Study on antibacterial and antitoxin activities of plant polyphenols

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A thesis submitted by

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TABLE OF CONTENTS

ABBREVIATIONS

AEEC: Attaching and effacing *Escherichia coli*

BSA: Bovine serum albumin

CDC: Center for Disease Control and Prevention

DHL: Deoxycholate Hydrogen Sulfide-lactose

E. coli: *Escherichia coli*

EHEC: Enterohaemorrhagic *Escherichia coli*

ETEC: Enterotoxigenic *Escherichia coli*

FDA: Food and Drug Administration

Gb3: Glycosphingolipid globotriaosylceramide

HUS: Hemolytic uremic syndrome

LB: Luria broth

PCR: Polymerase Chain Reaction

S. dysenteriae: *Shigella dysenteriae*

Shiga toxin: Stx

STEC: Shiga toxin producing *Escherichia coli*

TSA: Tryptic Soy Agar

TSB: Tryptic Soy Broth

VT: verotoxin

WHO: World Health Organization

Chapter 1. Introduction

1.1 Foodborne illness

Foodborne illness is caused by consuming contaminated foods. There are many causes of foodborne illness, including bacteria, viruses, fungi, protozoa and parasites. Among all causes of foodborne illness, bacterial pathogens are the most common cause of foodborne illness outbreaks. According to World Health Organization (WHO) assessment, there are about 600 million cases of foodborne illness and 420,000 deaths every year worldwide (World Health Organization, 2019). Center for Disease Control and Prevention (CDC) also estimates *Salmonella* infection cause 1.35 million foodborne illnesses, with 26,500 hospitalizations, and 420 deaths each year in United States (CDC, 2013). In 2019 in Indiana States, the number of cases of *Salmonella* infection was estimated approximately 137 foodborne illnesses, with 38 hospitalizations (Food and Drug Administration (FDA), 2019). Early 2020 in Republic of Korea, 36 foodborne illnesses, resulting in 36 hospitalizations and 4 deaths is thought to be associated with *Listeria monocytogenes* in the mushrooms (CDC, 2020).

Among all bacterial pathogens causing food poisoning, the prevalence of pathogenic *Escherichia coli* (*E. coli*) strains in foods are the most common cause of foodborne illness outbreaks. Most outbreaks of *E. coli* infection have been found to be associated with consumption of various foods, such as meat and meat products, dairy products, vegetables and fruits. *E. coli* O157:H7-contaminated undercooked meat as cause of several outbreaks of hemorrhagic colitis was investigated involving 47 cases of illness occurred in Oregon and Michigan in 1982 (Riley et al., 1983). The foodborne outbreaks of *E. coli* O157:H7 occurred in Washington and California in 1994 have been linked to eating dry fermented salami (Tilden et al., 1996). After this outbreak, the requiring least a 5-$\log_{10}$ unit reduction of *E. coli* O157:H7 during processing dry fermented salami was established

by the United States (US) Department of Agriculture Food Safety Inspection Service to ensure food safety (Chacon et al., 2006). Each year in US, the number of cases of *E. coli* O157:H7 infection was estimated to be approximately 73,500 cases (Lahmer et al., 2017). Of all multistate incidents of food poisoning by *E. coli* associated with fresh products occurred in US from 2010 to 2017, more than half of them were due to *E. coli* O157:H7 infections (Carstens et al., 2019). The outbreak of 167 cases of foodborne diseases and 85 hospitalizations in US and Canada in 2019 found to be linked to *E. coli* O157:H7 infection in salad has also been reported by FDA on January, 2020 (FDA, 2020). In the Isfahan province, Iran, the prevalence of *E. coli* strains in ready-to-eat food is 5%, among them the prevalence of Shiga toxin producing *E. coli* (STEC) strains is 2.63%, in which the prevalence of enterohaemorrhagic *E. coli* **(**EHEC) and attaching and effacing *E. coli* (AEEC) were 36.84% and 52.63%, respectively (Shahreza et al., 2017). In 1995, 70% of 40 patients were infected by *E. coli* O157:H7 linked to leaf lettuce in western Montana (Ackers et al., 1998). In the Piedmont region of Italy, the ratio of STEC contamination in dairy and meat products was 42% (Rantsiou et al., 2012). From April 2000 to July 2002, the prevalence of *E. coli* O157:H7 is 1.7% in beef cattle, 6.7% in dairy cattle, 0.3% in pig and none in chicken in Korea (Jo et al., 2004). Although prevalence of EHEC is less than that of enterotoxigenic *E. coli* (ETEC), there is significant risk for the *E. coli* O157:H7 infection by contaminated meat products from livestock animals (Kang et al., 2004; Cho et al., 2014).

1.2 Enterohaemorrhagic *Escherichia coli*

1.2.1 Introduction enterohaemorrhagic Escherichia coli

Escherichia coli is a bacterium found in the food, environment, and in the intestines of human and animals. Most strains of *E. coli* are vital part in the healthy human intestinal and harmless to humans. However, some strains such as STEC, ETEC and diarrheagenic *E. coli* can cause some foodborne disease. The categorization of pathogenic *E. coli* is

associated with their pathogenicity profiles such as clinical syndromes, and virulence factors and phylogenetic description. Six intestinal pathotypes of pathogenic *E. coli* are ETEC, EHEC, enteropathogenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli* and diffusely adherent *E. coli* (Kaper et al., 2004).

EHEC is known cause of severe intestinal infection in humans, including abdominal pain, diarrhea and bloody diarrhea. It may be transferred to humans by the consumption of contaminated food such as undercooked meat products, contaminated fresh vegetables and fruits (Berger et al., 2010). EHEC can grow in a wide range of temperature from 7^{o}C to 50^{o}C, in acidic food with pH of 4.4 and at minimum water activity of 0.95 (Balamurugan et al., 2015).

Shiga toxin (Stx), a strong toxin produced by EHEC, can give rise to hemorrhagic colitis and hemolytic uremic syndrome (HUS). EHEC was first recognized in 1982 after two outbreaks of hemorrhagic colitis and was identified to have resulted from eating undercooked ground beef contaminated with *E. coli* O157:H7 (CDC, 1982; Riley et al., 1983). Since then, the other serotypes of *E. coli* also have been associated with the outbreaks of hemorrhagic colitis in humans. Meng *et al*. reported that more than 600 different serotypes of STEC including 160 O serogroups and 50 H types have been isolated from patients with STEC infection (Meng et al., 2007). EHEC O157:H7 strain was reported to cause outbreaks of hemorrhagic colitis in many countries in the world such as Japan (Trofa et al., 1999), the United States (O'Brien et al., 1983), and Australia (McPherson et al., 2011). EHEC infections not only affect on public health but also impose a significant economic cost on society. The estimated total cost for each case of EHEC infection is AUD$3,132 in South Australia (McPherson et al., 2011)), $5,515 in United States (Frenzen et al., 2005).

1.2.2 Virulence factors of EHEC

An early event in EHEC infection is hemorrhagic colitis that is thought to be caused by various virulence factors including fimbrial and nonfimbrial adhesion, flagella, Stx, and the Type III secretion system (Foster, 2013), of which the adhesion and the production of Stx are the most important factors for the pathogenicity of EHEC.

1.2.2.1 The adhesion of EHEC

Some studies have described the adhesion mechanisms of EHEC. Jeter and Matthysse showed the characteristics of the binding of both non-pathogenic and pathogenic strains to various plant surfaces (Jeter & Matthysse, 2005). Contrary to non-pathogenic *E. coli*, EHEC O157:H7 strains showed strong adherence to spinach leaves, roofs of alfalfa sprout and tomato skin. The attachment to these plant surfaces is mediated by curli (Jeter & Matthysse, 2005). However, the production of curli on the surface of non-pathogenic strains has been shown to be sufficient to allow bacterial adhesion to roofs of alfalfa sprouts, but curli are not required for the attachment of pathogenic strains to plant surfaces, suggesting that other adhesion factors were involved together.

E. coli O157 and O26 strains use the filamentous type III secretion system (fT3SS) comprising EspA filaments for binding to salad leaves (Shaw et al., 2008). In addition, fT3SS-dependent protein secretion in *E. coli* O157 plays an important role in the adhesion of bacteria to mammalian cells (Garmendia et al., 2005). In contrast to attachment of mammalian hosts, the adhesion of *E. coli* O157 to salad leaves was independent of translocation of effector protein (Shaw et al., 2008). Flagella also play a role for binding of *E. coli* O157 to baby spinach and lettuce leaves as deletion of the fliC flagellin gene in EHEC significantly reduced the adhesion to the surface of leaves (Xicohtencatl-Cortes et al., 2009). The mutation of *escN* (ATPase) gene that linked to the function of fT3SS also significantly reduced the level of adhesion. This suggested that *E. coli* O157 used more than one mechanism for binding to host cells.

It has also been reported that both the fimbrial and non-fimbrial EHEC adhesins have been related to adhesion of EHEC to host cells. F9 fimbriae, a long polar fimbriae of EHEC O157:H7, can prevent or disrupt the adhesion of EHEC to human cells because mutants defective in F9 fimbriae increased the adhesion of EHEC to cultured epithelial cells (Low et al., 2006). *E. coli* common pilus, a pilus fimbrial structure produced by both pathogenic and non-pathogenic *E. coli*, promoted intercellular interactions in biofilms communities (Rendón et al., 2007; Martínez-Santos et al., 2012). The non-fimbrial EHEC adhesins that have been implicated in adhesion include the chromosomal genetic locus *efa1* (EHEC factor for adherence 1), plasmid-encoded *toxB*, calcium-binding antigen 43 homolog, outer membrane protein A, and the chromosomally-encoded adhesins (Tatsuno et al., 2001; Johnson et al., 2005; Torres et al. 2005)

1.2.2.2 Shiga toxin

Major virulence factor of EHEC strains is Shiga toxin (Stx) that causes some diseases in humans such as hemolytic uremic syndrome and hemorrhagic colitis. Stx was known as verotoxin (VT) because of its similarities to VT (Johnson et al., 1983) and the words "Shiga toxin" or "vero toxin" refer to the same toxin (Kaper & O'Brien, 2014). Based on the antigenic characteristics of the prototypical Stx produced by *Shigella dysenteriae* (*S. dysenteriae*) I, Stxs produced by STEC are subdivided into two major types, Stx1 and Stx2 (Strockbine et al., 1986; Scheutz et al., 2012). STEC strains can produce both Stx1 and Stx2 or either one. While Stx1 is a similar cytotoxin to Stx of *S. dysenteriae* I or differs by only one amino acid, Stx2 shares 56% amino acid sequence similarity with Stx1 (Kaper & O'Brien, 2014). Purified Stx2 has higher toxic activity than Stx1, and strains of STEC O157:H7 that express Stx2 are more likely to cause HUS than strains expressing Stx1 (Scotland et al., 1987; Louise & Obrig, 1995; Boerlin et al., 1999).

Stxs are the members of AB_5-toxin family, consisting of an enzymatically active A subunit linked with a pentameric B subunits and structure of Stx is shown in Figure 1-1.

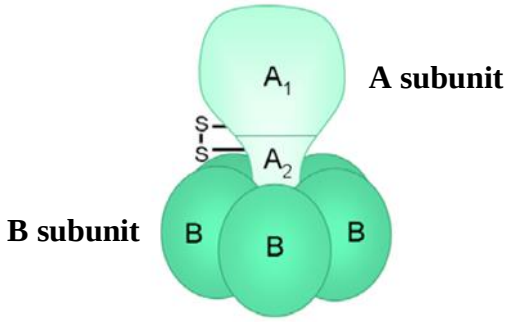


Figure 1-1. Structure of Stx from *E. coli* O157:H7 (Sandvig, 2001)

The A subunit comprised of two main domains, A1 (27.5 kDa) and A2 (4.5 kDa), that are linked together by disulfide bond between cysteines 242 and 261 (Garred et al., 1995). The A1 domain had an N-glycosidase activity responsible for the toxicity to the host cell. The activity of N-glycosidase on 28S rRNA of the 60S ribosomal subunit causes inhibition of protein synthesis and results in endothelial cell death. The A2 domain is essential for binding of the remainder of A subunit to B pentamer to create the holotoxin which is responsible for binding to glycosphingolipid globotriaosylceramide (Gb3) found on the surface of vascular endothelial cells and kidney epithelial cells. The chemical structure of Gb3 is shown in Figure 1-2.

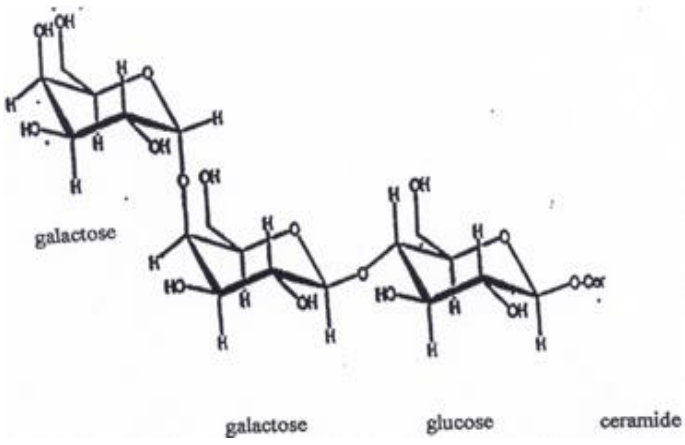


Figure 1-2. Chemical structure of glycosphingolipid globotriaosylceramide (Paton & Paton, 1998)

Stxs are encoded by *stx* genes found on lambdoid bacteriophages. At low iron concentrations, *stx*1 can be expressed in the absence of lytic phage replication, whereas the expression of *stx2* depends mainly on prophage induction (Wagner & Waldor, 2002; Tyler et al., 2004). Stx production of STEC strains is closely correlated with *stx*-encoding phages and could be significantly enhanced by DNA-damaging, antimicrobial and SOS-inducing agents such as mitomycin C, quinolones, furazolidone and trimethoprim (Al-Jumaili et al., 1992; Kimmitt et al. 2000; Wagner et al., 2001b). It has also been reported that Stx promoted EHEC adhesion to host epithelial cells by enhancing surface expression of two receptor candidates, nucleolin and phosphatidylethanolamine (Foster et al., 2000; Robinson et al., 2006).

1.2.2.3 Other virulence factors of EHEC

Other virulence factors of EHEC include the locus of enterocyte effacement (LEE) and the non-LEE encoded effectors. The LEE consists of five major polycistronic operons (LEE1-5) that encode the components of type III secretion system (TT3SS) and effector proteins (Mcdaniel et al., 1995). EHEC colonization was promoted by the LEE-encoded proteins, including Map, EspF, EspH, EspA ans EspG (Ritchie & Waldor, 2005; Campellone et al., 2007). In addition to the LEE, the non-LEE encoded effector proteins are also translocated into the host cell through the TT3SS (Connolly et al., 2015). Both the LEE and the non-LEE contribute to the modulation of host cell signaling to assist the replication and survival of bacteria, as well as host colonization and disease development.

1.2.3 Prevention of EHEC contamination in food

EHEC strains can survive and grow in many food products. Thus, the minimizing initial contamination and subsequent growth of EHEC in foods are essential to prevent foodborne disease. Some EHEC strains can induce stress responses to enhance their

resistance and growth. It has been reported that acid tolerance of *E. coli* O157:H7 enable the pathogen to survive in acidic conditions of gastrointestinal tract and finally, cause disease (Arnold & Kaspar, 1995; Diez-Gonzalez et al., 1998).

Furthermore, pathogenic *E. coli* can also survive all production stages of smear-ripened cheese for up to 70 days after manufacture (Maher et al., 2001). Hence, control steps in the food supply chain are necessary for the prevention of bacterial infection leading to the risk reduction to public health. In the industry, heat treatment is an effective method to eliminate EHEC from food products because of the sensitivity of EHEC to heat (Juneja et al., 1997; Li & Gänzle, 2016). Cooking at a high temperature can prevent *E. coli* O157:H7 infection in minced meat (Abong'o & Momba, 2009). The treatment of spinaches inoculated with *E. coli* O157:H7 and *Salmonella enterica* with the antimicrobial from lactic acid bacteria (LAB) was effective to reduce *E. coli* O157:H7 and *Salmonella* viable cells (Cálix-Lara et al., 2014). To reduce contamination with *E. coli* O157:H7 in apple cider, sodium benzoate was used in the some processes, including washing, brushing and preservation (Zhao et al., 1993). Some recent research demonstrated the effects of the treatment by bacteriophage to control *E. coli* O157:H7 in chicken, pork, tomato, spinach, beef and in both cooked and raw beef (Flynn et al., 2004; Abuladze et al., 2008; Hudson et al., 2013; Seo et al., 2016).

In addition, multiple hurdles may be applied during food processing to ensure safety of the processed food. For example, the combination of treatments with chloride and LAB before packaging and the packaging in modified atmosphere such as nitrogen (80% N_2, 20% O_2) or oxygen (80% O_2, 20% N_2) significantly reduced the survival of *E. coli* O157:H7 and *Clostridium sporogenes* in spinach (Brown et al., 2011). Combination of the lytic activity of bacteriophage BPECO19 and low temperature (4^{o}C) was effective to control *E. coli* O157:H7 in meat (Cho et al., 2011). Although many methods were used in the control of EHEC in food products, the use of natural antimicrobial compounds in food

industry is increasing due to consumer concerns about the adverse effects of chemically synthesized food preservatives.

1.3. Plant polyphenols

Polyphenols are organic compounds found in plant such as fruits, vegetables, cereals, tea, wine, beverages and dark chocolate. Their beneficial effect on human health is believed to be due to their antioxidant activity (Sato et al., 2002). Meta-epidemiological studies suggested that long term consumption of plant polyphenol-rich foods had an important role in human health, helps to protect against many disease such as cancers, lung damage, diabetes, osteoporosis, type 2 diabetes, cardiovascular and neurodegenerative illness (Pandey & Rizvi, 2009; Martín-Peláez et al., 2013; Xiao & Hogger, 2014; Fujiki et al., 2015). Over 8,000 types of polyphenolic compounds have been identified in many plants. Based on the number of phenol ring in polyphenol structure and the structural elements that bind these rings to each other, polyphenols were categorized into 4 main groups, including flavonoids, phenolic acids, stilbenes and lignans (Pandey & Rizvi, 2009). The basic structure of main groups of polyphenols is shown in Figure 1-3.

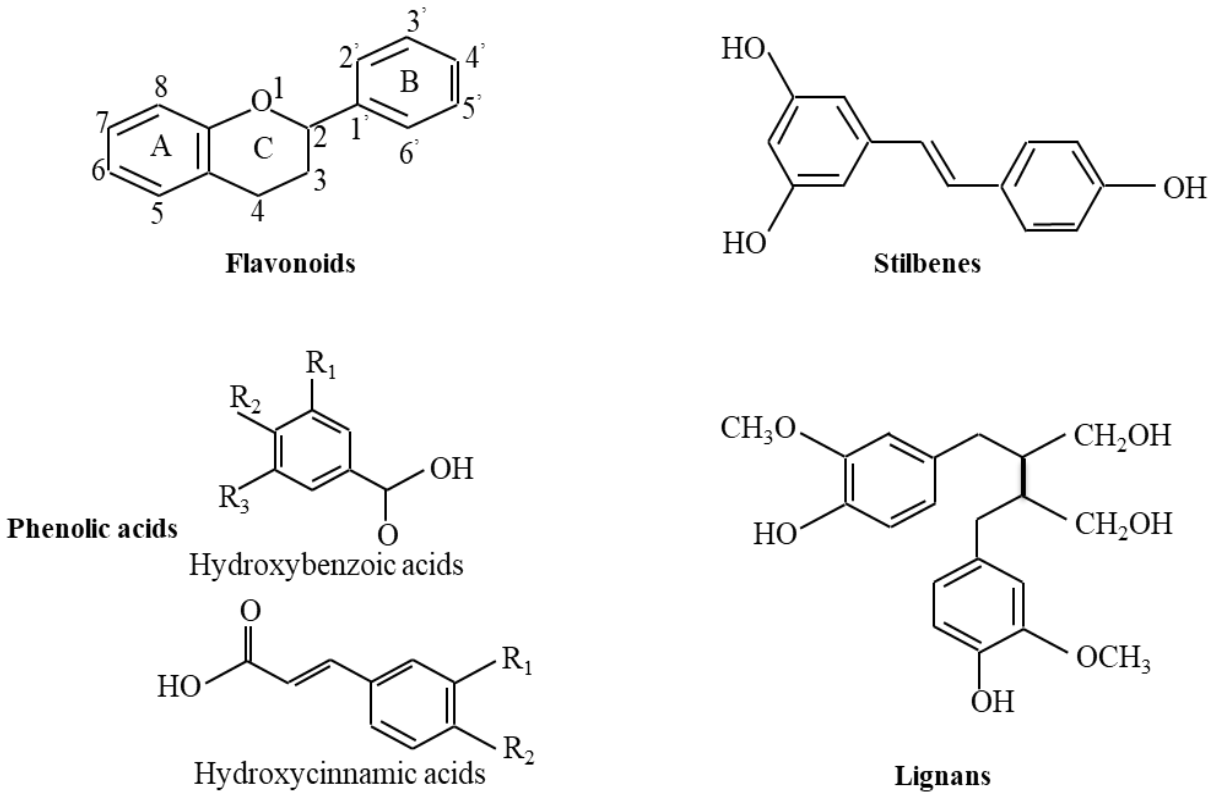


Figure 1-3. Basic structure of the different classes of polyphenols

1.3.1 Flavonoids

Flavonoids account for about 60% of all polyphenols and are found in various foods such as fruits, tea, vegetables, flowers and grains. They have a common basic structure consisting of two benzene rings (A and B) and a heterocyclic pyrane ring (C) (Fig. 1-3) (Brown, 1980). Flavonoids may be subdivided into six subgroups: flavones, flavonols, flavanones, anthocyanidins, flavanols and chalcones (Fig. 1-4).

