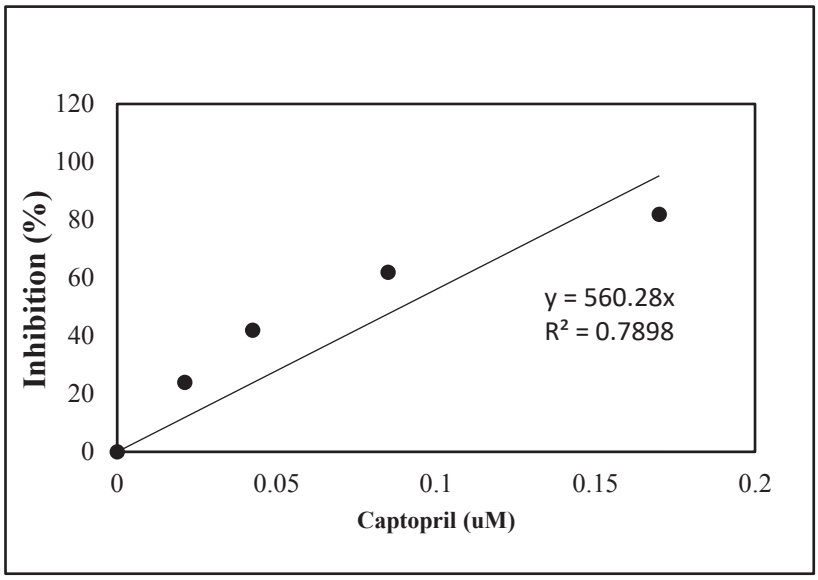

Figure A.5. Captopril standard curve for determination of antihypertensive activity

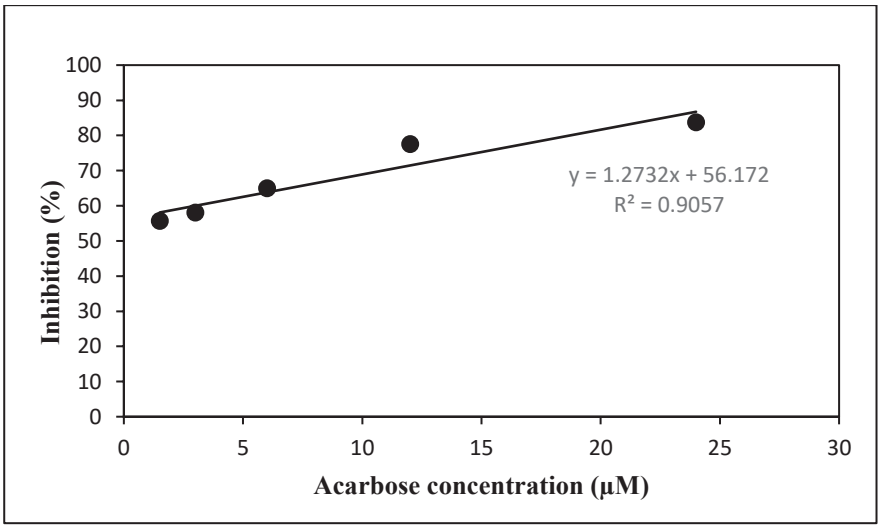

Figure A.6. Acarbose standard curve for determination of alpha-glucosidase (AGH) activity (used for samples with 55-85% inhibition)

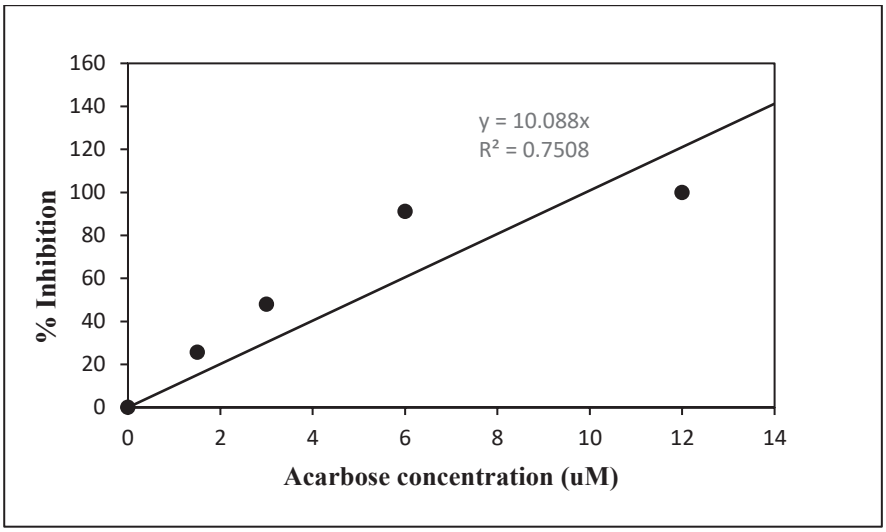

Figure A.7. Acarbose standard curve for determination of human saliva alpha-amylase (HSA) activity

