Inhibiting *Listeria monocytogenes*, *Vibrio parahaemolyticus* and *Morganella morganii* with Aqueous Methanol Extracts of *Punica granatum* and *Galla chinensis*

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ABSTRACT

Listeria monocytogenes, *Vibrio parahaemolyticus* and *Morganella morganii* are closely related to foodborne illnesses caused by the consumption of seafood and ready-to-eat (RTE) food. Traditional Chinese medicines (TCM) have been widely studied as complementary and alternative medicines, and many of them have been verified to have antimicrobial properties. The purpose of this research was to study antimicrobial effects of plant extracts as potential preservatives in seafood products and to identify the primary antimicrobial compounds in plant extracts.

Four plants, Pomegranate peel ("PP", *Punica Granatum* L.), Chinese gallnut ("CG", *Galla chinensis*), forsythia fruit ("FS", *Forsythia suspensa*) and Baikal skullcap root ("BS", *Scutellaria baicalensis*) were ground and extracted with 70% methanol, respectively. The extracts were diluted at tested for antimicrobial activities on *V. parahaemolyticus*, *L. monocytogenes* and *M. morganii* both in agar diffusion assay using tryptic soy agar (TSA), and in microdilution assay using tryptic soy broth (TSB). Both CG and PP extracts, with concentrations no lower than 1 mg/ml, significantly inhibited both *V. parahaemolyticus* and *L. monocytogenes* ($P<0.01$) and reduced the bacterial population by up to 4 logs. No significant inhibition was observed with FS and

BS extracts, except for BS at 5 mg/ml on *V. parahaemolyticus*. None of the extracts showed significant inhibition against *M. morganii*.

The antibacterial activities of CG and PP 70% methanol extracts were tested in ground raw tuna and cooked tail-on shrimp. The extracts were mixed in tuna with final concentration at 1.7 mg/ml, and applied as soaking treatments (5 mg/ml) for shrimp. Both CG and PP extracts inhibited *V. parahaemolyticus* on both food matrices while only CG significantly inhibited *L. monocytogenes*.

The 70% methanol crude extract of CG was analyzed by HPLC and LC-MS. Oligo-galloyl-O-glucose (nGG, n=1-10) are the major compounds in CG. The crude CG extract was fractionated using HPLC and the fractions were collected based on elution time and tested for their antimicrobial activities against *V. parahaemolyticus* and *L. monocytogenes* using agar diffusion methods. The fractions containing 3GG – 8GG were the most active antimicrobials on both bacteria.

DEDICATION

This dissertation is dedicated to Lina Zhou and Angelina Wu.

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CHAPTER 1:

INTRODUCTION

Seafood safety has been continuously improved during recent years; however, seafood related foodborne disease outbreaks remained to be a serious food safety issue. According to Center of Disease Control and Prevention (CDC), aquatic animal commodities (fish, mollusks and crustacean) are estimated to be responsible for more than 25% of all foodborne diseases outbreaks that have been reported with an implicated contaminated food or food ingredient (Gould et al., 2013).

Vibrio parahaemolyticus and *Listeria monocytogenes* are two important foodborne pathogenic bacteria. *V. parahaemolyticus* is responsible for the largest number of bacteria-caused outbreaks, and the number of foodborne illnesses caused by *V. parahaemolyticus* is increasing. In 2013, the highest number of illnesses associated with *V. parahaemolyticus* was observed since 1996. *L. monocytogenes* was responsible for the third largest number of foodborne deaths in the USA (CDC, 2010). According to CDC's foodborne outbreak online database, outbreaks caused by *V. parahaemolyticus* are specifically associated with consumption of raw shellfish and crustacean; outbreaks caused by *L. monocytogenes* are associated with ready-to-eat (RTE) food products. In both situations, pre-consumption heat treatment is either absent or not sufficient. Therefore, an alternative pathogen control strategy is necessary to ensure safety of related food commodities.