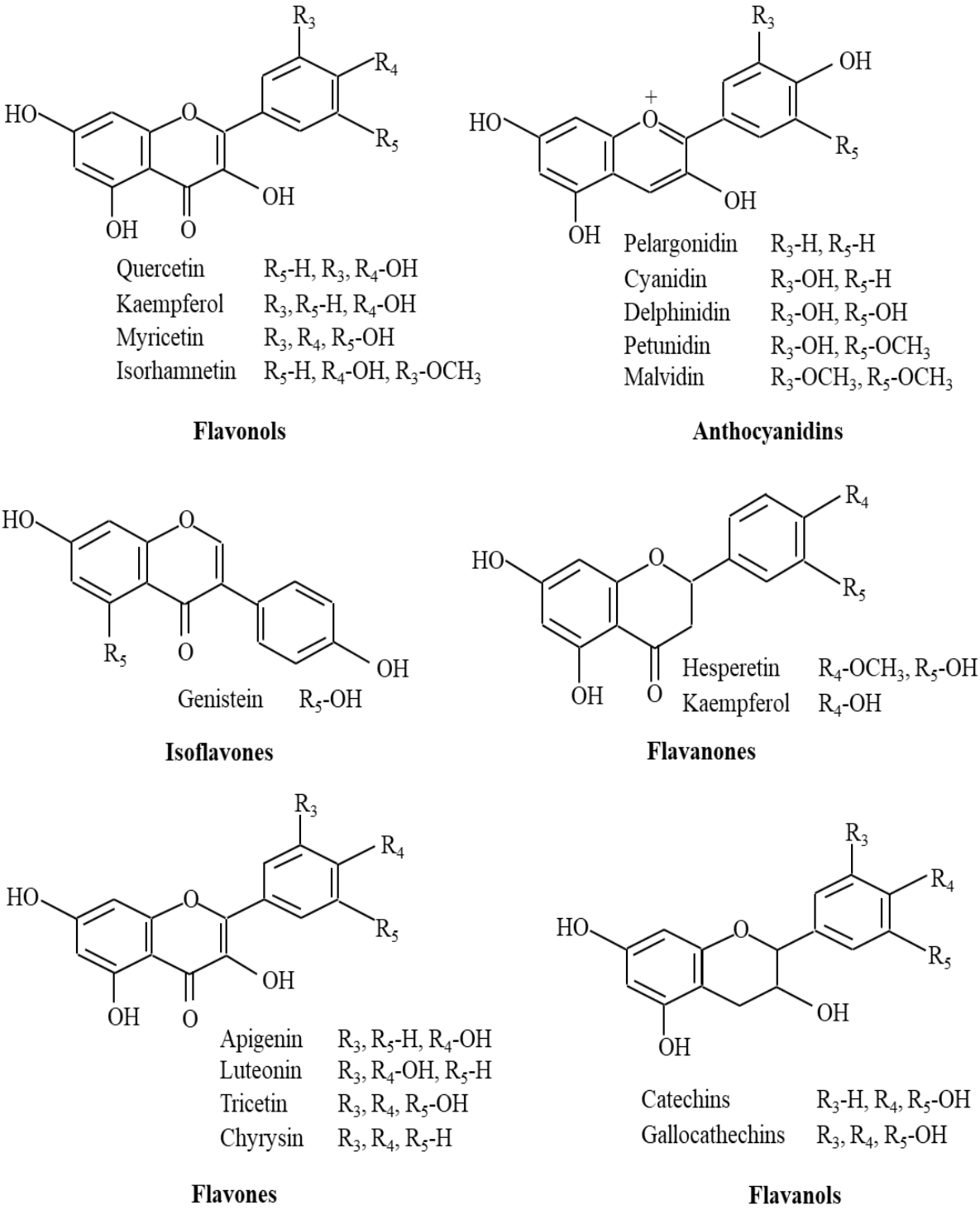


Figure 1-4. Structures of different classes of flavonoids

The health benefits of flavonoids in humans are associated with their antioxidant, anticarcinogenic anti-inflammatory and antibacterial activities. Many flavonoids show antioxidative activity, anticancer activity, preventing coronary heart disease and free radical scavenging activity, while some other flavonoids are shown to have functions of

anti-human immunodeficiency virus (Brown et al., 1998; Mishra et al., 2013). The antioxidant activity of flavonoids includes suppression of ROS generation, scavenging ROS, and upregulation of antioxidant defenses (Mishra et al., 2013). Hepatoprotective activities were also shown in several flavonoids such as rutin, catechin, naringenin, apigenin, quercetin, and venoruton (Tapas et al., 2008). It has been reported that liver damage was improved by treatment with flavonoids in histopathological examinations (Wu et al., 2006). According to the report of Cushnie and Lamb, some flavonoids (such as isoflavones, flavonol glycosides, apigenin, quercetin, flavone, chalcones and galangin) have been shown to have potent antibacterial activity (Cushnie & Lamb, 2005a). Catechin was reported to have antibacterial activity against *Vibrio cholerae*, *Shigella*, *Steptococcus mutans* and some other bacteria (Moerman, 1996). In addition, the anti-inflammatory activity of flavonoids was shown through their inhibitory effects on enzymes which are responsible for the inflammatory responses. For example, cyclooxygenase-2 that is responsible for the production of inflammatory mediators was inhibited by luteolin and quercetin (Kimata et al., 2000). Rutin effectively inhibited phospholipases A2 that can increase inflammatory response (Lindahl & Tagesson, 1997)). Hesperidin, a flavanone glycoside, inhibited induction of mammary and colon cancers in rats by azoxymethanol (Ren et al., 2003). The antiviral activity of various flavonoids has been reported, such as the inhibitory effect of baicalin on HIV-1 infection and replication (Li et al., 2000), and inhibition of DNA polymerases of HIV-1 by catechin (Cushnie & Lamb, 2005a). Furthermore, several flavonoids also showed the antitoxin activities. For example, the mixture of catechins inhibited the production and activity of Stx2 (Okubo et al., 1998). The extracellular release of both Stx1 and Stx2 from EHEC were inhibited by gallocatechin gallate (GCg) and epigallocatechin gallate (EGCg) (Sugita-Konishi et al., 1999).

1.3.2 Phenolic acids

Phenolic acids account for around 30% of all polyphenolic compounds. They are mostly found in vegetables and fruits and play a vital role in the formation of unique taste and flavor of food quality. Some common phenolic acids include caffeic acid, ferulic acid, sinapic acid and gallic acid. The basic structure of phenolic acids contains an aromatic ring and an organic carboxylic acid function (Fig. 1-3) (Brown, 1980). They were divided into two groups: hydroxycinnamic acid and hydroxybenzoic acid (Daglia et al., 2014). Phenolic acids have beneficial effects on human health because they act as antioxidants that significantly delay cellular damage by scavenging hydroxyl radical (Kukić et al., 2006). Some studies showed that phenolic acids have a major role in the prevention of many chronic illnesses because of their contribution to the intake of natural antioxidants in human diets (Balasundram et al., 2006; El-Seedi et al., 2012; Upadhyay and Rao, 2013). It has been reported that the antioxidant activity of phenolic acids is more effective than that of vitamin E or vitamin C (Tsao & Deng, 2004). Furthermore, phenolic acids are responsible for anticancer activity. Several phenolic acids (such as gallic acid, ferulic acid, and caffeic acid) showed high anticancer activities (Hwang et al., 2006; Madlener et al., 2007). The inhibitory effect of caffeic acid on human colon cancer cells was also reported (Jaganathan, 2012). The possible role of phenolic acid in inhibiting the toxin-production by microorganism has been reported. The toxin produced by *Clostridium botulinum* was inhibited by gallic acid (Reddy et al., 1982). Phenolic acids including chlorogenic, ferulic and p-coumaric acids showed the inhibitory effects on the growth and production of HT-2 and T-2 toxins by *Fusarium sporotrichioides* and *Fusarium langsethiae* (Ferruz et al., 2016a, 2016b).

1.3.3 Stilbenes

Stilbenes are found in many plant species such as sorghum, grapes, and peanut (Chong et al., 2009). They contain two phenyl moieties connected by a two-carbon methylene bridge (Fig. 1-3) (Brown, 1980). Most stilbenes work as antifungal phytoalexins that are synthesized in response to injury, infection, fungal attacks and UV irradiation (Reinisalo et al., 2015). Resveratrol, a stilbenoid polyphenol found largely in grapes, is a phytoalexin that acts against bacterial and fungal pathogens (Beekwilder et al., 2006; Wang et al., 2011; Li et al., 2015; Nawaz et al., 2017). Resveratrol was also shown strong inhibitory effects on VacA toxin producing by *Helicobacter pylori* (Tombola et al., 2003).

1.3.4 Lignans

Lignans are diphenolic compounds found in many plants and food, including wine, tea, nuts, grains, seeds and vegetables (Suzuki & Umezawa, 2007). The concentration of lignans found in sesame and flax seeds is higher than that in other foods (Landete, 2012). Lignans comprise of two propylbenzene unit linked by a β, β'-bond (Fig. 1-3) (Brown, 1980). Based on the cyclization pattern and the oxygen incorporation into the molecule, lignans may be subdivided into 8 subgroups: arynaphthalenes, dibenzylbutanes, furofurans, dibenzylbutyrolactones, aryltetralins, dibenzocylcooctadienes, and dibenzylbutyrolactols (Pan et al., 2009). Lignans express various biological characteristics, including antioxidant, antitumor and anti-inflammatory activity (Ionkova, 2011).

1.4 Antibacterial activity of polyphenols

Polyphenols are an important compound for the finding and the development of novel antibacterial drugs because of their strong antibacterial activities. In vitro, many studies reported that polyphenols have antibacterial activities against Gram-negative and Gram-positive bacteria. The mechanisms of the antibacterial activity of polyphenols are

being increasingly recorded. Due to the structural variety of polyphenol groups, it is impossible to predict the interaction between bacteria and polyphenol which are responsible for the antibacterial activities of polyphenol.

Based on the structural differences of some flavonoids (such as dihydrobiochanin A, darbergioidin and ferreirin) to evaluate their antibacterial activities by using paper disk agar diffusion assay, Osawa et al. (1992) suggested that 5,2,4-trihydroxyl-7-methoxyisoflavanone inhibited the growth of *Streptococcus cricetus*, *S. mutans*, *S. sobrinus*, and *S. rattus*, in which 5-hydroxyisoflavanones and 5-hydroxyflavanones with hydroxyl group at position 2' are important for their antibacterial activity. Tsuchiya et al. (1996) indicated that 5,7-dihydroxylation of the A ring and 2',4'- or 2',6'-dihydroxylation of the B ring in flavanone structures are essential to antibacterial activity against methicillin resistant *Staphylococcus aureus*, while substitution at the 6 or 8 position with a certain aliphatic group also enhanced this activity.

Cowan (1999) suggested that the binding of OH group or OH groups to both the aromatic rings and the oxygen substituted ring linked to the antimicrobial activities of polyphenols. It has been reported that the antibacterial activities of flavonoids are closely related to the number, positions and alkylation of OH groups, the backbone structure, and the presence of glycoside linkages (Liu et al., 2010; Wu et al., 2013b). Mori et al. (1987) suggested that myricetin, (−)-epigallocatechin and robinetin significantly inhibited DNA, protein and lipid syntheses in *Proteus vulgaris*. The inhibitory effects on these syntheses were attributed to the role of ring B of flavonoids in intercalation or hydrogen bonding with the stacking of nucleic acid bases (Mori et al., 1987).

Some flavonoids, including isoflavones, flavonol glycosides, apigenin, chalcones, quercetin, flavone, and galangin have been shown to have strong antibacterial activity (Cushnie & Lamb, 2005a). Taguri et al. (2004) demonstrated that 10 types of polyphenols including proanthocyanidins, hydrolyzable tannins, and catechins and their oxidation products,

inhibited the growth of four groups of foodborne bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, and *Vibrio*. The susceptibility of bacteria to polyphenol is related to the structure of both polyphenol and bacterial strain. Taguri et al. (2004) also suggested that 3,4,5-trihydroxyphenyl groups play an important role in the antibacterial activities of epigallocatechin, epigallocatechin-3-*O*-gallate, prodelphinidin and castalagin which show relatively strong antimicrobial activities.

Several studies proposed that EGCg inhibited the growth of bacteria by inhibiting the function of cytoplasmic membrane (Cushnie & Lamb, 2005b; Nakayama et al., 2008, 2013). Cushnie and Lamb (2005b) showed that galangin (3,5,7-trihydroxyflavone) induced a significant increase in potassium leakage in *Staphylococcus aureus* cells, which may be associated with the cytoplasmic membrane damage of the bacterium.

1.5 Antitoxin activity of polyphenols

Various bacterial toxins were inhibited by polyphenols. Polyphenols could directly inhibit the activities of toxin, or could block the binding of the toxins to the receptor. Resveratrol inhibited cholera toxin (CT) activity against Vero cell by suppressing the toxin activity and toxin internalization (Morinaga et al., 2010). Several plant polyphenols have been shown the inhibitory effects on CTA1 by directly inhibiting the catalytic activity of the toxin (Oi et al., 2002). Some polyphenol extracts inactivated heat-labile enterotoxin (LT) produced by enterotoxigenic *E. coli* by inhibiting the binding of LT to its intestinal receptor GM1(Verhelst et al., 2013). The inhibitory activity of EGCg on cholera toxin appear to specifically disrupt CT-GM1 interactions by its binding with toxin (Cherubin et al., 2016), in contrast to the inhibition of LT-GM1 interaction by toxin precipitation with EGCg (Verhelst et al., 2013).

Several polyphenols could aggregate bacterial toxins by sharing a common structural organization that contains catalytic A subunit and a cell binding B subunit

(Friedman, 2007). It has been reported that the inhibitory activity of EGCg against the cytotoxicity of Stx2 is due to forming a binding structure at the pocket of Stx2B subunit pentamer (Miyamoto et al., 2014). Caffeic acid protected Vero cells from the cytotoxicity of ochratoxin A by altering principally the lysosomal function in Vero cells (Cariddi et al., 2015). Yahiro et al. (2005) reported that the inhibitory activity of polyphenols against VacA toxin produced by *Helicobacter pylori* might be due to forming of a complex between polyphenol and toxin or inhibiting binding of toxin to cell receptors in the digestive tract.

Bacterial toxins are proteins and their virulence was determined by 3-D conformation. Thus, the change of the native structural integrity of these proteins could inactivate the activities of bacterial toxin by preventing molecular interactions with cell membrane receptors of host cells (Friedman, 2007). EGCg strong inhibited anthrax toxin produced by *Bacillus anthracis* by binding between phenolic OH groups of EGCg to the zinc atom linked to the metalloproteinase of toxins (Friedman et al., 1986; Benelli et al., 2002). Some polyphenols isolated from black tea also inhibited the botulinum neurotoxin produced by *Clostridium botulinum* by binding with the metalloproteinase part of toxin (Satoh et al., 2002). The inhibitory effects of catechins on the production and extracellular release of Vero toxins produced by *E. coli* O157:H7 appears to be caused by interference of the transfer of periplasmic proteins through the outer membrane of bacterial cells by catechins (Sugita-Konishi et al., 1999).

In addition, the specific structure of each polyphenol is also responsible for its antitoxin activity. EGCg inhibited CT, exotoxin A, diphtheria toxin and ricin, whereas epicatechin gallate which differs from EGCg by a single OH group only inhibited exotoxin A (Cherubin et al., 2016). Verhelst *et al*. (2013) suggested that the presence of at least two galloyl moieties in polyphenol structure is essential for the inhibitory effects of polyphenol on enterotoxin. Pentagalloyl glucose which contains more galloyl moieties than EGCg and GCg has stronger inhibitory activity on LT than EGCg and GCg (Verhelst et al., 2013).

1.6 Purpose of this study

EHEC causes foodborne illness, and if it becomes severe, it causes hemorrhagic colitis and potentially fatal hemolytic uremic syndrome due to the action of Stx produced by the bacterium. The beneficial effect of polyphenols on human health is believed to be due to their various properties such as antibacterial, antioxidant and antitoxin activities. They have been reported to inhibit the growth of various bacteria and the activities of toxins produced by bacteria. The combinations of polyphenols with other antimicrobial agents have also shown synergistic effects against many bacteria. In this study, to obtain basic evidences for the use of plant polyphenols for controlling EHEC, antibacterial activities of the tea polyphenol formulations were used to investigate the combined effects with some food additives and heat treatment. It has been shown that the cytotoxicity of Stx1 decreased after pre-incubation with GCg and EGCg. However, the cytotoxicity of Stx2 was not inhibited by preincubation with catechins and theaflavins. To find out plant polyphenols effective against both Stx1 and 2, phenolic compounds were screened by docking simulation based on the analysis of interaction between Stx B subunit pentamers and natural products from the natural compounds database. The effects of selected compounds on the cytotoxicity of Stxs were investigated. Since baicalein inhibited the cytotoxicity of both Stx1 and 2 among compounds selected by *in silico* screening, effects of baicalein on cytotoxicity and productivity of Stxs were investigated in detail.

Chapter 2. The combined effects of tea polyphenols and food additives against enterohaemorrhagic *Escherichia coli*

2.1 Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) is an important foodborne pathogen causing many diseases, and it can lead to hemolytic uremic syndrome (HUS) in humans (Kaper et al., 2004). *Escherichia coli* O157:H7, a serotype in EHEC, is one of the most important foodborne pathogens. *E. coli* O157:H7 can survive in acidic conditions (as low as pH 2.0) (Leyer et al., 1995; Diez-Gonzalez et al., 1998) and grow in TSB containing high concentration of salt up to 6.5% NaCl or at pH from 4.5 to 9.0 (Glass et al., 1992). Many antibiotics are contraindicated in treatment for *E. coli* O157:H7-infected patients because they might increase the risk of the hemolytic uremic syndrome (Wong et al., 2000).

The beneficial effect of tea polyphenols on health is believed to be due to their strong antioxidant activity (Forester & Lambert, 2011). Moreover, tea polyphenols also have anti-inflammatory, antiviral and anticancer activities (Serafini et al., 2011). Epigallocatechin gallate (EGCg), one of the green tea catechins, has been reported to inhibit the growth of both Gram-positive and Gram-negative bacteria, including *E. coli* O157:H7 (Nakayama et al., 2008). EGCg inhibited the growth of *E. coli* by direct interaction with proteins in the outer membrane of *E. coli* (Nakayama et al., 2013). Nakayama et al. (2008) also indicated that the antibacterial activity of green tea extract increased in the presence of 4% NaCl and have also shown its combined effects with various food additives against both *E. coli* O157:H7 and *Listeria monocytogenes*. Theaflavins (TF), a group of polyphenols, are formed from polymerization of catechins during the fermentation of black tea. Theaflavin (TF_1), theaflavin-3-gallate, theaflavin-3-gallate (TF_2A, TF_2B), and theaflavin-3,3-digallate (TF_3) are the major TF, specifically TF_3

has strongest antioxidant activity among all catechins and TF derivatives (Leung et al., 2001).