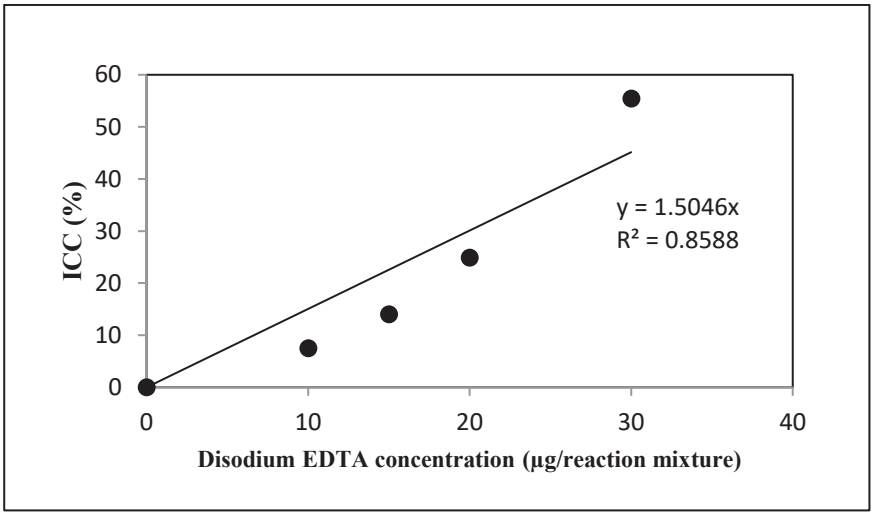

Figure A.8. Disodium EDTA standard curve for determination of iron chelation capacity