Many plant-source materials have been used in traditional Chinese medicine (TCM) for many centuries in China and other Asian countries. In recent years, TCM has been gradually accepted by the western world. In the USA, TCM is studied and used as a complementary and alternative medicine (CAM) as well as a dietary supplement. By the year 2000, more than 35,000 plant-source materials were screened for potential medical use by the National Cancer Institute (NCI) and the United States Department of Agriculture (Yuan. et al, 2000). Scientific research on the use of TCM has increased with thousands of functional compounds being isolated from TCM (Zhou. et al, 2011). For example, Chinese gallnut (CG, *Rhus chinensis* Mill.), pomegranate peel (PP, *Punica granatum* L.), forsythia fruit (FF, *Forsythia suspensa* (Thunb.) Vahl) and baikal skullcap root (BS, *Scutellaria baicalensis* Georgi.) are several of the most commonly used TCM. Research has been conducted to identify antimicrobial compounds contained in these plants. Phenolic compounds, such as essential oils, gallotannins and flavonoids, are the major compounds with antimicrobial activity found in these plants (Jiao et al. 2012). The antimicrobial properties were verified using antibacterial assays (Kang. et al. 2008, Tian et al. 2009, Fawole et al. 2012), and tested on food products (Taguri et al., 2004; Guillén and García, 2007; AL-Zoreky, 2009; González et al., 2010; Kanatt et al, 2010; Xi et al., 2012). However, little research has addressed on the application of TCM in seafood products; the inhibition of TCM on important foodborne pathogens, such as *Listeria monocytogenes* and *Vibrio parahaemolyticus,* deserve further studies and discussions.

The purpose of this research was to identify the potential inhibitory effects of plant extracts against *Listeria monocytogenes*, *Vibrio parahaemolyticus* and *Morganella*

morganii in shrimp and tuna products. Antimicrobial assays were conducted on pure cultures of selected bacterial strains both on agar plates and in broth. When the extracts showed bacterial inhibition, further studies were conducted in typical seafood product matrices including cooked shrimp and raw tuna. The extracts of CG and PP were added into shrimp and tuna meat with inoculated bacteria, and the inhibitory effects were studied. Furthermore, the major inhibitory compounds in CG were also fractionated and analyzed, and the antimicrobial activities of fractions were tested to see which compound(s) contributed to the total inhibitory effects against pathogenic bacteria.

References

Al-Zoreky, N. S. (2009). Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. *International Journal of Food Microbiology*, *134*(3), 244-248.

Centers for Disease Control and Prevention (CDC). (2010). Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food-10 states, 2009. *MMWR. Morbidity and Mortality Weekly Report*, *59*(14), 418-422.

Fawole, O. A., Makunga, N. P., & Opara, U. L. (2012). Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. *BMC complementary and alternative medicine*, *12*(1), 200-210.

González, M. J., Torres, J. L., & Medina, I. (2010). Impact of thermal processing on the activity of gallotannins and condensed tannins from *Hamamelis virginiana* used as functional ingredients in seafood. *Journal of Agricultural and Food Chemistry*, *58*(7), 4274-4283.

Gould, L. H., Walsh, K. A., Vieira, A. R., Herman, K., Williams, I. T., Hall, A. J., & Cole, D. (2013). Surveillance for foodborne disease outbreaks – United States, 1998-2008. *MMWR Surveill Summ, 62*(2), 1-34.

Guillén, G., & Montero García, P. (2007). Polyphenol uses in seafood conservation. *American Journal of Food Technology, 2*, 593-601.

Jiao, J., Fu, Y. J., Zu, Y. G., Luo, M., Wang, W., Zhang, L., & Li, J. (2012). Enzyme-assisted microwave hydro-distillation essential oil from *Fructus forsythia*, chemical constituents, and its antimicrobial and antioxidant activities. *Food Chemistry*, *134*(1), 235-243.

Kanatt, S. R., Chander, R., & Sharma, A. (2010). Antioxidant and antimicrobial activity of pomegranate peel extract improves the shelf life of chicken products. *International Journal of Food Science & Technology*, *45*(2), 216-222.