Food additives are substances added to food for various purposes such as inhibiting the growth of bacteria, preserving fresh attributes and nutritional quality etc. Some food additives have antibacterial activities against various foodborne pathogens. For example, sodium citrate (Na citrate) and sodium acetate (Na acetate) can inhibit the growth of several bacteria such as *Staphylococcus epidermidis, S. aureus* and *E. coli* (Lee et al., 2002; McWilliam & Stewart, 2002), sodium chloride (NaCl) inhibits the growth of *Pediococcus acidilactici* (Aina, 2017), *S. aureus* and *E.coli* (Omotoyinbo, 2016) by lowering the water activity. Moreover, ethylenediaminetetraacetic acid (EDTA) and ethanol are also capable of inhibiting the growth of bacteria (Farca et al., 1997; Brewer et al., 2002; Gill & Holley, 2003). Although widely used in food industry, food additives were viewed as a major risk to health because they could generate harmful chemical changes and should be considered as potentially toxic materials. Trasande et al. (2008) have been reported that certain food additives could cause endocrine disruption and other potential adverse effects on consumer health, especially children's health. In food industry, food additives can be used alone or in combination. The combination of food additives has been increasing interest because the combination of two or more food additives could increase their antibacterial effects and produce synergistic effects. For example, ethanol enhanced the sensitivity of *Listeria monocytogenes* to nisin, NaCl and organic acids (Barker & Park, 2001; Brewer et al., 2002), the combination nisin and EDTA also reduced the growth of *Salmonella* (Ukuku & Fett, 2004), and *E. coli* (Field et al., 2017). However, the antibacterial activity of food additives in combination with tea polyphenols on the growth of *E. coli* O157:H7 have been rarely reported.

In this study, the combined effects of EGCg and theaflavin with some food additives including EDTA, ethanol, Na acetate, Na citrate and NaCl on the growth of *E.*

coli O157:H7 were evaluated by determining minimum inhibitory concentration (MIC). The fractional inhibitory concentrations (FIC) index were further determined through the study on the modes of actions of the compounds using time kill assays. The synergistic effects of these combinations could be a way to overcome the risk of using food additives.

2.2 Materials and methods

2.2.1 Preparation of bacteria

E. coli O157:H7 No.28 strain was used in this study. The bacterium was inoculated into 5 mL of Tryptic Soy Broth (TSB) medium (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) and cultured overnight at 37^{o}C with shaking at 130 rpm to obtain cells in the stationary phase of growth. This culture was then adjusted by dilution in sterilized water to a final OD_{660} of 0.10 (bacterial concentration, ca. 10^8 CFU/mL). The bacterial cells were harvested by centrifugation (6,000 g, 5 min, 25^{o}C), and then were suspended in sterile water to attain a final concentration of ca. 10^8 CFU/mL for use in subsequent experiments.

2.2.2 Preparation of tea polyphenol and food additive solutions

The tea polyphenol products used in this study were food additive formulation including EGCg or theaflavins. Teavigo containing 95%(w/w) EGCg was purchased from DSM Nutrition Japan, Tokyo, Japan. TF40, a theaflavins extract containing 40%(w/w) theaflavins, was purchased from Yaizu Seisakusho Kogyo Co., Japan. To prepare tea polyphenol solutions, Teavigo and TF40 powders were dissolved in sterilized water and in 0.1 % dimethyl sulfoxide (DMSO), respectively. These solutions were sterilized by filtration through a Millex-GP 0.22 μm filter (Merck Millipore, Billerica, MA, USA).

Food additives used in this study were EDTA, NaCl, Na acetate, Na citrate and ethanol. All the reagents were of analytical grade. To prepare working solutions, reagents were dissolved in sterilized water and then autoclaved at 121^{o}C for 15 min.

After autoclaving, these solutions were stored at room temperature to use in the following experiments.

2.2.3 Determination of the minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) determinations were performed using broth dilution method in test tube as described previously (Wiegand et al., 2008). To 3.8 mL of glucose added ammonium (GA) medium (0.909 g/L $(NH_4)_2HPO_4$, 0.182 g/L KCl, 0.182 g/L $MgSO_4/7H_2O$, 0.182 g/L yeast extract and 9.091 g/L glucose, pH 6.5) in separate test tube, 100 µL of each compound with various concentration were added. And then, 100 µl of bacterial suspension with a final concentration of 2 x 10^5 CFU/mL was inoculated to these tubes. The inoculated tubes were incubated at 37^{o}C for 24 h. The MIC was defined as the lowest concentration of compounds which showed no visible bacterial growth.

Final concentrations of each compound were used as follows: 2000, 1500, 1000, 500 and 250 µg/mL for Teavigo, 1000, 750, 500 and 250 µg/mL for TF40, 0.1, 0.05, 0.025, 0.0125 and 0.00625 (%) for EDTA, 0.5, 0.25, 0.125, 0.0625 and 0.03125 (%) for Na acetate, 12, 8, 4, 2, 1, 0.5 (%) for Na citrate, and 25, 20, 15, 10, 5 and 1 (%) for ethanol.

2.2.4 Synergy testing of tea polyphenols with food additives using broth dilution method in test tube

To determine the synergistic interaction between tea polyphenols and food additives, the fractional inhibitory concentrations (FIC) index was calculated using formula:

$FIC = ([A]/MIC_A) + ([B]/MIC_B)$

[A] and [B] are the MIC of each compound when used together.

MIC_A and MIC_B are the MIC of each compound when used alone.

The interaction effects were determined as follows: $FIC \leq 0.5$, synergy; $0.5 < FIC \leq 0.75$, partial synergy; $0.75 < FIC \leq 1.0$, additive effect; $1.0 < FIC \leq 4.0$, indifference, and $4.0 < FIC$, antagonism (Berenbaum, 1981).

2.2.5 Time-kill assays

The time-kill assays were performed with a final concentration of bacterial inoculation about 2 x 10^5 CFU/mL in a final volume of 4 mL in a test tube. The samples and the control (a bacterial suspension in GA) were incubated at 37^{o}C. Viable cell count was determined at 0, 3, 6, 9 and 24 h incubation.

2.2.6 Viable cell count

Each sample was divided into 1 mL aliquots, and the sample was 10-times serially diluted with phosphate buffered saline (PBS). One hundred microliters of each dilution were spreaded onto tryptic soy agar (TSA, Becton, Dickinson & Co., Franklin Lakes, NJ, USA) plate. After cultivation of the plates for 48 h at 37^{o}C, the number of formed colonies was counted, and then the viable cell counts were calculated.

2.2.7 Statistical analysis

Viable cell counts were determined for three separate experiments and analyzed in Microsoft Excel 2010 (Microsoft, Seattle, WA, USA). Statistical significance of the viable counts was determined by Student's T-test. Multiple comparisons of the viable cell count between differently treated sample groups were performed using the Tukey-Kramer's multiple comparison post hoc test followed by the one-way ANOVA by Statcel 3 (Yanai, 2011), which is an add-in application in Microsoft Excel.

2.3 Results

2.3.1 Antibacterial effect of tea polyphenols and food additives alone

The antibacterial effect of the test compounds against *E. coli* O157:H7 was evaluated by MIC determinations. The MIC values of 2 tea polyphenol formulations and 5 food additives against *E. coli* O157:H7 are summarized in Table 2-1. For 2 tea polyphenol

formulations, TF40 showed the stronger inhibitory effect with MIC value about 2.7-fold lower compared to that of Teavigo. Among 5 food additives, EDTA exhibited the highest inhibitory effect against *E. coli* O157:H7 (MIC: 0.025%), whereas ethanol was the weakest inhibitor (MIC: 10%). The order of strong antibacterial activity was EDTA > Na acetate > NaCl > Na citrate > ethanol.

Table 2-1. Minimum inhibitory concentration (MIC) of tea polyphenols and food additives against *E. coli* O157:H7

Compounds	MIC
Teavigo	2000 mg/L
TF40	750 mg/L
Na citrate	8%
Na acetate	0.50%
EDTA	0.025%
NaCl	4%
Ethanol	10%

2.3.2 Combined antibacterial effect of tea polyphenols and food additives

To evaluate whether the antibacterial activities of tea polyphenol formulations can be improved by the combined use with food additives, FIC determinations were carried out. When 5 food additives used in this study were combined with 2 tea polyphenols formulations, synergistic effects on the inhibition of growth of *E. coli* O157:H7 were observed. The combined effects of food additives and Teavigo or TF40 against *E. coli* O157:H7 were shown in Table 2-2. In general, there were 2 synergistic effects that observed in the combination of Teavigo with EDTA or Na citrate, whereas the synergistic effect was only observed in the combination of TF40 with EDTA. The partial synergistic

effect was observed in the combination of Teavigo and ethanol, NaCl or Na acetate against *E. coli* O157:H7. However, the combination of TF40 with ethanol or Na citrate also showed the partial synergistic effect. There was no combined effect of TF40 and Na acetate or NaCl in this study.

Table 2-2. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) index of tea polyphenol formulations and food additives in combination against *E. coli* O157:H7

Tea polyphenol formulations	Food additives	MIC		FIC index	Interpretation
		Food additive (%)	Tea polyphenol formulation (mg/L)		
Teavigo	Ethanol	2.0	1000	0.70	Partial synergy
	Na acetate	0.25	500	0.75	Partial synergy
	Nacitrate	0.25	500	0.28	Synergy
	EDTA	0.0005	500	0.25	Synergy
	NaCl	1.0	1000	0.75	Partial synergy
TF40	Ethanol	4.0	500	0.73	Partial synergy
	Na acetate	0.25	500	1.17	Indifference
	Nacitrate	0.25	500	0.7	Partial synergy
	EDTA	0.001	250	0.37	Synergy
	NaCl	2.0	500	1.17	Indifference

The concentration of Teavigo required for inhibition of the growth of *E. coli* O157:H7 in the presence of food additives decreased as follows: 1/2 MIC in the presence of ethanol or NaCl, and 1/4 MIC in the presence of Na acetate or Na citrate or EDTA. The combination of TF40 with food additives, the concentration of TF40 required for inhibition

of the growth of *E. coli* O157:H7 decreased to 2/3 MIC in the presence of EDTA and 1/3 MIC in the presence of other 4 food additives (NaCl, ethanol, Na citrate or Na acetate).

2.3.3 Time kill assay of the combined use

To examine the mode of combined effect of tea polyphenol formulations and food additives in more detail, effects of tea polyphenol formulations and food additives on the growth of *E. coli* O157:H7 were determined.

For TF40, the combination of 500 mg/L TF40 and 0.01% EDTA reduced the viable cell counts of *E. coli* O157:H7 to below detection limit after 24 h incubation. The antibacterial activity of combinations of 500 mg/L TF40 and 0.001 % EDTA, 250 mg/L TF40 and 0.01 and 0.001% EDTA were more effective than that of single use (Fig. 2-1A). When Teavigo (500 and 1000 mg/L) was used in combination with EDTA (either at 0.01% or at 0.001%), the viable cell counts decreased to below lower detection limit after 24 h incubation (Fig. 2-1B). Teavigo and TF40 reduced EDTA concentration to inhibit the growth of *E. coli* O157:H7 to 1/50 and 1/25, respectively (Table 2-1 and 2-2).

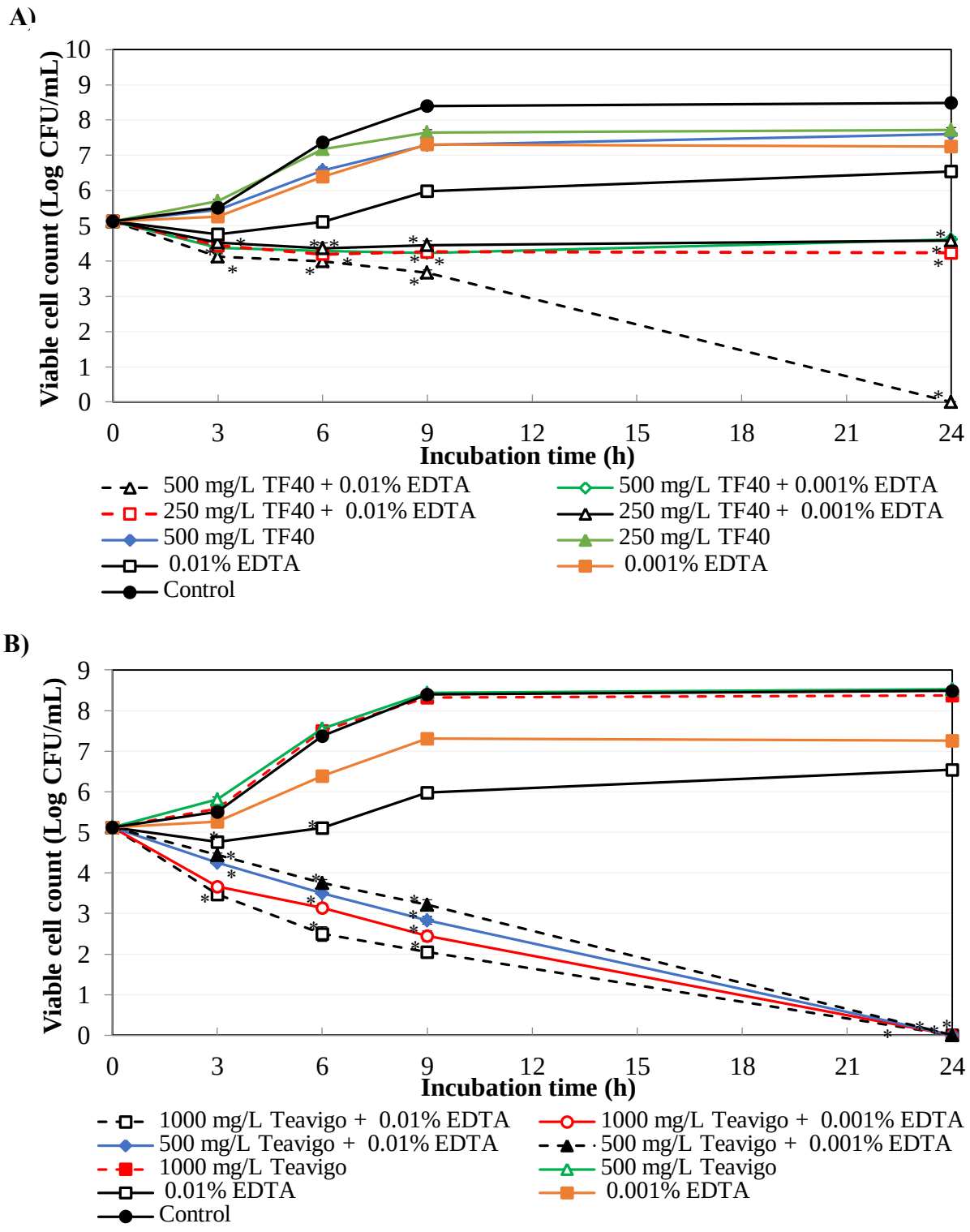


Figure 2-1. Combined effect of tea polyphenol formulations and EDTA on the growth of *E. coli* O157:H7. The mixtures of EDTA and TF40 (A) or Teavigo (B) were added to *E. coli* O157:H7 culture and incubated at 37°C. Viable cell count was determined at 0, 3, 6, 9 and 24 h incubation. Values are average ± SD for 3 separate experiments. *, $P < 0.05$.

Similar to the combination with EDTA, inhibitory effects of Na citrate were observed in both the combination with TF40 and Teavigo (Fig. 2-2 A and B). A rapid decrease of the viable cell counts was observed in the combination of 1000 mg/L Teavigo and 0.5% Na citrate. The viability of *E. coli* O157:H7 decreased to below lower detection limit after incubation for 9 h (Fig. 2-2B). After 24 h incubation, the combination of 1000 mg/L Teavigo and 0.25% Na citrate, 500 mg/L Teavigo and Na citrate (0.5% or 0.25%), and the combination of 500 mg/L TF40 and 0.5% Na citrate also reduced the viable cell counts to below lower detection limit. This combined effect was not observed in the combination of 500 mg/L TF40 and 0.25% Na citrate, TF40 250 mg/L and Na citrate (0.5% or 0.25%), but their combination effects against *E. coli* O157:H7 were stronger than that of single use (Fig. 2-2 A and B). There is a 16 and 32-fold reduction of Na citrate concentration for inhibition of the growth of *E. coli* O157:H7 in the combination with Teavigo and TF40, respectively (Table 2-1 and 2-2).

Figure 2-3 shows the time-kill curves of tea polyphenol formulations, NaCl and their combination against *E. coli* O157:H7. Although the combination of TF40 and NaCl did not show a significant combined effect, the combination of 500 mg/L TF40 and 3% NaCl showed bactericidal effects after 24 h incubation (Fig. 2-3A). When the concentration of TF40 was further decreased to 250 mg/L, bactericidal effect was not observed in the combination with 2% and 3% NaCl. However, the growth of *E. coli* O157:H7 was inhibited by the combination of 500 mg/L TF40 and 2% NaCl, and 250 mg/L TF40 and 3% NaCl. The combination of Teavigo (1000 mg/L or 500 mg/L) and NaCl (2% or 3%) reduced viable cell counts to below lower detection limit after 24 h incubation (Fig. 2-3B).

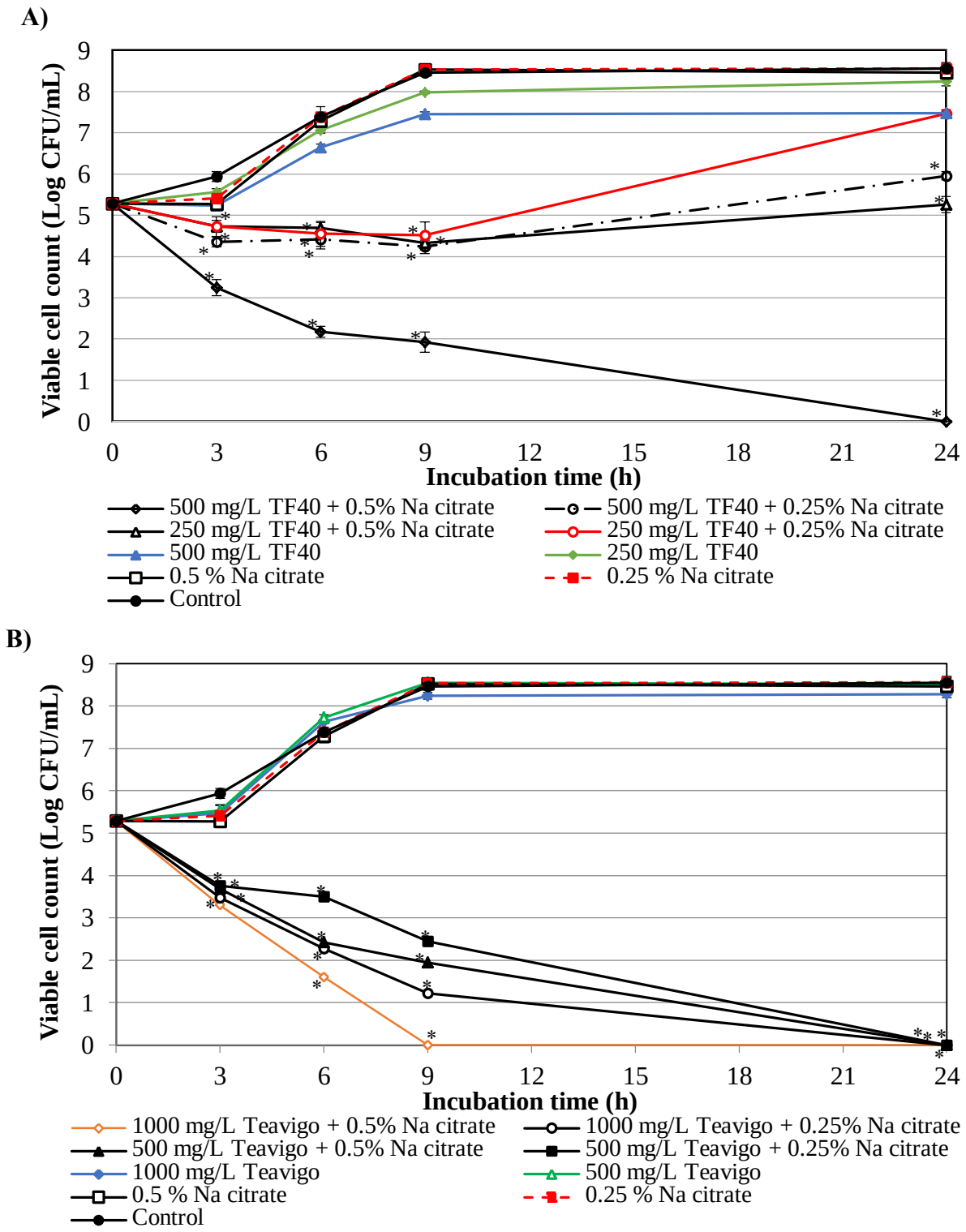


Figure 2-2. Combined effect of tea polyphenol formulations and Na citrate on the growth of *E. coli* O157:H7. The mixtures of Na citrate and TF40 (A) or Teavigo (B) were added to *E. coli* O157:H7 culture and incubated at 37°C. Viable cell count was determined at 0, 3, 6, 9 and 24 h incubation. Values are average ± SD for 3 separate experiments. *, $P < 0.05$.