APPENDIX B

INHIBITION OF GTE ON CACO-2 GROWTH

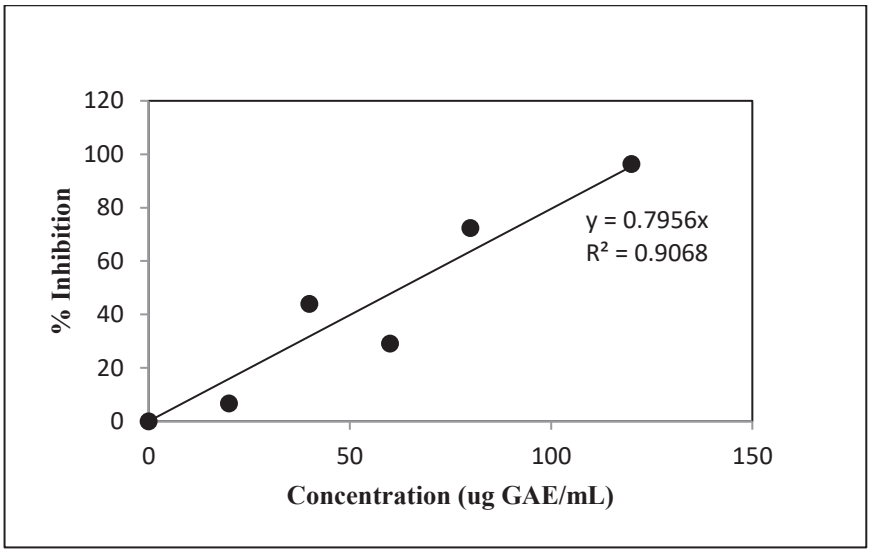

Figure B.1. Inhibition caused by GTE solution series against Caco-2 cells

APPENDIX C

PHOTOGRAPHS OF THE GELS

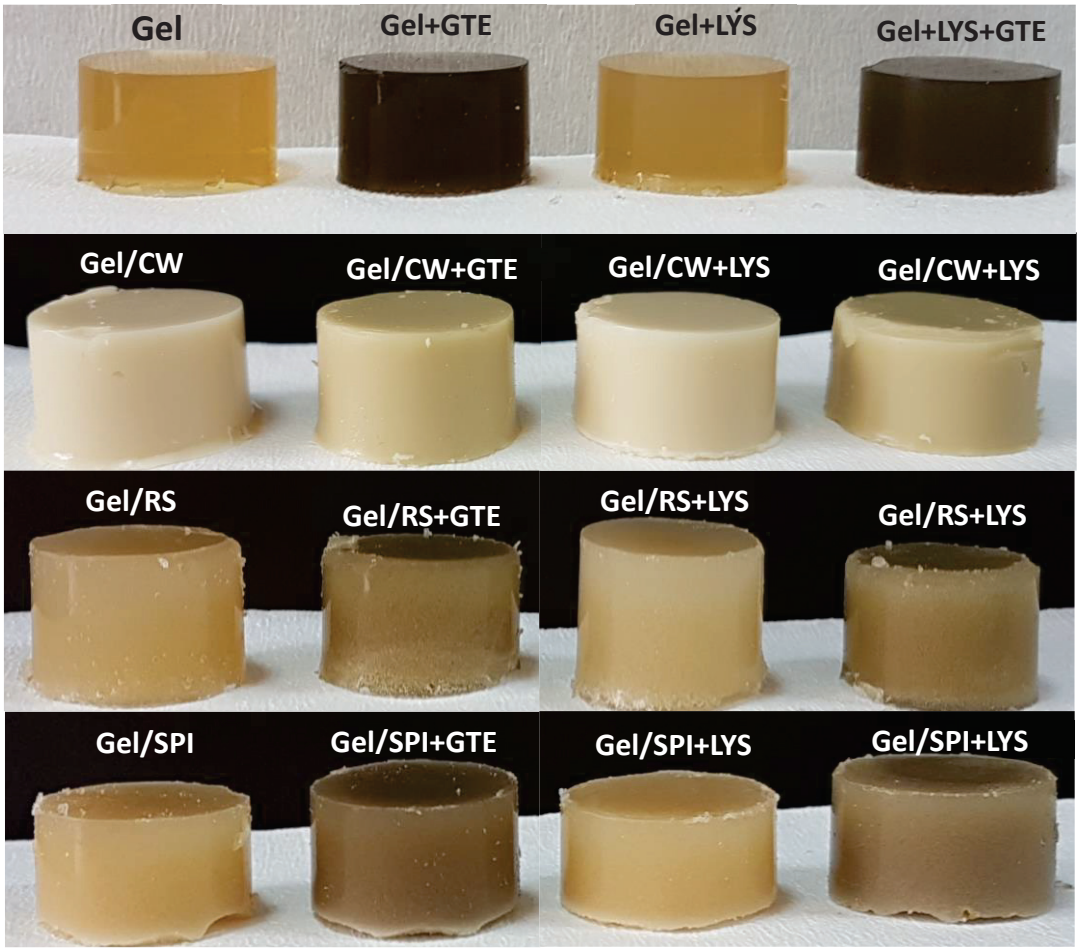

Figure C.1. Photographs of Gel, Gel/CW, Gel/RS and Gel/SPI without any active agent and with GTE (1%), LYS (1%) or GTE (1%)+LYS (1%).

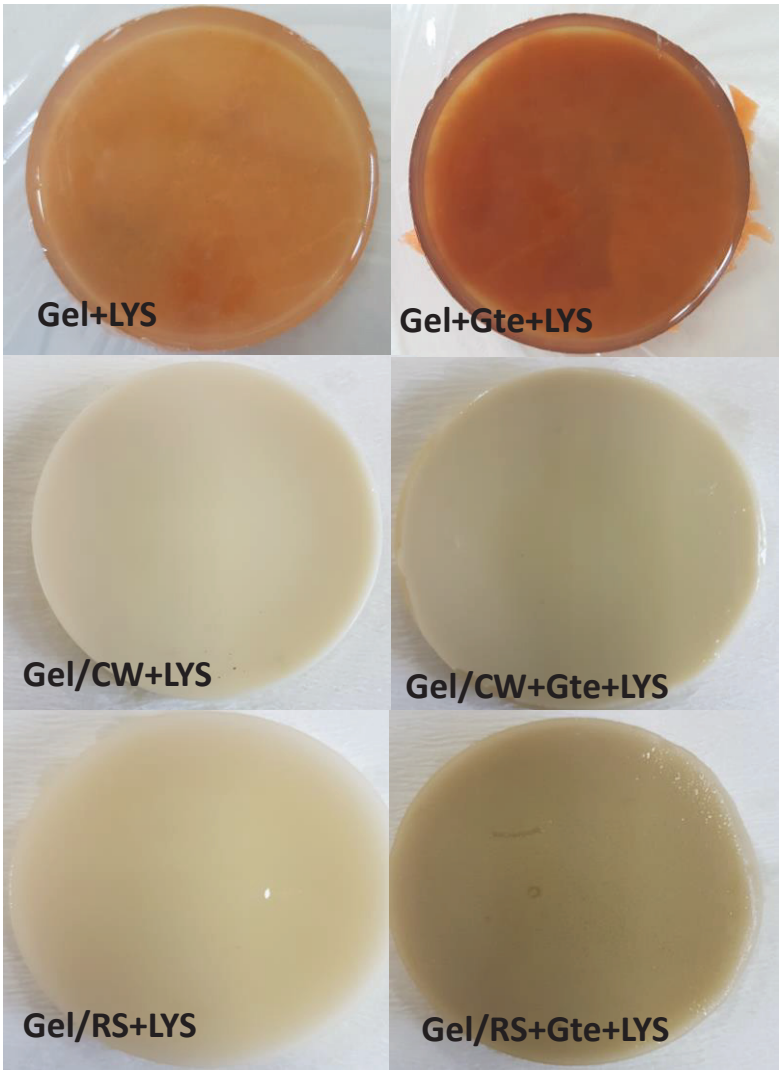

Figure C.2. The gels with LYS (1%) and GTE (1%)+LYS (1%) coated on 10 g of smoked salmon samples (diameter: 6.6 cm, thickness: 0.5 cm).

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PUBLICATIONS

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