Kang, M. S., Oh, J. S., Kang, I. C., Hong, S. J., & Choi, C. H. (2008). Inhibitory effect of methyl gallate and gallic acid on oral bacteria. *The Journal of Microbiology*, *46*(6), 744-750.

Taguri, T., Tanaka, T., & Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biological and Pharmaceutical Bulletin*, *27*(12), 1965-1969.

Tian, F., Li, B., Ji, B., Yang, J., Zhang, G., Chen, Y., & Luo, Y. (2009). Antioxidant and antimicrobial activities of consecutive extracts from *Galla chinensis*: The polarity affects the bioactivities. *Food Chemistry*, *113*(1), 173-179.

Xi, D., Liu, C., & Su, Y. C. (2012). Effects of green tea extract on reducing *Vibrio parahaemolyticus* and increasing shelf life of oyster meats. *Food Control*, *25*(1), 368-373.

Yuan, R., & Lin, Y. (2000). Traditional Chinese medicine: an approach to scientific proof and clinical validation. *Pharmacology & Therapeutics*, *86*(2), 191-198.

Zhou, J., Xie, G., & Yan, X. (2011). *Encyclopedia of Traditional Chinese Medicines-Molecular Structures, Pharmacological Activities, Natural Sources and Applications: Vol. 6: Indexes* (Vol. 6). Springer.

CHAPTER 2:

REVIEW OF LITERATURE

1. Seafood Related Pathogens and Safety Concerns

In the United States, an estimated 48 million foodborne illnesses occur annually (Scallan et al., 2011). An outbreak is defined as “the occurrence of two or more cases of a similar illness resulting from ingestion of a common food” (CDC, 2014a). While only a small portion of all illnesses cases are involved in outbreaks, investigation and study of foodborne disease outbreaks can provide more useful information for public health officials, regulatory agencies and the food industry (CDC 2014a, 2014b). In recent years (1998-2008), food safety and outbreaks are serious issues in the USA. The numbers of reported outbreaks increased from the previous years 1973 to 1997, which may suggest improved surveillance. The number of annual reported outbreaks shows a declining trend (Gould et al., 2013).

1.1. Current concerns of seafood safety

According to a report from the Centers for Disease Control and Prevention (CDC), 889 foodborne diseases outbreaks have been reported from 1998 – 2008 associated with aquatic animals (Gould et al., 2013). This is a large portion of all outbreaks reported from a food source, compared to outbreaks attributed to land animals (1585) and plants (790). From an etiological perspective, *Vibrio parahaemolyticus* and scombrotoxin (histamine) have been the leading bacterial and chemical agents,

responsible for the majority of illnesses and outbreaks from seafood products. *V. parahaemolyticus* is associated with more than 50% of all seafood related outbreaks caused by bacterial agents, and scombrotoxin has caused more than half of all seafood related outbreaks from chemical agents (Gould et al., 2013; CDC, 2014a and 2014b).

According to CDC (Gould et al., 2013), there were 353 illnesses associated with outbreaks from *Listera monocytogenes* from 1998 – 2008, of which 61% of people were hospitalized. From 1998 – 2008, *L. monocytogenes* was responsible for 25% of all deaths associated with foodborne illnesses.

1.2. *Vibrio spp.*

Vibrio spp. is a Gram-negative, facultative anaerobic bacteria which occurs naturally in the marine environment. *Vibrio* spp. have a curved-rod shape and are motile with flagella (Thompson et al., 2004). There are more than 80 species in the genus and at least 12 of them can cause human illnesses (Thompson et al., 2004), among them are 8 species are associated with foods (Doyle and Buchanan, 2011). *Vibrio* spp. usually require a small amount of salt for growth and survival, but some species are also found in fresh water and lakes (Abbott et al., 2006). *Vibrios* grow optimally at neutral and slight alkaline environment (7.5-8.5), but growth can still occur in acid conditions at pH 4.5 – 5.0 (Adams and Moss, 2008). The optimal temperature for *Vibrio* growth is approximately 37°C, but *Vibrio* spp. can survive and grow at temperatures between 10 and 43°C.