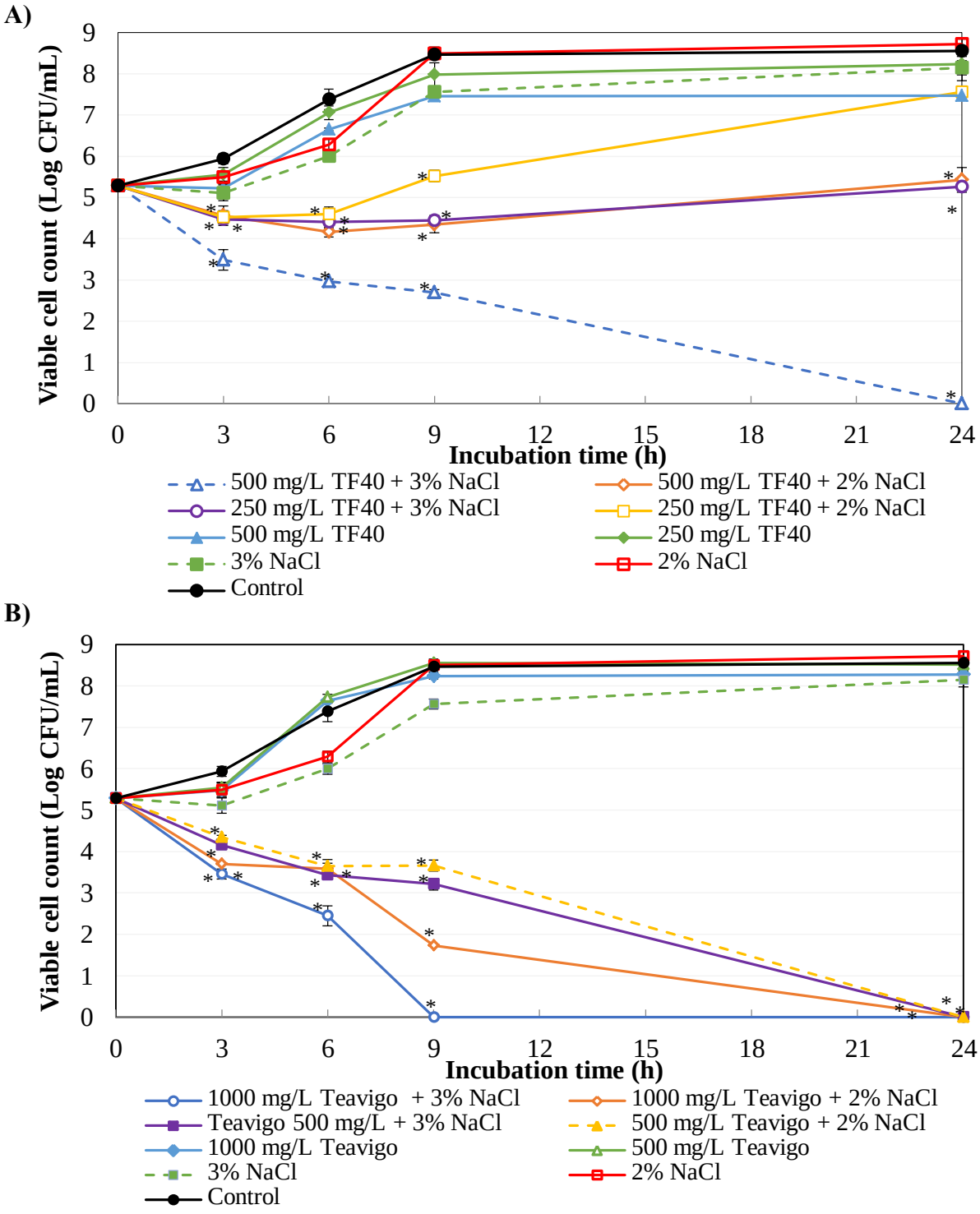


Figure 2-3. Combined effect of tea polyphenol formulations and NaCl on the growth of *E. coli* O157:H7. The mixtures of NaCl and TF40 (A) or Teavigo (B) were added to *E. coli* O157:H7 culture and incubated at 37°C. Viable cell count was determined at 0, 3, 6, 9 and 24 h incubation. Values are average ± SD for 3 separate experiments. *, $P < 0.05$.

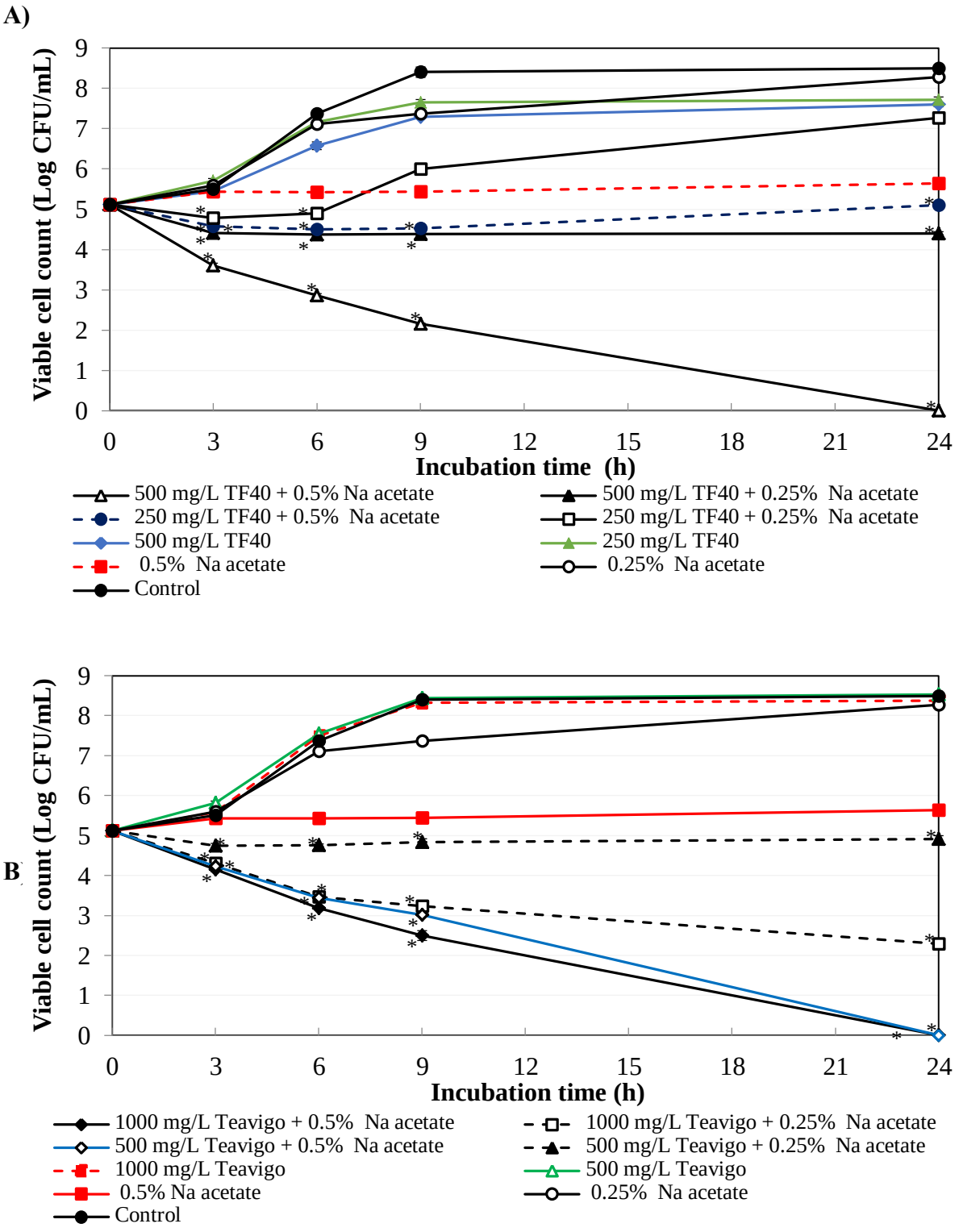


Figure 2-4. Combined effect of tea polyphenol formulations and Na acetate on the growth of *E. coli* O157:H7. The mixtures of Na acetate and TF40 (A) or Teavigo (B) were added to *E. coli* O157:H7 culture and incubated at 37°C. Viable cell count was determined at 0, 3, 6, 9 and 24 h incubation. Values are average ± SD for 3 separate experiments. *, $P < 0.05$.

Figure 2-4 shows the combined effect of the tea polyphenol formulations and Na acetate against *E. coli* O157:H7. The growth of *E. coli* O157:H7 was observed in the combination of 250 mg/L TF40 and 0.25% Na acetate, while the combination of 250 mg/L TF40 and 0.5% Na acetate, and 500 mg/L TF40 and 0.25% Na acetate slightly inhibited the growth of *E. coli* O157:H7 (Fig. 2-4A). In the combination of 500 mg/L TF40 and 0.5% Na acetate, the viable cell counts were decreased to below lower detection limit after 24 h incubation (Fig. 2-4A). However, the combined antibacterial effects of TF40 and Na acetate were not significantly strong compared with those of the single use (Table 2-2). The viable cell counts slightly decreased in combination of 1000 mg/L Teavigo and 0.25% Na acetate. Teavigo inhibited the growth of *E. coli* O157:H7 and significantly reduced the viable counts to below detection limit at both 1000 mg/L and 500 mg/L in the combination with 0.5% Na acetate after 24 h incubation (Fig. 2-4B).

Combined effect of the polyphenol formulations and ethanol on the growth of *E. coli* O157:H7 is shown in Figure 2-5. The growth of *E. coli* O157:H7 was observed in the combination of 250 mg/L TF40 and 4% ethanol. The combinations of 500 mg/L TF40 and 4% ethanol, and 250 mg/L TF40 and 6% ethanol slightly inhibited the growth of *E. coli* O157:H7. After 24 h incubation, the viability of *E. coli* O157:H7 was decreased to below lower detection limit in the combination of 500 mg/L TF40 and 6% ethanol (Fig. 2-5A). The combinations of 1000 mg/L Teavigo and ethanol at both 4% and 6%, and 500 mg/L Teavigo and 6% ethanol also reduced the viable cell count to below lower detection limit after 24 h incubation (Fig. 2-5B). The growth of *E. coli* O157:H7 was inhibited by the combination of 500 mg/L Teavigo and 4% ethanol.

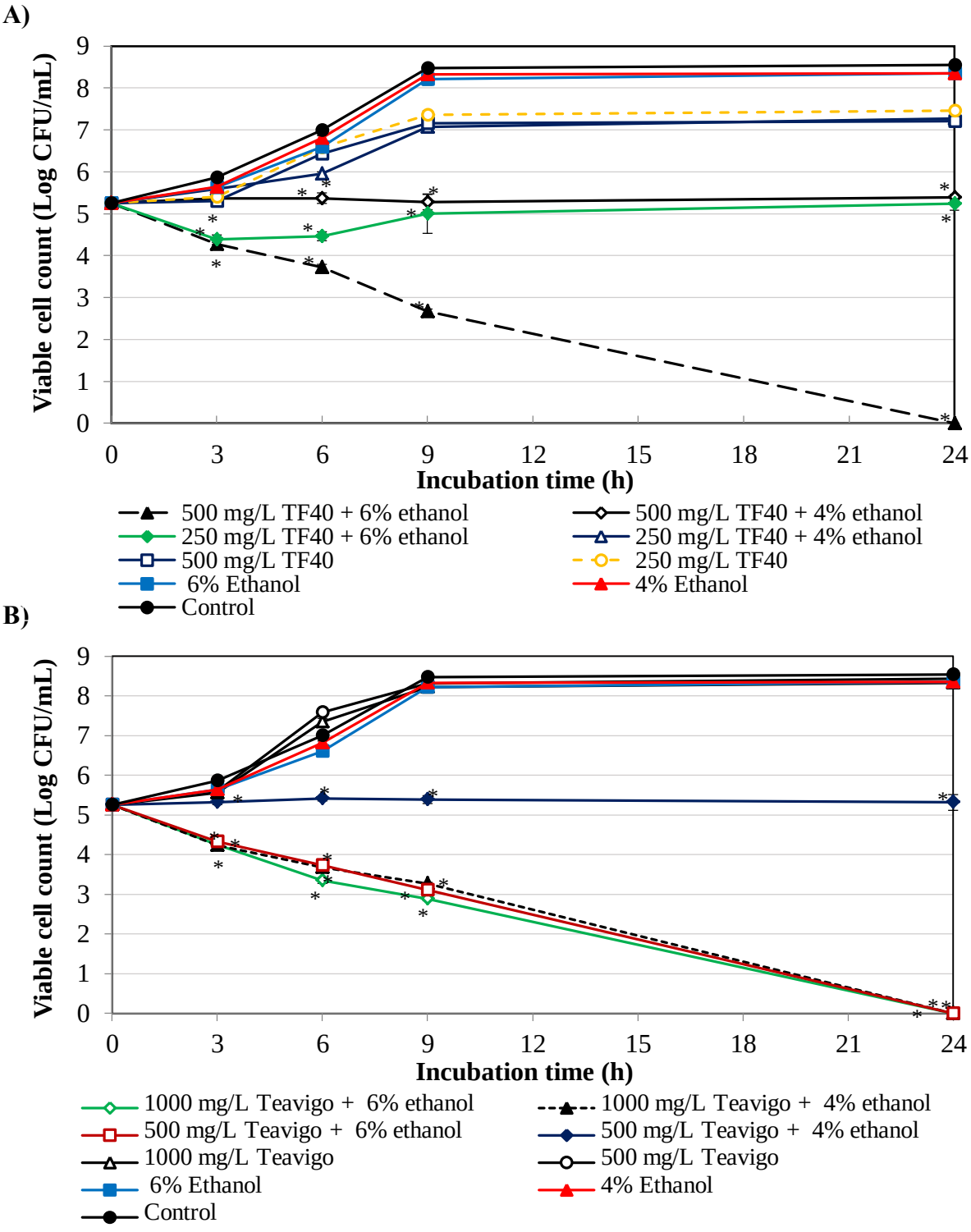


Figure 2-5. Combined effect of tea polyphenol formulations and ethanol on the growth of *E. coli* O157:H7. The mixtures of ethanol and TF40 (A) or Teavigo (B) were added to *E. coli* O157:H7 culture and incubated at 37°C. Viable cell count was determined at 0, 3, 6, 9 and 24 h incubation. Values are average ± SD for 3 separate experiments. *, $P < 0.05$.

2.4 Discussion

Tea polyphenols, including EGCg and theaflavin, have been reported to inhibit the growth of both Gram-positive and Gram-negative bacteria (Bansal et al., 2013; Reygaert, 2014). Both EGCg and theaflavin inhibit the growth of *Fusobacterium nucleatum* by their iron-chelating activities and damage the bacterial cell membrane integrity (Lagha et al., 2017). Moreover, EGCg and theaflavin have also been reported to inhibit the growth of *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis* and *Prevotella intermedia* (Bedran et al., 2015; Fournier-Larente et al., 2016). Interestingly, the effect of two conventional antibiotics used in periodontal therapy, including metronidazole, and tetracycline, were enhanced by EGCg and theaflavin (Bedran et al., 2015; Fournier-Larente et al., 2016). EGCg and theaflavin were thought to increase permeability of bacterial cell membrane (Lagha et al., 2017; Reygaert, 2018). The results in this study showed the combination of 2 tea polyphenol products including tea polyphenol formulations showed stronger antibacterial effects against *E. coli* O157:H7 in combination with 5 food additives than those of the single use (Table 2-1 and 2-2). The MIC values of these compounds in combination were lower than that of the compounds alone. The results of time-kill assay confirmed the combined effects of tea polyphenol formulations and food additives against *E. coli* O157:H7.

Many food additives have antibacterial activities against various foodborne pathogens. Increased antibacterial effects by the combination of two or more food additives have been demonstrated. EDTA showed synergistic effect in combination with other compounds such as nisin (Delves-Broughton, 1993; Field et al., 2017), and antimicrobial peptide AA230 (Umerska et al., 2018). It has been reported that the effect of nisin against Gram-negative bacteria was enhanced in the presence of EDTA (Delves-Broughton, 1993). The results in our study showed that a significant reduction of MIC (25-fold and a 50-fold) of EDTA obtained when used in the combination with TF40 and

Teavigo, respectively. Importantly, the synergistic and partial synergistic effects were also observed in combination with EDTA in Teavigo and TF40. Marvin et al. (1989) reported that EDTA induced damage in outer membrane of *E. coli*. EDTA treatment caused rapid release of lipopolysaccharide from cell wall layer of *E. coli* also resulted in an increase of permeability of the membrane (Leive, 1968). The activity of antibiotics against *E. coli* O157:H7 was improved by the combination with EDTA (Leive, 1968; Weiser, 1968). It has been also reported that the synergistic effect between EDTA and some antibiotics are due to the changes in the permeability of bacterial cell membrane by EDTA (Leive, 1968; Weiser, 1968). The synergistic effect of nisin and EDTA against *Pseudomonas fluorescens* is induced by the action of EDTA removing Mg^{2+} and Ca^{2+} ion from the cell wall of Gram-negative bacteria, leading to the release of lipoprotein and phospholipid, which in turn allows possibly increase in the sensitivity of bacterial cell membrane against nisin (Delves-Broughton, 1993). In addition, EDTA evidently causes the loss of substantial amounts of lipopolysaccharide of *E. coli* which increase the sensitivity of the cells against the action of many antibacterial substances (Hancock, 1984; Nikaido & Vaara, 1985; Tubbing et al., 1994). It seems that the damage in the outer membrane of Gram-negative bacteria by EDTA improved antibacterial activity of Teavigo and TF40 against *E. coli* O157:H7.

The antibacterial effects of sodium salts of organic acids, including Na citrate and Na acetate, have been indicated (Figrue 2-2 and 2-4). The previous studies showed that Na citrate and Na acetate showed antibacterial activity against some foodborne pathogens such as *E. coli, Listeria monocytogenes, Clostridium botulinum, Staphylococcus aureus* and *Yersinia enterocolitica* (Anders et al., 1989; Qvist et al., 1994; Lee et al., 2002; McWilliam & Stewart, 2002). At the concentrations from 0.4 to 51.2 mg/mL, Na citrate inhibited the growth of many bacteria such as *Prevotella histicola, Prevotella melaninogenica, Prevotella atypica, Prevotella dispar, Prevotella parvula* (Watanabe et al., 2011). Na citrate and Na acetate inhibited the growth of various bacteria by both inhibiting

metabolism and decreasing pH (Jay, 2000). In the range of pH from 5.0 to 8.0, Na citrate did not inhibit the growth of *Streptococcus pneumoniae* (Nagaoka et al., 2010). The antibacterial activity of Na acetate was shown at low pH (5.0-6.0) (Nerbrink et al., 1999). Ibrahim Sallam (2007) reported that the antibacterial activity of Na acetate is stronger than that of Na citrate at acidic pH. It has been reported that Na citrate inhibited the growth of *Streptococcus pneumoniae* by disrupting the outer membrane of the bacterium to disturb cation uptake (Nagaoka et al., 2010; Watanabe et al., 2011). It was due to the chelating activity of citrate. Na acetate showed partial synergistic effect in combination with Teavigo, while the synergistic effect was not observed in the combination with TF40. In contrast, synergistic effects were observed in both TF40 and Teavigo in combination with Na citrate. Especially, there is a significant reduction of MIC from 16 to 32-fold in Na citrate in combination with tea polyphenol formulations. In this study, combined effects were determined in GA medium at pH 6.5. It seems that antibacterial effects of organic acid were not observed in Na acetate because of the pH of the medium. On the other hand, Na citrate acted as chelating agent like EDTA at the pH and showed combined effects with Teavigo and TF40.

In this study, the combination of NaCl and Teavigo or TF40 against *E. coli* O157:H7 was more effective than single uses and their combinations showed the partial synergistic effect (Fig. 2-3). NaCl, a common food additive, is believed to play a vital antibacterial role in many foods. The antibacterial activity of NaCl is due to its ability to decrease intracellular water activity of bacteria leading to poor growth (Jamshidi et al., 2008). Measures reported that NaCl can only enter juried bacterial cells (Measures, 1975). Many studies have demonstrated that tea polyphenols including EGCg and theaflavin inhibit the growth of many bacteria by damaging bacterial cell membrane (Lagha et al., 2017, Bedran et al., 2015, Bansal *et al*., 2013). Damage in the cell membrane caused by

Teavigo and TF40 may support NaCl to enter easily bacterial cell of *E. coli* O157:H7, leading to growth inhibition.

Ethanol has been used as antibacterial agents in food industry. However, some people are sensitive to ethanol at high concentration, while 20% ethanol (v/v) had no adverse effect on human skin (Lachenmeier, 2008). It has been reported that ethanol increases permeability of bacterial cell membrane, and destabilizes the structure of nucleic acids and proteins, and increase solubility of the hydrophobic compounds. As a result, bacterial cells reduced metabolism and subsequently growth (Ingram & Buttke, 1985; Huffer et al., 2011). Ethanol has also shown to inhibit the uptake of glucose (Leao & Van Uden, 1982; 1984) and caused the leakage of cellular constituents of *E. coli* O157:H7 and *Salmonella* Enteritidis (Phongphakdee & Nitisinprasert, 2015). The combination of ethanol and low pH or nisin was found to inhibit the growth of *E. coli* O157:H7 (Jordan et al., 1999; Phongphakdee & Nitisinprasert, 2015). It has been reported that ethanol could remove receptors on the membrane, leading to increase in the nisin sensitivity to cell membrane (Phongphakdee & Nitisinprasert, 2015). According to Tsuchiya (2001), polyphenols showed their biological activity through their interactions with bacterial cell membrane. The combination of ethanol and tea polyphenol formulations showed stronger antibacterial effect on *E. coli* O157:H7 than single uses (Fig. 2-5). It seems that the damage in outer membrane caused by ethanol facilitated the antibacterial action of Teavigo and TF40 against *E. coli* O157:H7.

2.5 Summary

The combined effects of 2 tea polyphenol formulations and 5 food additives on the growth of *E. coli* O157:H7 were evaluated by MIC and FIC index. The 2 tea polyphenol formulations showed stronger antibacterial effects against *E. coli* O157:H7 in combination with 5 food additives than those of the single use. The combination of Teavigo with EDTA or

Na citrate, TF40 with EDTA showed the synergistic effect. The partial synergistic effect was observed in the combination of Teavigo with ethanol, NaCl or Na acetate, and the combination of TF40 with ethanol or Na citrate. There was no combined effect of TF40 and Na acetate or NaCl in this study. The results of time-kill assay confirmed the combined effects of the tea polyphenol formulations and food additives against *E. coli* O157:H7. It is possible to take advantage of the different antibacterial activity of these compounds and the application of combined use of plant polyphenols and additives in food preservation is feasible.

Chapter 3. Combined effects of epigallocatechin gallate and heat treatment against enterohaemorrhagic *Escherichia coli*

3.1 Introduction

Epigallocatechin gallate (EGCg), one of the green-tea catechins, has been reported to inhibit the growth of both Gram-positive and Gram-negative bacteria, including *Escherichia coli*. The antimicrobial activity of EGCg on Gram-negative bacteria was lower than that on Gram-positive ones (Yoda et al., 2004). Except for antibacterial activity, beneficial effects of EGCg on human are derived from antioxidant (Du et al., 2012), anticancer (Fujiki et al., 2018) and anti-inflammatory activities. On the antibacterial mechanism for EGCg, it has been reported that EGCg inhibits the major functions of the porin proteins, leading to the growth inhibition of *E. coli* (Nakayama et al., 2013). Furthermore, EGCg has also been reported to cause the disruption of the cell membrane and the inhibition of the activities of some enzymes (Sirk et al., 2008, 2009).

Heat treatment is widely used to reduce the risk of foodborne illness in the food industry. Its basic purpose is to reduce or destroy bacteria, both pathogenic and spoilage, to make the food safe and having reasonable shelf-life. However, heat treatment with excessively long times and high temperatures for the inactivation of bacteria might induce the loss of food quality such as the change of flavors, color and degradation of nutritional ingredient. Mild heat treatment is widely applied to minimize changes in food quality. The combination of mild heat treatment and other antimicrobial treatments have been reported to show a synergistic effect. Combined ultraviolet light irradiation and mild heating at 65^{o}C showed synergistic effect on the inhibition of growth of *E. coli* O157:H7 and *Salmonella* Typhimurium on red pepper powder (Cheon et al., 2015). The combined use of lauryl arginate ethyl and mild heating demonstrated synergistic effects on inactivation of *E. coli* O157:H7 and *Listeria innocua* (Yang et al., 2019).

Heat-induced inactivation and injury of bacteria are associated with damage of membrane, leakage of intracellular compounds, and oxidative stress etc. (Semanchek & Golden, 1998; Lee & Kaletunç, 2002; Ebrahimi et al., 2018). Bacterial cells have catalase and superoxide dismutase activities as a mechanism to counter heat stress (Mackey & Seymour, 1987). Heat resistance of *E. coli* and *Lactobacillus plantarum* were attributed to stability of the ribosomal subunit against by heating (Lee & Kaletunç, 2002). The damage of the outer membrane of *E. coli* by heat treatment at 55^{o}C was enhanced by the presence of various substances such as Tris-hydrochloride buffer after the heat treatment (Tsuchido et al., 1985).

It has been recognized that not all bacterial cells are destroyed after heat treatment, but a large number of surviving bacterial cells may be injured (Iandolo & Ordal, 1966). Heat-injured cells cannot recover under the lack of active oxygen scavenger in heat-treated cells (Murano & Pierson, 1993). However, the recovery of heat-injured cells was found in some strains of *Salmonella* after heat treatment at 55^{o}C for 30 min (Dabbah et al., 1969). It has been reported that heat-injured cells of *E. coli* can recover under anaerobic conditions (Murano & Pierson, 1993). Wu reported that for recovery of heat-injured cell, the impaired function of membrane permeability and metabolic activities are important (Wu, 2008). However, some heat-injured cells, which still have pathogenicity may recover and grow after heat treatment, increase the risk of food poisoning (Johnson & Busta, 1984). According to Rowe and Kirk, expression of three virulence factors of *E. coli* O157:H7, including Shiga toxins 1 and 2, and the attaching and effacing gene (*eae)* product, were unaffected by starvation and heat stress (Rowe & Kirk, 2000). The virulence factors that remain in the normal intact cells were even less than those in the recovery of heat-injured cells (Hassani et al., 2009).

To date, no research has demonstrated the combined effect of EGCg and mild heat treatment against *E. coli*. Thus, this study was performed to test whether the presence of

EGCg in heating medium promotes the heat injury in *E. coli*. The viability, membrane potential and leakage of intracellular compounds of *E. coli* after heat treatment in the presence of EGCg were determined.

3.2 Materials and methods

3.2.1 Bacterial strains and culture condition

Escherichia coli CP4 strain, which possesses high antimicrobial resistance, was used in this study. This strain was isolated from pork in the laboratory of Food Hygienic Chemistry, Kyushu University and resistant to piperacillin, cefazolin, minocycline, sulbactam:ampicillin and kanamycin. To obtain cells in the stationary phase of growth, the bacterium was cultured overnight in 5 mL of Tryptic Soy Broth (TSB) at 37^{o}C with shaking at 130 rpm. This culture was then diluted with sterilized water and adjusted to a final OD_{600} of 0.6 (about 10^9 CFU/mL). The bacterial cells were harvested by centrifugation (6,000 x *g*, 5 min, at 25^{o}C), and suspended in sterilized water to prepare the cell suspension with 1.0 x 10^7 CFU/mL. This bacterial suspension was used in the following experiments.

3.2.2 Heat treatment and recovery

To each test tube, 4.8 mL of 50% Luria broth (LB) (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), and 100 μL of Teavigo (containing 95% EGCg) solution, were added into this culture, 100 μL of bacterial suspension were inoculated to attain a final concentration of 2 x 10^5 CFU/mL. The mixtures were incubated in a 25^{o}C water bath for 10 min, and then heating in a water bath at 55^{o}C for 5, 10, 15, 20 min, with shaking at 80 rpm. After incubation in a water bath for 10 min at 25^{o}C, the mixtures were used for the determination of viable counts by conventional plating method using Tryptic soy agar

(TSA) and Deoxycholate Hydrogen Sulfide-lactose (DHL) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan)

To prepare Teavigo solution, Teavigo powder was dissolved in sterilized water with concentration 50,000 mg/L. This solution was sterilized by filtration through a Millex-GP filter (pore size: 0.22 μm, Merck Millipore, Billerica, MA, USA) and then diluted with sterilized water. The final concentrations of Teavigo were 1000, 500, 250 and 125 mg/L.

To determine the recovery of *E. coli* after heat treatment, these mixtures were incubated at 37^{o}C for various periods, and then viable cell counts were determined. The viable cell counts determined by the plating method using TSA and DHL agar were defined as total of the intact and injured but recoverable cells, and the intact cells of *E. coli*, respectively.

3.2.3 Determination of viable counts

From each test solution, 1 mL aliquots were withdrawn and 10-fold serially diluted with phosphate buffered saline (PBS). One hundred microliters of each dilution were spreaded onto TSA and DHL agar. The plates were cultured at 37^{o}C for 48 h, the number of colonies formed was counted, and then the viable cell counts were calculated. The viable cell count determined on TSA was defined as the sum of intact and injured cells, and the viable cell count on DHL agar was defined as intact cells only. The difference between the viable counts of *E. coli* determined by the plating method using TSA and DHL were defined as the number of the injured but recoverable cells.

3.2.4 Measurement of protein leakage

The leakage of protein was determined using Bradford method (Bradford, 1976). Bacterial cells of *E. coli* were heat-treated at 55^{o}C for difference times in the presence and absence of Teavigo. After the heat treatment, the suspensions were incubated at 37^{o}C for 1 and 4 h. Two mL of bacterial suspension were centrifuged at 25^{o}C, 10,000 x *g* for 10 min. and the supernatant was recovered. To 200 μL of the supernatant, 800 μL of Coomassie

brilliant blue solution were added and kept at room temperature for 5 min in the dark. The absorbance was measured at a 590 nm to calculate the concentration of protein. Bovine serum albumin (BSA) was used as a standard.

3.2.5 Measurement of nucleic acid leakage

The leakage of nucleic acid was directly measured absorbance at 260 nm using a UV spectrophotometer as previously described (Chang et al., 2009). Bacterial cells of *E. coli* were heat-treated at 55^{o}C for difference periods in the presence and absence of Teavigo. After incubation at 37^{o}C for 4 h, 4 mL of bacterial suspension was centrifuged at 25^{o}C, 10,000 x *g* for 10 min. The supernatant was recovered and filtered twice using a Millex-GP 0.22 μm filter to remove any remaining planktonic cells. Then, 1 μL of this supernatant was used to determine the leakage of nucleic acid by measuring the absorbance at 260 nm, with a UV spectrometer (Nanodrop ND-1000, Thermo Fisher Scientific, USA).

3.2.6 Statistical analysis

Viable cell counts were determined for three separate experiments and analyzed in Microsoft Excel 2010 (Microsoft, Seattle, WA, USA). Significant differences between the viable counts on TSA and DHL agar were determined by Student's T-test. Multiple comparisons of viable cell count on TSA or DHL agar between differently treated sample groups were performed using the Tukey-Kramer's multiple comparison post hoc test followed by the one-way ANOVA by Statcel 3 (Yanai, 2011), which is an add-in application in Microsoft Excel.

3.3 Results

3.3.1 Combined effect of Teavigo and heat treatment on the growth of E. coli

Figure 3-1 shows the viability of *E. coli* after heat treatment in the presence of Teavigo. The viable counts of *E. coli* decreased with increase in both the concentration of

Teavigo and heating time. The viable counts by both TSA and DHL agar of *E. coli* heated in the presence of Teavigo were lower than those in the absence of Teavigo. The viable count of *E. coli* significantly decreased in the presence of Teavigo after heat treatment at 55°C for 10, 15, and 20 min. The experiments were carried out to determine the recovery of injured bacterial cells.

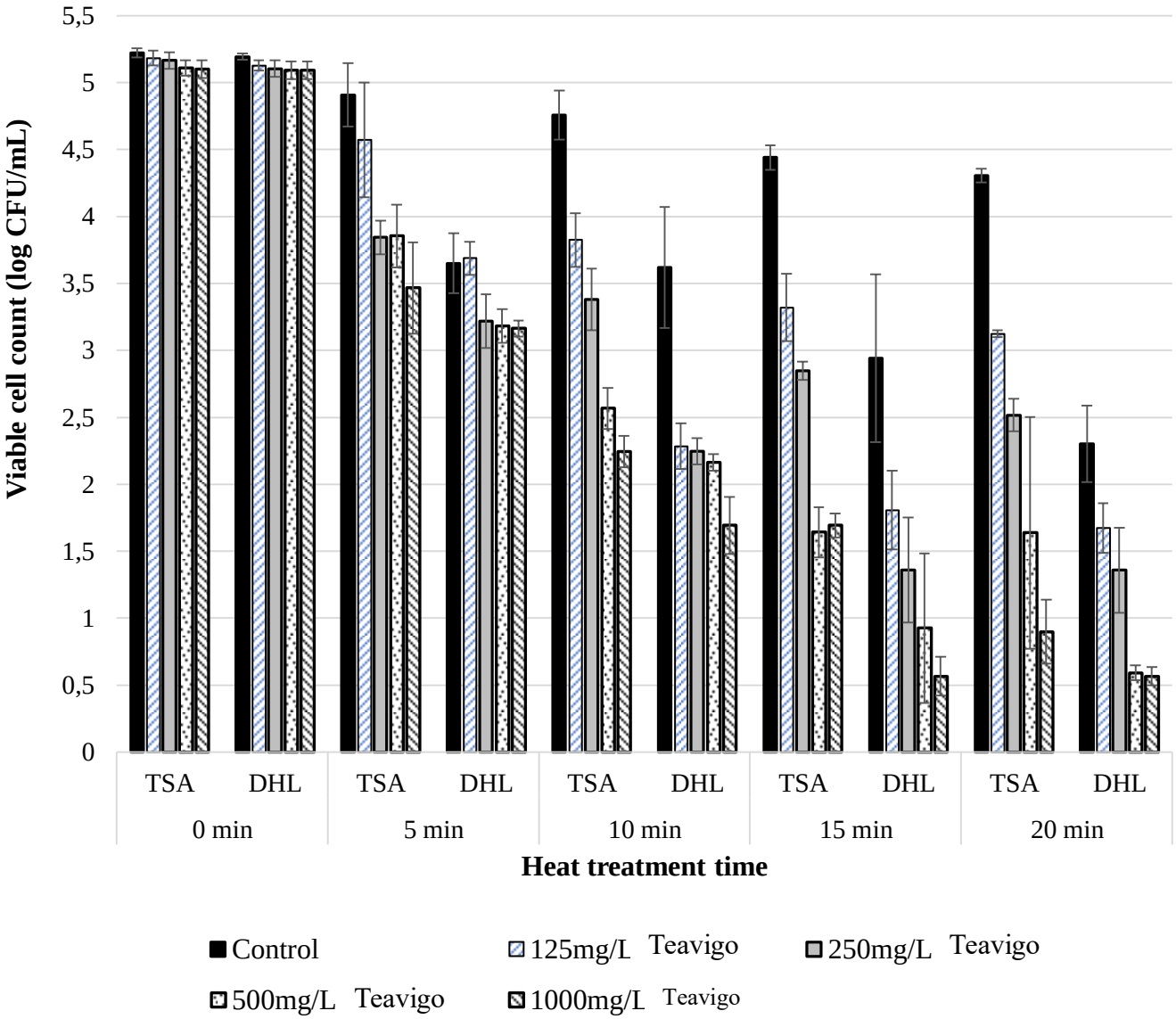


Figure 3-1. Effect of Teavigo on viability of *E. coli* after the heat treatment. *E. coli* CP4 in 50% LB medium was heat-treated at 55°C for different time in the absence and presence of Teavigo. After the heat treatment, viable cells were determined by the plating method using TSA and DHL agar. Values are average ± SD of 3 separate experiments.

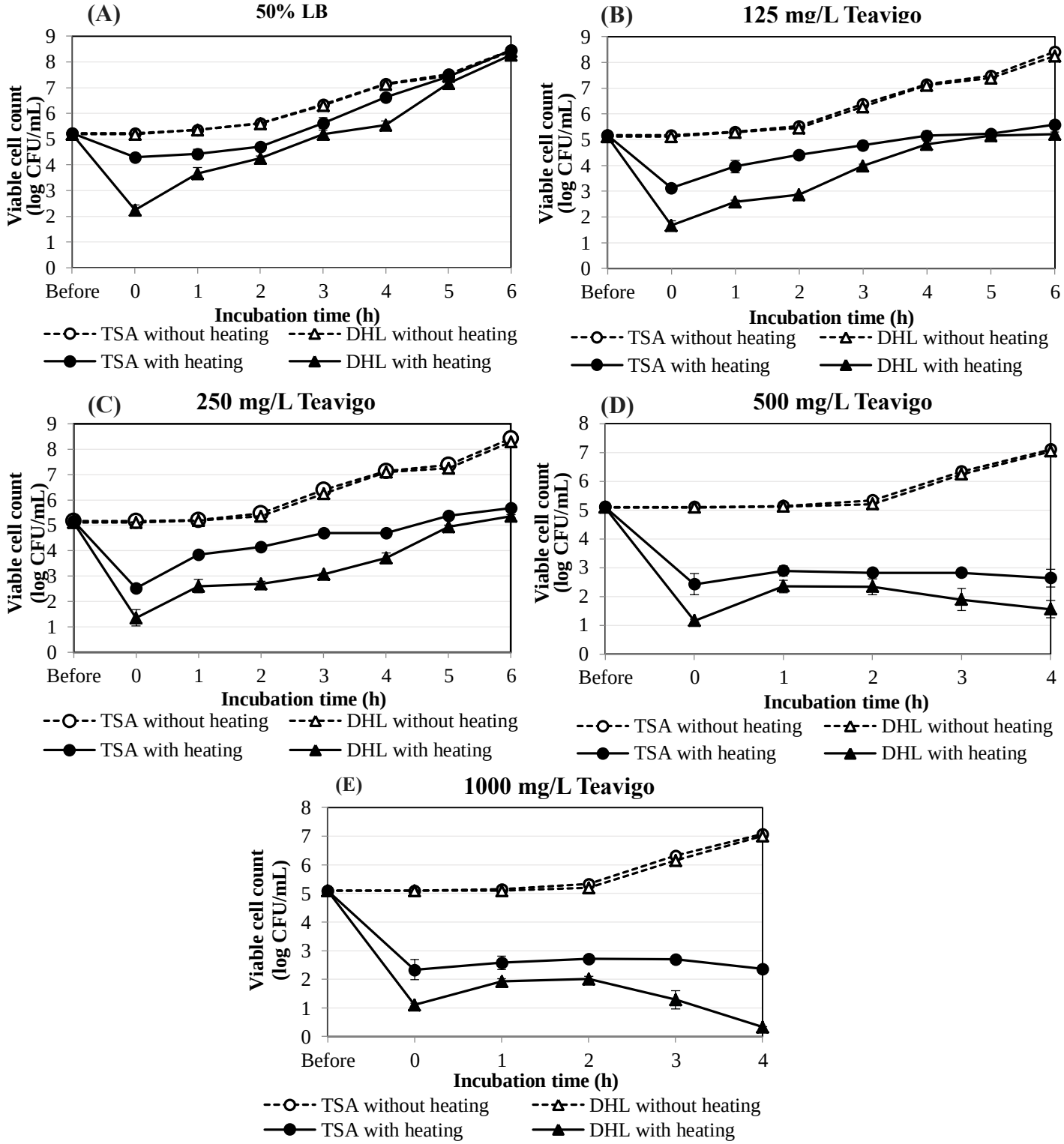


Figure 3-2. Effects of Teavigo on viability of *E. coli* after heat treatment and during recovery. *E. coli* CP4 in 50% LB medium was not heat-treated and heat-treated at 55^{o}C for 20 min in the absence of Teavigo (A), and the presence of various concentration of Teavigo at 125 mg/L (B), 250 mg/L (C), 500 mg/L (D) and 1000 mg/L (E). After heat treatment, *E. coli* was incubated at 37^{o}C for different periods. The viability of the cells was determined before by the plating method using TSA and DHL agar. Values are average ± SD of 3 separate experiments.

Figure 3-2 shows the effects of Teavigo on the viability of *E. coli* after heat treatment and subsequent recovery in the same media. In the absence of Teavigo, the viability of *E. coli* determined on both TSA and DHL agar increased during incubation and the viable count determined on DHL agar reached a similar level to that on TSA after 2 h incubation (Fig. 3-2A). In the presence of Teavigo at 125 and 250 mg/L, the viable counts after heat treatment also increased during incubation (Fig. 3-2 B and C). In the presence of Teavigo at higher concentrations (500 and 1000 mg/L), the viable counts largely decreased after heat treatment. The counts on both TSA and DHL agar slightly increased at 1 h incubation then decreased, indicating injured *E. coli* cells did not recover and the number of damaged bacteria increased leading to the cell death in the presence of Teavigo at 500 and 1000 mg/L (Fig. 3-2 D and E).