The isolation of *Vibrio* spp. is based on traditional microbiological techniques (DePaola et al., 2004). Alkaline peptone water is used for enrichment since *Vibrio* spp.

are more tolerant to alkaline conditions compared with other microorganisms (Adams and Moss, 2008). The most commonly used medium for isolation and differentiation of *Vibrio* spp. is thiosulfate-citrate-bile salt-sucrose agar (TCBS). Identification of *Vibrio* spp. is achieved through a group of biological tests (Alsina and Blanch, 1994). Given the specific combination of the results, the species are determined.

Vibriosis refers to the illness caused by *Vibrio* spp. other than *V. cholerae*. Typical symptoms of vibriosis includes watery diarrhea, abdominal cramps, nausea, vomiting, fever and chills (Doyle and Buchanan, 2011). According to CDC's estimation (Scallan et al., 2011), there are approximately 80,000 vibriosis cases, 500 hospitalizations and 100 deaths. Among all pathogenic *Vibrio* spp., *V. parahaemolyticus, V. cholera, and V. vulnificus* are the most species of concern according to CDC's Cholera and Other Vibrios Illness Surveillence (COVIS) annual summary (2011).

V. parahaemolyticus is the leading bacterial cause of foodborne illnesses associated with consumption of seafood (CDC, 2010; Doyle and Buchanan, 2011), mostly from consumption of raw or undercooked fish and shellfish. In the United States, gastrointestinal illnesses caused by *V. parahaemolyticus* are usually associated with ingestion of shellfish such as oysters, crab, shrimp, and lobsters; while in Japan, the consumption of raw fish is the major cause (Adams and Moss, 2008), since raw fish is more commonly consumed in Japan than in the United States. *V. parahaemolyticus* is more often found in shallow coastal area associated with shellfish (Hackney et al. 1980), although fish can also be cross-contaminated during transportation, and in the kitchen. The absence of adequate heat treatments, inadequate cooking, and post-

processing contamination are all possible paths for foodborne illness from *V. parahaemolyticus* (Doyle and Buchanan, 2011).

V. parahaemolyticus associated outbreaks from 1973 to 1998 were summarized by Daniels et al. (2000). A typical large outbreak occurred in 1978, when 1133 persons were infected after eating dinner in Port Allen, LA. The food was boiled shrimp. The raw shrimp was purchased and held in a wooden box, boiled and put back into the same boxes where the raw shrimp was held. The shrimp was transported unrefrigerated and held without refrigeration for several hours before being served. Although food handling practices have been improved over the years, *V. parahaemolyticus* remains a major foodborne pathogen. The CDC's data (2010) shows the increase of foodborne illnesses associated with *V. parahaemolyticus* since 2000.

1.3. *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, facultative anaerobic bacterium with motility via flagella. The genus *Listeria* contains eight species, and six of them are closely related (Doyle and Buchanan, 2011). Among the six species, only *L. monocytogenes* and *L. ivanovii* are considered as human pathogens, and the only important human pathogen is *L. monocytogenes* (Doyle and Buchanan, 2011). There are 13 serotypes of *L. monocytogenes*, but the serotypes of concern are 1/2a, 1/2b, and 4b (Seeliger and Höhne, 1979). The isolation of *L. monocytogenes* requires serotyping (Doyle and Buchanan, 2011). Methods of isolation include phage-typing, molecular typing (ribotyping, enzyme electrophoresis, DNA microrestriction, etc.), and recently developed PCR-based sub-typing methods (Doyle and Buchanan, 2011 and Doumith et

al., 2005). Rapid detection methods are also available based on PCR and DNA probe principles (Ingianni et al., 2001).