3.3.2 Combined effects of Teavigo and heat treatment on protein leakage from E. coli

The amount of protein released from *E. coli* cells after heat treatment in the absence and the presence of Teavigo was determined by Bradford assay. As shown in Figure 3-3, the amount of leaked protein was compared among control cells, and heat-treated cells in the absence and the presence of Teavigo. Protein leakage was observed in the presence of Teavigo at 500 and 1000 mg/L. Both in the presence and absence of Teavigo, the protein leakage from *E. coli* cells during heat treatment increased with increase of time of heat treatment (Fig. 3-3 A and B). The protein leakage from heat-treated *E. coli* cells was significantly increased by the presence of Teavigo. Heat treatment at 55^{o}C for 15 and 20 min in the presence of Teavigo, the protein leakage from heat-treated *E. coli* cells was significantly higher than that from cells without Teavigo. During incubation from 1 to 4 h, protein leakage from heat-treated cells at 55^{o}C for 20 min increased from 0.563 to 4.6842 mg/mL in the absence of Teavigo, and from 3.159 to 13.1941 mg/mL in the presence of

500 mg/L Teavigo, and from 4.199 to 14.6411 mg/mL in the presence of 1000 mg/L Teavigo, respectively (Fig. 3-3 A and B).

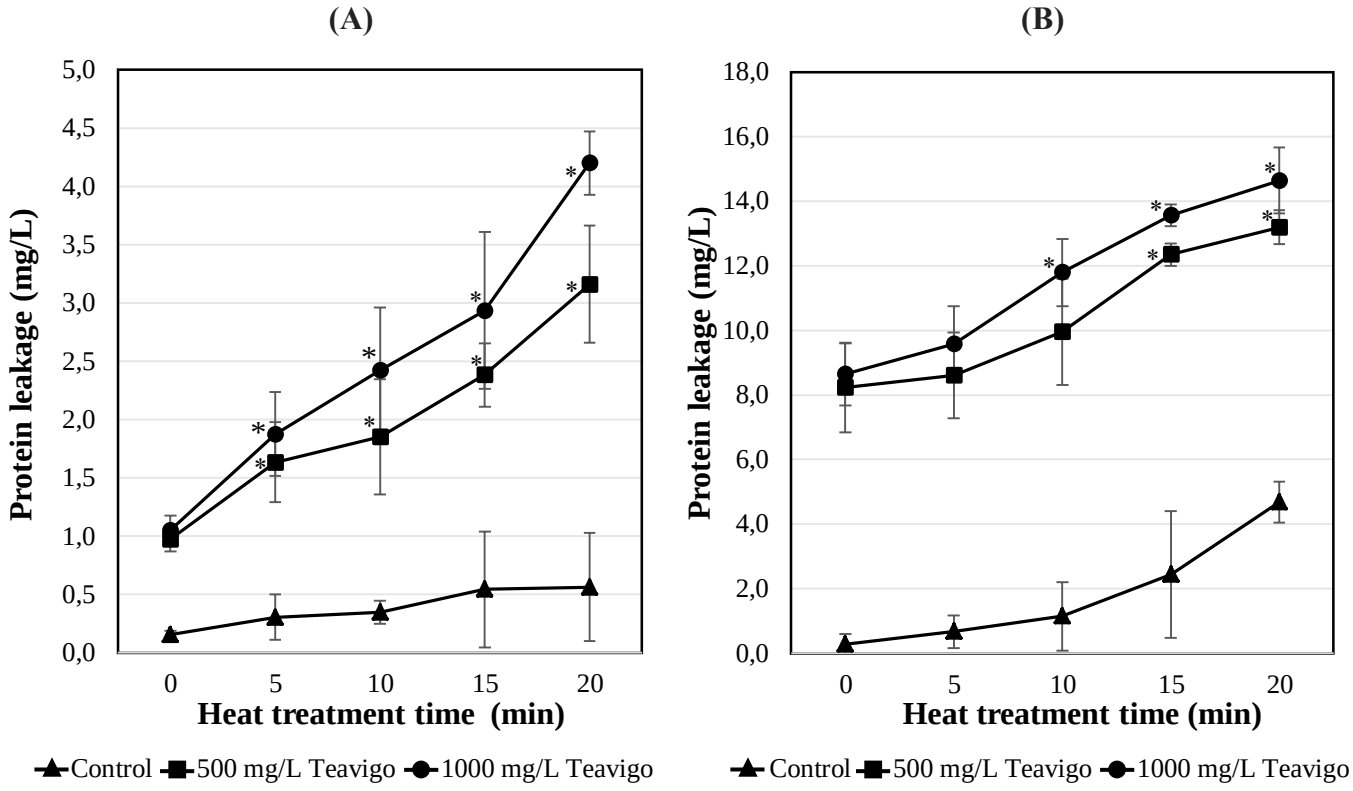


Figure 3-3. Effects of Teavigo on protein leakage from *E. coli* by heat treatment. *E. coli* CP4 was heat-treated at 55°C in the absence (▲), and presence of Teavigo at 500 mg/L (■) and 1000 mg/L (●). After incubation at 37°C for 1 h (A), and 4 h (B) of the heat-treated cells, protein in the supernatant was determined by Bradford method. Values are average ± SD of 3 separate experiments. *, $P < 0.05$.

3.3.3 Combined effects of Teavigo and heat treatment on nucleic acid leakage from E. coli

Figure 3-4 shows the leakage of nucleic acid from *E. coli* cells after heat treatment in the absence and presence of Teavigo. Nucleic acid leakage increased both in the presence and absence of Teavigo with an increase in the time of heat treatment. However, without Teavigo, there was no significant increase in the amount of nucleic acid leaked from the cells with increasing heating time. However, in the presence of Teavigo, nucleic acid leakage increased with increasing Teavigo concentration and increasing time of heat treatment.

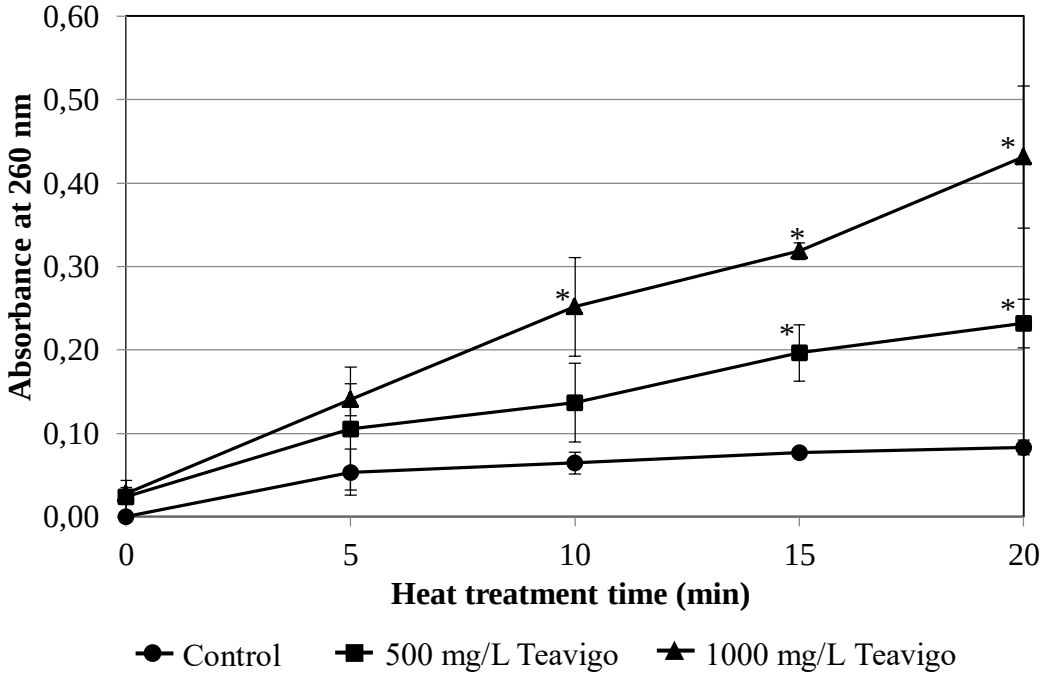


Figure 3-4. Effects of Teavigo on nucleic acid leakage from *E. coli* by heat treatment. *E. coli* CP4 was heat-treated at 55°C in the absence (●), and presence of Teavigo at 500 mg/L (■) and 1000 mg/L (●). After incubation at 37°C for 4 h of the heat-treated cells, nucleic acids in the supernatant was determined by measuring absorbance at 260 nm. Values are average ± SD of 3 separate experiments. *, $P < 0.05$.

3.4 Discussion

Heat treatment is widely used in food industry for decontamination of spoilage and pathogenic bacteria because of its capacity to inactivate various bacteria. However, not all bacterial cells are killed after heat treatment, but a large number of contaminating bacterial cells in food may be injured but recoverable (Iandolo & Ordal, 1966). In this study, the viable count of *E. coli* immediately after heat treatment decreased with an increase in both the concentration of Teavigo and heating time (Fig. 3-1). Thus, the presence of Teavigo during heating increased the effect of heat treatment against the growth of *E. coli*. Bacterial cells are injured as a result of the exposure to physical or chemical treatment. Heat-induced injury of *E. coli* cells has been reported by Russell and Harries (Russell & Harries, 1967).

In addition, the state of heat injury of bacterial cells is considered to be temporary because these cells are able to repair their injuries in the appropriate environmental conditions, and therefore they can grow again (Mackey, 2000). Depending on the degree of cell injury, the injury may be reversible or irreversible. When the extent of cell injury exceeds the cell's capability to repair by itself, the cells will die (Cobb et al., 1996). In this study, there were the recovery of injured cells after heating both in the absence and the presence of Teavigo at 125 and 250 mg/L. Thus, the presence of low concentration of Teavigo was not enough to inhibit the recovery of heat-injured *E. coli* cells (Fig. 3-2 A, B, and C). However, at higher concentration of Teavigo (500 and 1000 mg/L), the viable counts on DHL increased for 1 h, but there after decreased gradually (Fig. 3-2 D and E). The decrease in the viable counts might be due to the unrecoverable deep heat-damage on the structure or metabolism of the cells in the presence of EGCg at high concentration.

According to Wesche et al. (2009), heat treatment can lead to the loss of cell functions and the damage of cell structures. Heat treatment at 55^{o}C in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and Tris- HCl magnesium sulfate at pH 8, the permeability barrier function of the outer membrane of *E. coli* cells was partially disrupted (Tsuchido et al., 1985). The cytoplasmic membrane of *Lactobacillus bulgaricus* was injured after heating at 64^{o}C (Teixeira et al., 1997). Some studies demonstrated that the membrane integrity of bacterial cells decreased after heating (Kramer & Thielmann, 2016; Marcén et al., 2017). EGCg was also found to cause damages in the bacterial cytoplasmic membrane (Arakawa et al., 2004). Horner and Anagnostopoulos (1975) reported that *Staphylococcus aureus* gradually lost its capability to recover by adding sucrose, glycerol or NaCl to recovery medium after heat treatment. Increase in the degree of sensitivity of heat-treated *E. coli* cells to hydrophobic antibiotics resulted in the changes of cell structure, indicating cell

membrane damage (Mackey, 1983). The above-mentioned reports suggested that *E. coli* cells after heat treatment increased the susceptibility of *E. coli* cells to EGCg. Especially, the presence of EGCg at high concentration more than 500 mg/L resulted in the irreversible damage of cells by mild heating.

Furthermore, the heat-injured cells can become osmotically sensitive and lose some of their cellular compounds via leakage into the surrounding medium. After the heat treatment of *E. coli* at 55^{o}C for 20 min in the absence and presence of Teavigo, both protein and nucleic acid leakages were significantly higher than that of control without heating. The leakage of protein and nucleic acid from *E. coli* cells after heating in the presence of Teavigo was significantly higher than those in the absence of Teavigo (Fig. 3-3 and 3-4). However, there were no significant increase in both protein and nucleic acid leakages from the cells after the same heat treatment without Teavigo (Fig. 3-3 and 3-4). It has been reported that heat-injured *Staphylococcus aureus* cells released some intracellular materials, leading to the weakening of the cell membrane (Iandolo & Ordal, 1966; Allwood & Russell, 1968). Heating at 55^{o}C for 40 min, the leakage of 260 nm and 280 nm absorbing materials in heat-treated *E. coli* cells had no significant changes compared to the untreated cells (Robert, 1978). Ikigai et al. (1993) also indicated that EGCg initially cause the injury of the bacterial membranes, ultimately leading to the rapid leakage of 5,6-carboxyfluorescein from phosphatidylcholine liposomes. It has been reported that the generation of hydrogen peroxide by EGCg within the lipid bilayer of the bacterial membrane can lead to the leakage of intracellular materials (Arakawa et al., 2004). These facts and results suggested that the mild heat treatment in the presence of EGCg at the concentrations without growth inhibition caused the significant damage in the membrane

leading to the leakages of cellular protein and nucleic acid related substances and subsequent death in *E. coli* cells.

3.5 Summary

The combined effects of Teavigo and heat treatment against of *E. coli* O157:H7 were investigated. Heat treatment at 55 ^{o}C for 10, 15, and 20 min significantly decreased the viable count of *E. coli* in the presence of Teavigo compared to those in the absence of it. After heating at 55^{o}C for 20 min, there was the recovery of injured cells after heating both in the absence and the presence of Teavigo at 125 and 250 mg/L. However, at higher concentration of Teavigo (500 and 1000 mg/L), the injured *E. coli* O157:H7 did not recover. In addition, after the heat treatment of *E. coli* at 55 ^{o}C for 20 min, both protein and nucleic acid leakages increased with increasing heating time and Teavigo concentration. These results suggested that the mild heat treatment in the presence of EGCg at the concentrations without growth inhibition in the single use caused the significant damage in the membrane leading to the leakages of cellular protein and nucleic acid related substances and subsequent death in *E. coli* cells.

Chapter 4. Effects of baicalein on the cytotoxicity, production and secretion of Shiga toxins of enterohaemorrhagic *Escherichia coli*

4.1 Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) causes foodborne illness, and it can lead to hemorrhagic colitis (bloody diarrhoea) and potentially fatal hemolytic uremic syndrome (HUS) (Croxen & Finlay, 2010). The important virulence factor of EHEC is the Shiga toxin (Stx), also known as verocytotoxin or vero toxin. Two subgroups of Stx, namely, Stx1 and Stx2 are found in various combinations in EHEC isolates (Nataro & Kaper, 1998). Stx is one of the AB-5 family of toxins, consisting of a pentameric B subunit, noncovalently bound to an enzymatically active A subunit. Stx receptors are globotriaosylceramides (Gb3s) found on the surface of kidney epithelial cells. The Stx B subunit interacts with Gb3 and induces membrane invagination, leading to the internalization of the toxin. The internalized Stx toxin inhibits protein synthesis, leading to cell death.

Bacterial infections are commonly treated with antibiotic. However, antibiotic therapy in treatment EHEC infection is still controversial because of the restricted use of antibiotics in the therapy. The risk of HUS development is one of the concerns about use of antibiotics in treatment EHEC infection by increasing Stx production and secretion (Holtz et al., 2009; Smith et al., 2012; Agger et al., 2015). Some drugs commonly used in the clinic to treat for EHEC–infected patients are not recommended such as antibiotics, antimotility agents, narcotics, and non-steroidal anti-inflammatory (Tarr et al., 2005). In addition, there is an increase of EHEC isolated from patients that show the variety antimicrobial resistance (Hiroi et al., 2012). So, the demand for alternatives to the antibiotic therapy of EHEC infections disease is increasing.

Plant polyphenols and plant extracts, including polyphenols, have been reported to inhibit cholera (Morinaga et al., 2005), and Stx2 toxins (Quiñones et al.,

2009), among others. Furthermore, the polyphenols have almost no toxicity to human and some natural phenolic compounds could develope as effective drugs. For example, the extracts of plants and mushrooms are cost effective alternative for antibiotics to enhance innate immunity in chickens (Lee et al., 2010). Phytochemicals including plant polyphenols have shown inhibited effects on the verocytotoxin producing *E.coli* O157:H7 (Doughari et al., 2009). The combination of phenolic compounds and antibiotics have also shown synergistic effects against both *Staphylococcus aureus* and *Escherichia coli* (Sanhueza et al., 2017). Further studies in this area will be helpful to develope the new methods for treatment of bacterial infections.

It has been previously investigated the effects of green tea polyphenols against Stxs at low concentration by using purified catechins. In the previous study (Miyamoto et al., 2014), they have shown that the cytotoxicity of Stx1 decreased after preincubation with gallocatechin gallate (GCg) and epigallocatechin gallate (EGCg). In contrast, the cytotoxicity of Stx2 was not inhibited by preincubation with catechins and theaflavin (Miyamoto et al., 2014). These results suggested that the tertiary structure of gallocatechin, which comprises of the galloyl group, is important for Stx1 cytotoxicity inhibition.

In this study, I examined the effects of phenolic compounds screened by docking simulation from the natural compounds database, on the cytotoxicity of Stx. Among the compounds selected by the *in silico* screening, baicalein reduced the cytotoxicity of Stx1 and Stx2. The effects of baicalein, which showed the strongest inhibitory activity against Stx, were investigated on the protection of Vero cells against Stx, productivity of Stx by EHEC in addition to the effect on cytotoxicity.

4.2 Materials and methods

4.2.1 Analysis of interaction between StxB pentamers and natural products

The interaction between the compounds in the MEGxp, library of plant-derived natural products, and the Stx2 B subunit pentamer was analysed using the CDOCKER

module of the Discovery Studio software (Accelrys, Inc.), on a Windows XP PC. The crystal structure of the Stx2 B subunit pentamer (Fraser et al., 2004) registered in the Protein Data Bank as PDB ID: 3MXG was used in the calculations. Docking simulations were conducted with the compound positioned in the center of the Stx2 B subunit pentamer pore. Docking was performed using molecular dynamics simulations. The conditions used for the screening of the natural products were as follows: the molecular weight was less than that of the EGCg (MW: 458.4), and the lowest intramolecular energy (strain energy + electrostatic energy) was less than 2 kcal/mol. Structural analysis of the binding between the B subunit pentamers and the compounds was performed using the free energy minimization method (Brooks et al., 1983). The compounds selected from the *in silico* screening were obtained from NAMIKI SHOJI Co., Ltd., Tokyo, Japan.

4.2.2 Preparation of Stx1 and Stx2

The Stx1 and Stx2 preparations were respectively prepared from the cultures of *E. coli* O157:H7 No.33 (*stx1*+, *stx2*-) and O157:H7 No.184 (*stx1*-, *stx2*+), following the previously described procedure by Miyamoto et al. (2014). For preparation without Stx, the culture of O157:H7 No.37 (*stx1*-, *stx2*-) was used (Miyamoto et al., 2014). These bacteria were cultured in Luria Broth (LB, Becton, Dickinson and Company) at 37^{o}C for 24 h with shaking, then polymyxin B was added to the culture and incubated at 37^{o}C for 1 h with shaking. The final concentration of polymyxin B in the culture was 5000 U/mL. After centrifugation at 3,300 x *g* for 15 min at 4^{o}C of twenty-five mL of the culture, the supernatants were recovered. The supernatants were filtered by using Millex-GP 0.22 μm filter (Merck Millipore, Billerica, MA, USA) and the filtrates were used as Stx preparations.

The titers of Stx in the preparations were determined by using the VTEC-RPLA Seiken test (Denka Seiken Co., Ltd., Tokyo, Japan), following the manufacturer's

instructions. The titers of Stx1 and Stx2 were determined to be 256 and <2 in the Stx1, and <2 and 1024 in the Stx2 preparations, and <2 and <2 in the preparation without Stx, respectively. According to the manufacturer's instruction, titer of 128 is obtained for both purified Stx1 and Stx2 at 100 ng/mL. The concentrations of Stx1 and Stx2 were calculated according to the manufacturer's instructions.

4.2.3 Determination of Stx-inhibitory activity of baicalein

The effects of baicalein (NAMIKI SHOJI Co., Ltd., Tokyo, Japan) on the cytotoxicity of Stx were determined according to the previously reported method (Miyamoto et al., 2014), using Vero cells purchased from Cell Bank (RIKEN BioResource Center, Tsukuba, Ibaraki, Japan). The viability of the Vero cells was determined using the MTT Cell Proliferation Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the supplier's instruction. Medium was removed and 100 μL of fresh 5% FBS-MEM-E medium were added to the well. Ten μL of the 12 mmol/L MTT stock solution was added to each well. For a negative control, 10 μL of the MTT stock solution were added to 100 μL of medium alone. After incubation at 37^{o}C for 4 h, medium was removed and 100 μL of Cell-Based Assay Buffer was added to resolve MTT formazan crystals formed in the well. Absorbance at 595 nm was measured using Microplate Reader Model 680 (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the value was used to show the cell viability.

To determine the inhibitory activity of baicalein, various concentrations of the Stx preparations were incubated with baicalein before addition to the Vero cell culture. The final concentration of baicalein in the culture was 0.027 and 0.13 mmol/L. Three replicates of the treatments were carried out per experiment throughout this study and values were shown as an average ± SD of A_{595}. Statistical significance was determined by the Student's T-test.

4.2.4 Determination of protective effect of baicalein on Vero cells against Stx

The protective effects of baicalein on Vero cells against Stx were determined. Vero cell culture (0.1 mL) was seeded at 2×10^4 cells per well to each well of 96-well microtiter plate and cultured 24 h. One hundred microliter of baicalein at 0.08 and 0.4 mmol/L in PBS were added to each well and incubated for 1 h at 37°C. Stx preparations (0.1 mL) were added to each well and further cultured for 48 h at 37°C in 5% CO_2 incubator. The final concentrations of polyphenol in the culture were 0.027 and 0.13 mmol/L. For control, PBS was used instead of the polyphenol. The viability of the Vero cells was determined using the MTT Cell Proliferation Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the supplier's instruction.

Three replicates of the treatments were carried out per experiment throughout this study and values were reported as an average ± SD of A_{595}. Statistical significance was determined by the Student's T-test.

4.2.5 Determination of transcription of stx, production and secretion of Stx

To determine the effects of baicalein on transcription of *stx*, production and secretion of Stx, *E. coli* O157:H7 No.33 (*stx1*+, *stx2*-) and O157:H7 No.36 (*stx1*-, *stx2*+) were used. These bacteria were inoculated into LB and cultured overnight at 37°C with shaking. This bacterial culture was diluted in sterilized water to attain OD_{660} = 0.1 (bacterial concentration of ca. 10^8 CFU/mL). One mL of the culture was centrifuged at 60,000 x *g* for 5 min at 25°C and the supernatants were recovered. The precipitate was resuspended in 1 mL sterile water and then 10-dilutied to a final concentration of ca. 10^6 CFU/mL for use in subsequent experiments. One mL of 0.8 mmol/L baicalein solution was mixed with 1 mL of 2×LB and 100 μL of each of the diluted cultures in a test tube and the mixture was incubated at 37°C with shaking at 130 rpm. The final concentration of baicalein in the mixture was 0.38 mmol/L. For control, sterile water was used instead of

baicalein solution. To know the effects of Mitomycin C (MMC, Wao Pure Chemicals, Inc, Tokyo, Japan), LB (2 mL) and each of the diluted cultures (100 μL) were mixed and then incubated at 37°C with shaking to reach OD = 0.1 before adding with MMC (final concentration of 0.2 mg/mL). To determine the effect of baicalein and MMC on transcription of *stx*, cells were harvested at $OD_{660} \approx 0.6$. To determine the effects of baicalein on production and secretion of Stx, the mixtures were incubated for 24 h.

For determination of the amounts of transcript of *stx*, cells were harvested by centrifugation at 8,000 × *g* for 5 min at 4°C. The precipitates were suspended in sterile water and the suspension was centrifuged at 8,000 × *g* for 5 min at 4°C and the supernatants were removed. This process was repeated three times to wash cells. Mixture of 700 μL Dw-saturated phenol and 700 μL TES buffer pre-warmed at 65°C was added to the cell precipitate. After vortexing, the cell suspension was incubated for 40 min at 65°C with occasional vortexing. The cell suspension was then placed on ice for 2 min and centrifuged at 17,860 × *g* for 10 min at 4°C. Eight hundred μL of TRIzol® LS was added to the aqueous phase, and the mixture was vortexed and placed on ice for 5 min. After 200 μL of chloroform was added to the mixture, the mixture was vortexed, placed on ice for 5 min and centrifuged at 17,860×*g* for 10 min at 4°C. To the aqueous phase recovered, 600 μL of chloroform was added, and the mixture was vortexed, placed on ice for 5 min and centrifuged at 17,860 × *g* for 10 min at 4°C. Six hundred μL of isopropanol was added to the aqueous phase recovered, and the mixture was vortexed, placed on ice for 10 min and centrifuged at 17,860 × *g* for 10 min at 4°C. The precipitate was dissolved in 200 μL of DEPC- treated deionized water. Six hundred μL of 99% ethanol and 20 μL of 3 M NaOAc (pH 5.2) were added, and the mixture was stored at -80°C for 60 min. After centrifugation (17,860×*g*, 15 min, 4°C), the precipitate was dried at room temperature for 10 min and dissolved in 20 μL of DEPC-treated deionized water. Quality and quantity of total RNA were checked with spectrophotometer. The RNA samples were treated with RNase-Free

DNase set (QIAGEN), purified and concentrated by using the RNeasy Mini Kit (QIAGEN) according to the supplier's instruction. The purified RNA samples were reverse transcribed by using the ReverTra Ace® qPCR RT Master Mix (TOYOBO) according to the supplier's instruction. For *stx1*, primers Stx1-F-n2 (5'-gttgcgaaggaatttacc-3') and Stx1-R-n2 (5'-gtctgtaatggagtacctattg-3'), and for *stx2*, Stx2-F-n2 (5'-cgacccaacaaagttatg-3'), Stx2-R-n2 (5'-gggtgtggttaataacag-3') were designed on the basis of the nucleotide sequences of *stx* genes (accession number: CP017444.1, CP012802.1). For housekeeping gene, primers rrsA-F (5'-aggccttcgggttgtaaagt-3') and rrsA-R (cggggatttcacatctgact) were designed on the basis of the nucleotide sequence of *rrsA* gene (accession number: J01859.1). PCR mixture (20 μL) was consisted of 10 μL of THUNDERBIRD® SYBR qPCR Mix (TOYOBO), 0.04 μL of 50 × ROX reference dye, 0.6 μL of 10 μmol/L forward primer, 0.6 μL of 10 μM reaverse primer, 2 μL of 25 ng/μL cDNA, and 6.76 μL of RT-PCR grade water. Real-time PCR was conducted in the condition of initial denaturation at 95°C for 60 s, 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s, and 1 cycle of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and denaturation at 95°C for 30 s. All PCR reactions were run on Mx3000P® Real-Time PCR System and results were analyzed by MxPro™ Software version 3.00 (Stratagene, La Jolla, CA, USA).

To determine the effect of baicalein on production and secretion of Stx, the mixtures were centrifuged at 3,300 × *g* for 15 min at 4°C after incubation for 24 h. The supernatants were filtered through a Millex-GP filter (0.45 μm) and the filtrate was used as an extracellular sample. The precipitate was washed once with PBS by centrifugation and suspended with 2 mL of PBS. The suspension was then centrifuged at 3,300 × *g* for 15 min at 4°C. The precipitate was suspended with 2 mL of PBS. To the suspension, polymyxin B (Pfizer Japan Inc.) was added to the culture to attain a final concentration of 5000 U/mL. The culture was incubated for 30 min at 37°C with occasional vortex and centrifuged at

3,300 × *g* for 15 min at 4°C. The supernatant was filtered by Millex-GP filter (0.45 μm) and used as an intracellular sample. Extracellular samples were used for Stx sample in case of *E. coli* O157:H7 treated with MMC.

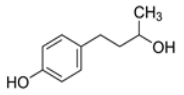
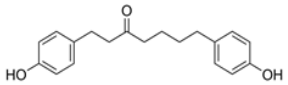
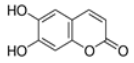
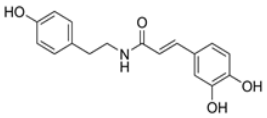
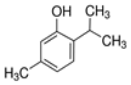
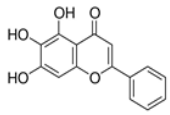
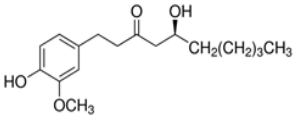
The titers of Stx in the samples were determined by using the VTEC-RPLA Seiken test kit and the concentrations of Stx1 and Stx2 were calculated as described above. For cytotoxicity test of the samples, Vero cell culture (0.1 mL) was seeded at 2×10^4 cells per well to each well of 96-well microtiter plate and cultured 24 h. Extracellular and intracellular samples at a various mixing ratio in PBS (200 μL) were added to each well and cells were cultured for 48 h at 37°C in 5% CO_2 incubator. The viability of the Vero cells was determined using the MTT Cell Proliferation Assay Kit as described above. Three replicates of the treatments were carried out per experiment throughout this study and values were reported as average or average ± SD of A_{595}. Statistical significance was determined by the Student's T-test.

4.3 Results

4.3.1 Effects of baicalein on the cytotoxicity of Stx

The natural compounds selected from the *in silico* screening are listed in Table 4-1. Viability of the Vero cells was determined after treatment with Stx, which had been incubated with or without each of the compounds at 100 mg/L. The cytotoxicity of Stx1 and Stx2 decreased after preincubation with baicalein, in contrast to preincubation with the other compounds (Table 4-1).

Table 4-1. Candidate compounds for Stx inhibitors selected from MEGxp collection by docking simulation

ID	Name	MW	Interaction with Stx2B pentamers obtained by the docking model		Structure
			No. of Hydrogen bond	Intramolecular energy (cal/mol)	
NP-000438	Rhododendrol	166.22	3	< 2	
NP-000814	Acerogenin G	298.38	3	< 1	
NP-001362	Esculetin	178.14	4	< 2	
NP-003423	N-caffeoyltyramine	299.32	4	< 2	
NP-003587	Thymol	150.22	0	< 2	
NP-003729	Baicalein	270.24	2	< 1	
NP-003855	[6]-Gingerol	294.39	2	< 1	

The effects of baicalein on the cytotoxicity of Stx1 and Stx2 were further investigated in detail. Baicalein was mixed with the Stx preparations with different Stx concentrations and incubated for 1 h. The mixture was then added to the Vero cell culture. Figure 4-1 shows the effects of baicalein on the cytotoxicity of Stx1 and Stx2. In the absence of baicalein, the viability of Vero cells decreased with an increase in the concentration of Stx1 and Stx2. However, the cytotoxicity of both Stx1 and Stx2 was significantly reduced (P<0.01) by the preincubation with baicalein (Fig. 4-1A and B). The results clearly demonstrate that baicalein inhibits the cytotoxicity of both Stx1 and Stx2.

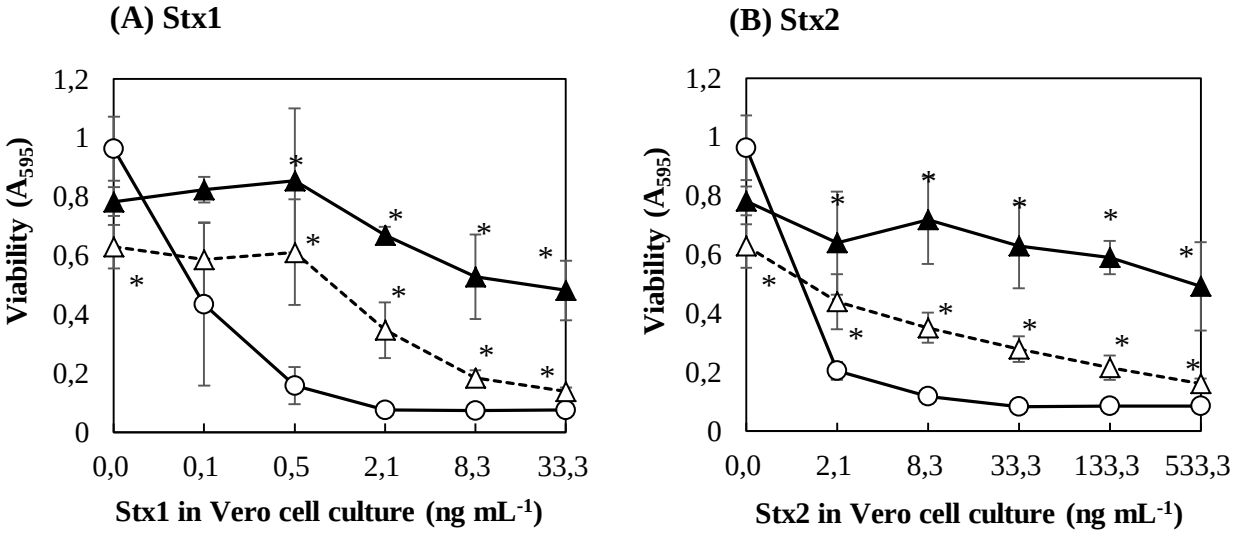


Figure 4-1. Effects of baicalein on the cytotoxicity of Stx1 and Stx2. Stx1 (A) and Stx2 (B) preparations respectively containing Stx1 and Stx2 were mixed with baicalein and incubated for 1 h at 37 °C. After the incubation, the mixture was added to the culture of Vero cells. The final concentrations of baicalein in the culture were 0 (○), 0.027 (△), and 0.13 (▲) mmol/L. Cell viability was determined by using MTT Cell Proliferation Assay after the cultivation at 37°C for 48 h. Values are average ± SD for 3 separate experiments. *, $P < 0.01$.

4.3.2 Protective effects of baicalein on Vero cells against Stx

To know the protective effects of baicalein on Vero cells against Stx, Vero cells were preincubated with baicalein and then Stx preparations were added to the cells. Figure 4-2 shows the effects of Stx preparations on viability of Vero cells pretreated with baicalein. In the absence of baicalein, the viability of Vero cells decreased with an increase in the concentration of Stx1 and Stx2. However, even in the presence of Stx1 or Stx2, the viability of Vero cells pretreated with baicalein was significantly higher than that of control without the pretreatment (Fig. 4-2A and B). It seems that baicalein protected Vero cells from the cytotoxicity of both Stx1 and Stx2.

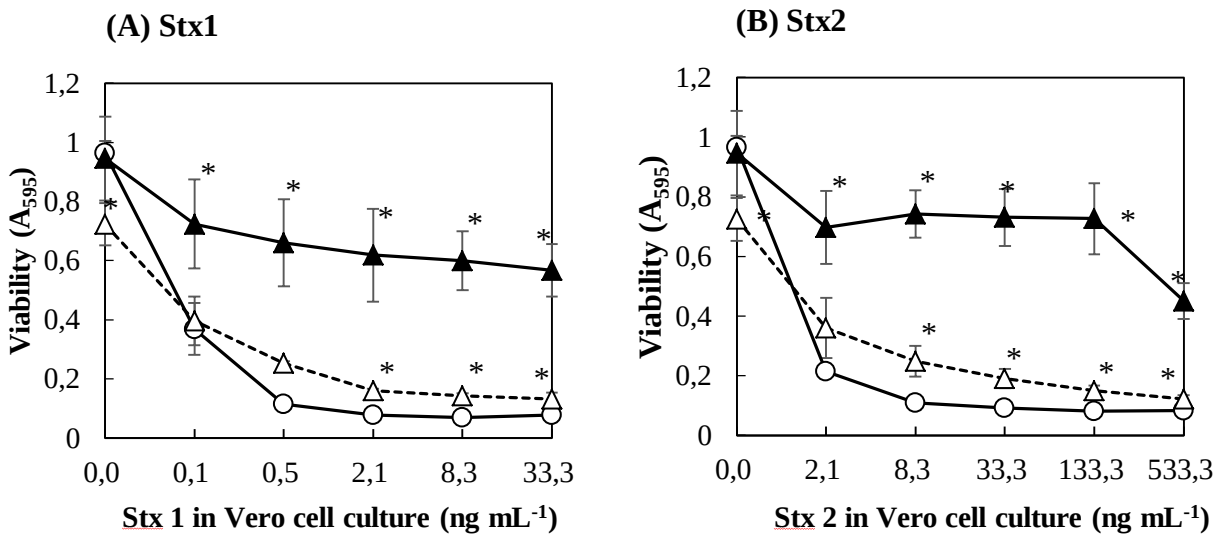


Figure 4-2. Effects of Stx1and Stx2 on viability of Vero cells pretreated with baicalein. Baicalein was added to Vero cell culture and incubated for 1 h at 37°C. After the incubation, Stx1 (A) and Stx2 (B) preparations were added to the culture. The final concentrations of baicalein in the culture were 0 (○), 0.027 (△), and 0.13 (▲) mmol/L. After the cultivation at 37°C for 48 h, cell viability was determined by using MTT Cell Proliferation Assay. Values are average ± SD for 3 separate experiments. *, $P < 0.01$.

4.3.3 Effects of baicalein on productivity of Stx by EHEC

The transcriptional levels of *stx* were evaluated by real-time qPCR. Relative quantity of transcripts of *stx1* and *stx2* was compared between cells treated with and without baicalein and MMC. Figure 4-3 shows the effects of baicalein and MMC on transcription of *stx* genes. After treatment with baicalein, the transcription level of *stx1* was enhanced to 2.9-fold of that of negative control (Fig. 4-3A), while no significant difference in the transcription level of *stx2* was obtained (Fig. 4-3B). However, in both of the extracellular and intracellular samples prepared from *E. coli* O157:H7 treated with baicalein, amounts of both Stx1 and Stx2 were similar to those of the samples without baicalein treatment (Table 4-2). For positive control, MMC induced transcription level of both *stx1* and *stx2* by 17.8 and 6.8 fold compared to negative control, respectively (Fig. 4-3A and B).

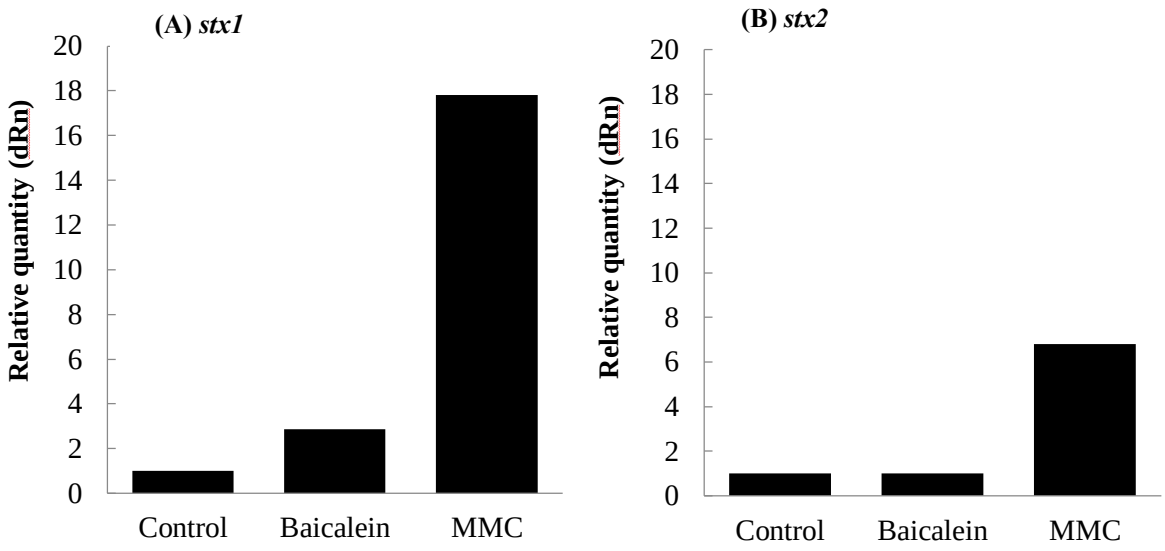


Figure 4-3. Effects of baicalein on the transcription of *stx* in *Escherichia coli* O157:H7. *E. coli* O157:H7 No.33 (*stx1*+, *stx2*-) and O157:H7 No.36 (*stx1*-, *stx2*+) strains were cultured until $OD_{660} \fallingdotseq 0.6$ in the presence and absence of baicalein at 0.38 mmol/L. Amounts of transcripts of *stx1* (A) and *stx2* (B) were determined by Real-time qPCR assay. Values are average of 2 separate experiments.

Table 2 Effects of baicalein on Stx production after 24 h incubation

Samples		Stx concentration (ng/mL)**		
		Control	Baicalein	Mitomycin C
Stx1	Extracellular	400	400	1600
	Intracellular	50	50	ND
Stx2	Extracellular	200	200	204800
	Intracellular	3	3	ND

**: Stx concentration was determined by RPLA assay

Values are average of 2 separate experiments. ND: not determined

To evaluate the effects of baicalein on the secretion of Stx in detail, cytotoxicity of the extracellular and intracellular Stx preparations was investigated. As shown in Figure

4-4, the viability of Vero cells decreased with an increase in the concentration of extracellular and intracellular Stx1 and Stx2 (Fig. 4-4A and B). There was no significant difference in viability between Vero cells in the presence of Stx prepared from *E. coli* O157:H7 cultured in the presence and absence of baicalein. The results suggested that baicalein had no effects on the secretion of both Stx1 and Stx2.