L. monocytogenes can survive and grow in a wide temperature range, from 0 °C to 42°C, with optimal growth between 30°C and 35°C (Adams and Moss, 2008). It is the most heat resistant vegetative foodborne pathogen. *L. monocytogenes* is inactivated at temperatures less than 0°C or higher than 50°C (Doyle and Buchanan, 2011). *L. monocytogenes* grows in foods with pH between 5.5 and 9.6; however, given optimal nutritional environment, bacterial growth of some strains occur at pH 4.4 (Doyle and Buchanan, 2011). The optimal water activity (a_w) for *L. monocytogenes* is 0.97; however, some strains can grow at $a_w = 0.90$, and survive at $a_w = 0.83$ (Shahamat et al., 1980). *L. monocytogenes* can also survive and grow in a high salt environment (10%-12%), and can grow to high population levels at moderate salt content (6.5%) (Doyle and Buchanan, 2011). Large inoculum size (106-107 cfu/ml) facilitates the survival and growth in adverse environment (Koutsoumanis and Sofos, 2005)

Listeria monocytogenes is ubiquitous in the environment and humans are frequently exposed to *L. monocytogenes* (Adams and Moss, 2008). However, the occurrence of infection is low. It is suggested that only when susceptible individuals are exposed to the dose of 10^3 cfu/g or higher does infection occur (Adams and Moss, 2008).

The illness listeriosis is usually caused by consumption of RTE foods contaminated with *L. monocytogenes* (Schlech et al., 1983). Listeriosis is a serious food safety concern for RTE foods in the USA (Silk et al., 2012). The most susceptible groups of people are older adults, pregnant women, infants, and people with weak or

compromised immune system (Adam and Moss, 2008). Listeriosis in pregnant women can cause miscarriage, stillbirth, premature delivery and life-threaten infections to newborn infants (Jackson et al., 2010). The typical symptoms of listeriosis in people other than pregnant women results in flu-like symptoms, symptoms similar to meningitis such as headache, stiff neck, loss of balance, and so on (Adams and Moss, 2008).

The monitoring of listeriosis outbreaks began in 1981, on a listeriosis outbreak in Canada (Schlech et al., 1983). Since then, more than 10 major listeriosis outbreaks have been traced to various food products (Doyle and Buchanan, 2011). According to CDC's estimation (Scallan et al., 2011), on an annual basis there are approximately 1600 cases of listeriosis and about 260 deaths in the United States.

L. monocytogenes can grow at refrigeration temperatures (Doyle and Buchanan, 2011). According to the US Food and Drug Administration (USFDA)'s risk assessment (2003), RTE meat (Deli, non-reheated frankfurters, etc.), dairy products, smoked seafood and RTE crustaceans were among the high risk foods associated with foodborne listeriosis. (CDC, 2011).

1.4. Histamine (scombrotoxin) poisoning and *Morganella morganii*

Histamine is a naturally occurred organic nitrogenous compound in humans. It is involved in local immune responses as well as regulating physiological function in the gut and acting as a neurotransmitter by binding to receptors on the cellular membrane (Lehane and Olley, 2000). When present at high levels (>500 ppm), histamine can cause poisoning thus is considered as a chemical hazard in food primarily associated with consumption of temperature abused scombrotoxic fish. The symptoms of histamine

poisoning can mimic salmonellosis (Russell and Maretic, 1986) and food allergies (Taylor, 1985). Histamine fish poisoning (HFP), also referred as scombroid fish poisoning. It is the second leading cause of foodborne illness from consumption of seafood (Olsen et al., 2000; Lynch et al., 2006). According to CDC's data (2000 and 2006), HFP outbreaks accounted for more than half of all foodborne illnesses and outbreaks caused by chemical agents. HFP is both a public health concern (Sanchez-Guerrero et al., 1997) and an issue in trade (Anonymous, 1998).

Histamine is produced by bacterial decarboxylases in scombroid fish that have high concentrations of histidine in their muscle tissue (Love, 1980). When scombrotoxic fish are stored for long periods of time above 4.4°C histamine is formed from free histidine in the fish muscle (Sapin-Jaloustre and Sapin-Jaloustre, 1957; Eitenmiller et al., 1982). Lehane and Olley (2000) summarized the factors affecting the occurrence of HFP, including post-catch contamination, temperature abuse on-board fishing vessels, inadequate chilling, freezing and thawing procedures, temperature abuse during drying and smoking procedures, improper canning, improper fermentation procedures, and temperature abuse during storage.