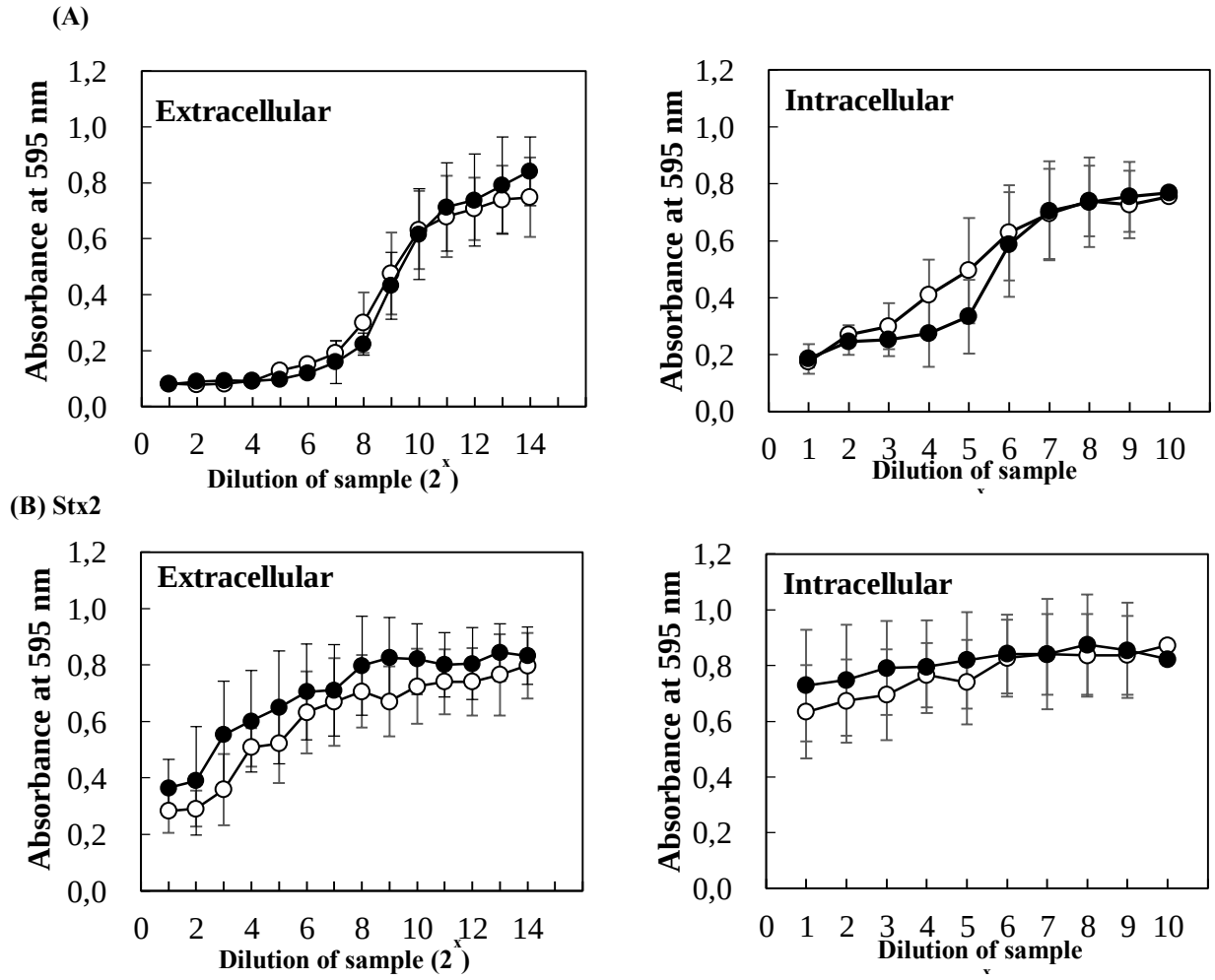
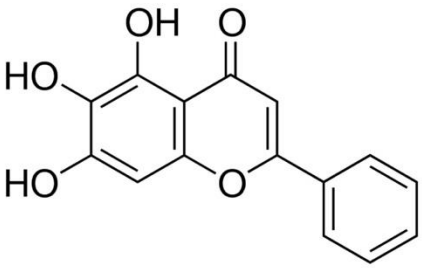


Figure 4-4. Effects of baicalein on secretion of Stx by *Escherichia.coli* O157:H7. *E. coli* O157:H7 No.33 (*stx1+*, *stx2-*) and O157:H7 No.36 (*stx1-*, *stx2+*) cultures were cultured in the presence (●) and absence (○) of baicalein for 24 h at 37°C. After the incubation, extracellular and intracellular Stx1 (A), extracellular and intracellular Stx2 (B) preparations were respectively prepared from the cultures. Cytotoxicity of the preparations was determined on Vero cells. Cell viability was determined by using MTT Cell Proliferation Assay. Values are average of 3 separate experiments.

4.3.4 Interaction of baicalein with Stx1B and Stx2B pentamers

Figure 4-5 shows the docking models of baicalein bound to the pockets of Stx1B and Stx2B pentamers. According to the conformation of the Stx1B and Stx2B pentamers, the potential site for binding to baicalein was estimated to be from Trp33 to Gly46 (Trp-Asn-Leu-Gln-Ser-Leu-Leu-Leu-Ser-Ala-Gln-Ile-Thr-Gly) in the Stx1B monomer and from Trp32 to Gly45 (Trp-Asn-Leu-Gln-Pro-Leu-Leu-Leu-Ser-Ala-Gln-Leu-Thr-Gly) in the Stx2B monomer (Stein et al., 1992; Fraser et al., 2004). According to the plain view of conformation of Stx1B and Stx2B pentamers predicted from their crystal structure, A-E and A-J were named of each the monomers of Stx1B and Stx2B pentamers, respectively (Miyamoto et al., 2014). The models showed that baicalein formed 2 hydrogen bonds with the side chains of amino acids facing inside the pocket of the Stx1B pentamer, with the lowest intramolecular energy (strain energy + electrostatic energy) of 1.8 kcal/mol at ①Ser42 and ②Ser42 of Monomer B (Fig. 4-5A). Similarly, in the case of Stx2B pentamer, baicalein formed 1 hydrogen bond with the lowest intramolecular energy of 0.2 kcal/mol at ①Ser41 of Monomer J (Fig. 4-5B). These results indicate that baicalein forms a stable structure with both the Stx1B (pocket size: 778$Å^3$) and Stx2B pentamers (pocket size: 475$Å^3$).

Baicalein



(A) Stx1B and Baicalein (B) Stx2B and

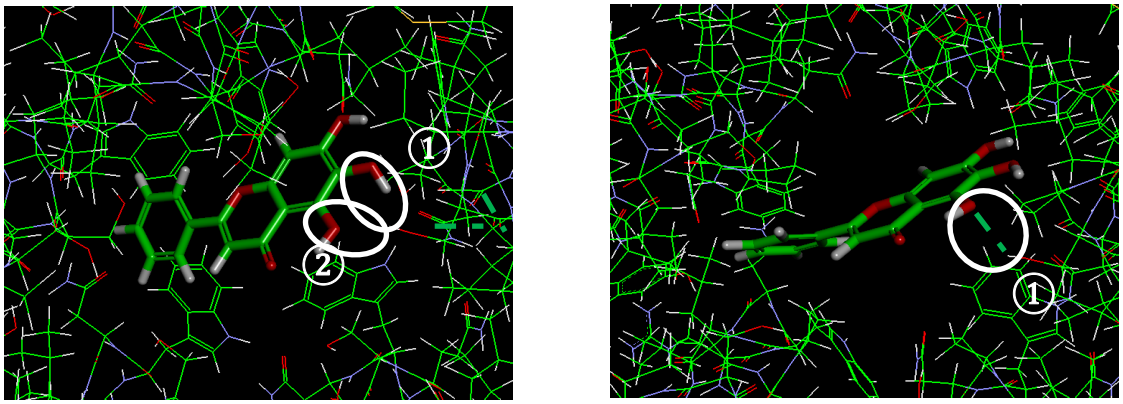


Figure 4-5. Docking models of baicalein bound to the pockets of Stx1B and Stx2B pentamers. Hydrogen bonds formed between baicalein and amino acids facing inside the pockets of Stx1B and Stx2B pentamers are enclosed with white circles and numbered. (A) Model showing 2 hydrogen bonds formed between baicalein and side chains of amino acid facing inside the pocket of the Stx1B pentamer at Ser42 and Ser42 of Monomer B. (B) Model showing 1 hydrogen bond formed between baicalein and side chain of amino acid of the Stx2B pentamer at Ser41 of Monomer J.

4.4 Discussion

Baicalein is a major component of *Scutellaria baicalensis*, a Chinese herb. In addition to its inhibitory effect on lipoxygenase and reverse transcriptase, it was found to have antioxidant, neuroprotective, antibacterial, antiviral, and antifungal activities (Ono et al., 1990; Ishige & Schubert D, 2001; Wu et al., 2001). Baicalein has also been reported to inhibit the biofilm formation of *Candida albicans* (Cao et al., 2008), and to induce apoptosis in a variety of human cancer cell lines (Kuntz et al., 1999; Pidgeon et al., 2002;

Ma et al., 2005; Van Leyen et al., 2006). Baicalein has been reported to induce cancer cell death and proliferation retardation by inhibiting CDC2 kinase and surviving (Chao et al., 2007). Baicalein has shown to inhibit enzyme of cytochorome P450 system (CYP2C9) that metabolizes drugs in the body by binding to the substrate site of the enzyme (Si et al., 2009). There are, however, no reports on the inhibition of cytotoxicity of both Stx1 and Stx2 by baicalein to the best of our knowledge. Although further studies with the purified Stx are required to confirm the results of the *in silico* study, this is the first report that demonstrated that baicalein inhibits the cytotoxicity of both Stx1 and Stx2, possibly by forming a stable binding structure at the pocket of the Stx1B and Stx2B pentamers.

The extent of the inhibitory activity of baicalein against the cytotoxicity of Stx1 was similar to that of EGCg, which did not inhibit the cytotoxicity of Stx2 (Miyamoto et al., 2014). Docking simulation suggested that baicalein formed 2 hydrogen bonds with the lowest intramolecular energy of 1.8 kcal/mol, with amino acids inside the pocket of the Stx1B pentamer, but it formed only 1 hydrogen bond with the lowest intramolecular energy of 0.2 kcal/mol with the Stx2B pentamer (Fig. 4-5). It has been previously reported that EGCg formed 6 hydrogen bonds with the lowest intramolecular energy of 5.2 kcal/mol with the Stx2B pentamer (Miyamoto et al., 2014). Since the molecular weight of baicalein (MW: 270.2) was much lower than that of EGCg (MW: 458.4), baicalein seemed to form a stable hydrogen bond with the side chain of amino acid facing inside the pocket of the Stx2B pentamer. This bond formed with the lowest intramolecular energy when compared to the bonds of EGCg with the Stx2B pentamer, even though the number of hydrogen bonds with the pentamer was less than that of EGCg.

Furthermore, baicalein protected Vero cells from cytotoxicity of both Stx1 and Stx2. It has been reported that baicalein protects human hepatoma cells against the attack by cancer cells by its ability to prevent the adhesion, migration, and invasion of cancer cells (Chiu et al., 2011). It has also been reported that baicalein protected human

skin cells againts oxidative stress by Ultraviolet B (UVB) via preventing reactive oxygen species and absorbing UVB radiation (Oh et al., 2016). In addition, Cariddi et al. (2015) reported that caffeic acid, a polyphenol, protected Vero cells from the cytotoxicity of ochratoxin A by altering principally the lysosomal function in Vero cells (Cariddi et al., 2015). It seems that baicalein protects Vero cells from cytotoxicity of Stx by binding to the surface of the cytoplasmic membrane of the cell and altering the function of the membrane. At 0.13 mmol/L, the preincubation with bacialein reduced Vero cells sensitivity to Stx1 and Stx2 by approx. 6 and 8 fold, respectively (Fig. 4-2). The cell protection against Stx of other compounds has also been reported in many studies. For exemple, the protective effect of 4 phenylacetyl-Arg-Val-Arg-4-amidinobenzylamide at concentration of 25 µmol/L on Hep-2 cells againsts Stx increased by 6 fold (Becker et al., 2012), while chloroquine at concentration of 25 µmol/L reduced Hep-2 cells sensitivity to Stx2 by 20 fold (Kavaliauskiene et al., 2017). Though these compounds protected cells at the lower concentration, however, they have not been used in humans beacause of their toxicity.

In this study, treatment with baicalein slightly increased the transcription of *stx1*, however no significant change was recorded for *stx2* compared to negative control. Stx production of EHEC is reported to be controlled by several factors such as growth phase, reactive oxygen species, quorum sensing, H_2O_2 and neutrophils (Konowalchuk et al., 1978; Wagner et al., 2001a; Bergholz et al., 2007). Specifically, Stx production by EHEC increase at the stationary phase (Konowalchuk et al., 1978; Bergholz et al., 2007). It is also known that Stx production was regulated by phage through the amplification of gene copy number and toxin release (Neely & Friedman, 1998). Wagner et al. (2002) suggested that damage of bacterial cell could lead to the release of Stx by the absence of phage-mediated lysis in *E.coli* O26:H19 (Wagner et al., 2002). Moreover, baicalein inhibits growth of *Staphylococcus aureus* by the aggregation of bacterial cells and damaging the bacterial cell membrane (Yun et al., 2012). There was no significant difference in viability between

Vero cells in the presence of Stx prepared from *E. coli* O157:H7 cultured in the presence and absence of baicalein at 0.38 mmol/L (Fig. 4-4). Yuk and Marshall (2006) have shown that changes in the ratio of extracellular to intracellular Stx might be due to changes in membrane fluidity (Yuk & Marshall, 2006). It has been reported in another study that *E. coli* O157:H7 adapted to pH 8.3 exhibited the greatest Stx concentrations at approximately 10^8 CFU/mL. It has also been reported that the ratio of extracellular to intracellular Stx concentration decreased at acidic pH, possibly due to the decrease of membrane fluidity (Yuk & Marshall, 2006). It has also been shown that baicalein exhibited antibacterial activity against *E.coli* by lowering membrane fluidity of the cells (Wu et al., 2013a). It seems that baicalein did not affect the growth and membrane fluidity at 0.38 mmol/L, though it reduced cytotoxicity of Stx and protected Vero cells at the lower concentration (0.13 mmol/L).

In this chapter, treatment with MMC strongly increased the transcription of both *stx1* and stx2, while baicalein slightly increased the transcription of *stx1* but not *stx2* (Fig. 4-3). Furthermore, some studies suggested that baicalein may be developed as a potential candidate for treatment of various disease models such as diabetes, cardiovascular diseases, inflammatory bowel diseases, gout and rheumatoid arthritis, asthma, encephalomyelitis, and carcinogenesis, neurological diseases, Alzheimer's and Parkinson's disease (Dinda et al., 2017; Li et al., 2017). Javed & Ojha reported that oral administration of baicalein for treatment Parkinson's disease is safe to human (Javed & Ojha, 2019). It has also been reported that mixture of baicalein in the dietary metabolic management of osteoarthritis is safe for human consumption (Burnett et al., 2007). Together with these facts, it was suggest that baicalein is one of the potential candidates for antivirulence strategies against EHEC infection. However, further researches are still required to carefully evaluate for application of baicalein in the clinic.

4.5 Summary

The effects on cytotoxicity of both Stx1 and Stx2 from *E. coli* O157:H7 were investigate on 7 compounds selected by *in silico* screening of the natural compound database according to the structural properties of EGCg which inhibited cytotoxicity of both Stx1. Among them, baicalein inhibited the cytotoxicities of both Stx1 and Stx2 against Vero cells, after preincubation at 0.13 mmol/L. Baicalein also reduced the susceptibility of Vero cells against both Stx1 and Stx2. Real-time qPCR showed that baicalein increased transcription of *stx1* but not of *stx2*. However, baicalein had no effects on both the intracellular Stxs production and the secretion of Stxs. Docking simulation suggested that baicalein inhibited cytotoxicity of both Stx1 and Stx2 due to the formation of stable complex at the pocket of the Stx1B and Stx2B pentamers.

Chapter 5. Conclusions

Enterohaemorrhagic *Eschericia coli* (EHEC) causes foodborne illness, and if it becomes severe, it causes hemorrhagic colitis and potentially fatal hemolytic uremic syndrome. Stx, a strong toxin produced by EHEC, is an important factor of EHEC that can give rise to hemorrhagic colitis and hemolytic uremic syndrome. EHEC infections not only affect on public health but also impose a significant economic cost on society. Bacterial infections are commonly treated with antibiotics. However, antibiotic therapy in treatment of EHEC infection is still controversial because of the restricted use of antibiotics in the therapy. The beneficial effect of polyphenols on human health is believed to be due to their various properties including antibacterial, antioxidant and antitoxin activities. They have been reported to inhibit the growth of various bacteria and the activities of toxins produced by bacteria. The combination of polyphenols with other antimicrobial agents has also shown synergistic effects against many bacteria. In this thesis, antibacterial and antitoxin activity of plant polyphenols were investigated to obtain basic evidences for the use of plant polyphenols for controlling EHEC.

Chapter 2: In this chapter, the combined effects of 2 tea polyphenol formulations (Teavigo including 95% EGCg and TF40 including 40% theaflavins) and 5 food additives (EDTA, NaCl, Na acetate, Na citrate and ethanol) on *E. coli* O157:H7 were evaluated by determining minimum inhibitory concentration (MIC), the fractional inhibitory concentrations (FIC) index and time kill assays. The combination of Teavigo and EDTA or Na citrate, and TF40 and EDTA showed the synergistic effects on the growth of *E. coli* O157:H7. The partial synergistic effect was observed in the combination of Teavigo and ethanol, NaCl or Na acetate, and the combination of TF40 and ethanol or Na citrate. The combination of tea polyphenols with food additives, the concentrations of these substances showed synergistic effect for inhibition of the growth of *E. coli* O157:H7 were lower than

those of the corresponding MIC value of the single use. The results of time-kill assays showed that the growth of *E. coli* O157:H7 was inhibited by the combination of 2 tea polyphenol formulations and 5 food additives at the concentrations without growth inhibition by the single use. The results suggest that taking advantage of using natural polyphenol formulations and compounds with different antibacterial action and their combination tested is feasible in food preservation. However, the mechanism of the combined effects still was unclear. Hence, the further researches are needed to indicate this combined effect.

Chapter 3: Effects of tea polyphenols on heat sterilization effect against *E. coli* O157:H7 were investigated in this chapter. The presence of Teavigo during heating increased the effect of heat treatment against *E. coli* O157:H7. On heat treatment at 55^{o}C for 10, 15, and 20 min, the viability of *E. coli* O157:H7 immediately after heating significantly decreased in the presence of Teavigo. After heating at 55^{o}C for 20 min, the viable count of *E. coli* O157:H7 decreased and the injured *E. coli* O157:H7 cells did not recover in the presence of Teavigo at 500 and 1000 mg/L during heating. The concentration was 1/4 MIC for the single use. The significant leakages of protein and nucleic acid related substances from *E. coli* O157:H7 cells were observed after heating in the presence of Teavigo at the concentrations, but not without Teavigo. It seems that the irreversible damages in the membrane caused by the heating in the presence of Teavigo during heating might be one of the reasons of inactivation of *E. coli* O157:H7. These results suggested that the mild heat treatment in the presence of EGCg can inactivate *E. coli* O157:H7 in food.

Chapter 4: In this chapter, effects of polyphenols on cytotoxicity of Shiga toxins from *E. coli* O157:H7 were investigated. To find out a polyphenol inhibiting cytotoxicity of both Shiga toxins 1 and 2 (Stx1 and Stx2), *in silico* screening of the natural compound database was done according to the structural information of EGCg that had been shown

the inhibitory effect on cytotoxicity of Stx1. Among 7 compounds selected by *in silico* screening of the natural compound database, baicalein inhibited the cytotoxicity of both Stx1 and Stx2 against Vero cells, after preincubation with the toxins at 0.13 mmol/L. On the other hand, baicalein also reduced the susceptibility of Vero cells to both Stx1 and Stx2. Real-time qPCR showed that baicalein increased transcription of *stx1* but not of *stx2*. In addition, baicalein had no effects on the secretion of both extracellular and intracellular Stxs and on their production. Docking models suggested that baicalein could stably formed complex with StxB pentamer with low intramolecular energy. The results demonstrated that inhibitory activity of baicalein against the cytotoxicity of both Stx1 and Stx2 might be due to forming a binding structure at the pocket of the Stx1B and Stx2B pentamers. The results suggested that baicalein is one of the potential candidates for antivirulence strategies against EHEC infection.

The findings in this study showed the antibacterial and antitoxin effects of plant polyphenols for controlling pathogenic EHEC. The combinations of polyphenols and food additives or heating were more effective than the single use against EHEC. Polyphenols are also the potential candidates for inhibiting Stx1 and Stx2 from EHEC. Future researches could be evaluated for application of polyphenols and their combination in food preservation and medical purposes.

Natural polyphenols are highly safe as they have experience in eating. Natural polyphenols are expected to be one of the means to contribute to the reduction of health risks derived from foods. For this purpose, it is necessary to further verify the combined effect with various additives and naturally derived antibacterial substances, elucidate its mechanism, and systematically elucidate the inhibitory effect against various bacterial toxins.

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