Many bacterial species of *Enterobacteriaceae* family are known to produce histidine decarboxylase (HD). Taylor et al. (1978) have identified 112 species of these histidine decarboxylating bacteria (HDB). *Morganella morganii*, some strains of *Klebsiella pneumonia* and *Hafnia alvei* are considered the most productive histamine formers (Stratton and Taylor, 1991). *Morganella morganii* is a Gram-negative, facultative anaerobic bacterium found in the gastrointestinal system of human and vertebrate animals as natural flora (O'Hara et al., 2000). *M. morganii* possesses HD, and can

produce histamine when fish are held at temperatures greater than 4°C (Stratton and Taylor, 1991). Since it is a part of the natural flora in fish gills, guts and external surfaces, the contact of HD and histidine in fish muscle is very likely to occur. (FDA, 2011).

1.5. Current processing strategies to ensure seafood safety

Thermal processing, low-temperature preservation, high-pressure processing, control of water activity, modified-atmosphere packaging, and chemical preservation are the most commonly used methods used to minimize the biological and chemical hazards in seafood (Adams and Moss, 2008; FDA, 2011). To ensure seafood safety, usually a combination of several preserving methods is applied.

Research has shown that proper thermal processing will destroy or deactivate pathogens (FDA, 2011). However, some seafood products are consumed raw without heat treatment or with mild heat treatment that are not sufficient to destroy pathogens (Awuha et al., 2007). For those products, additional processing methods alternative to heat treatments have been developed, such as high hydrostatic pressure processing, freezing, dehydration and ionizing radiation. Morris et al. (2007) summarized and discussed the features of several novel non-thermal processing methods for food products.

High hydrostatic pressure processing is widely used in shellfish products (Murchie et al., 2005). Studies shown that HPP can successfully inactivate pathogenic bacteria (Farkas and Hoover, 2000) and some viruses (Kinsley et al., 2002), extending the shelf life of seafood (Hurtado et al., 2000; Considine et al., 2008). It is commonly used for

shellfish processing (Voisin, 2002). However, HPP has its limitations. The processing is insufficient to sterilize the seafood (Murchie et al., 2005) by itself. Moreover, the efficacy is influenced by the chemical environment around microorganisms (Alpas et al., 2000). Furthermore, possible adverse effects on sensory properties may be a problem (Gudmundsson and Hafsteinsson, 2002).

Irradiation includes gamma ray radiation, electron beam (EB), ultraviolet (UV) radiation, and x-rays radiation (Morris et al., 2007). It is effective in reducing bacteria, parasites and insects in food, and irradiation has been approved by FDA for controlling *V. parahaemolyticus* and *V. vulnificus* in molluscan shellfish (FDA, 2011), and crustaceans (FDA 2014). At higher doses, it can cause generation of carcinogens in certain food (Morris et al., 2007). Individual quick freezing (IQF) processing is usually combined with extended frozen storage period to control parasites and some bacterial strains, such as *V. parahaemolyticus* and *V. vulnificus*.

The addition of chemical antimicrobials is another important preservation strategy. Common chemical preservatives can be roughly divided in traditional antimicrobials and naturally occurred antimicrobials (Doyle and Buchanan, 2011). The traditional antimicrobials include organic acids and derivatives, phosphoric acid and derivatives, lactoferrin and lactoferricin, nitrates, sulfites, lysozyme and so on. There are also natural occurring antimicrobials such as chitosan from animal sources, and essential oils and phenolic compounds from plant source (Doyle and Buchanan, 2011). The following sections describe the use of plant-sourced antimicrobials for potential use in seafood products.

2. Plant-Source Antimicrobial Compounds

There are more than 250,000 species of plants on the earth (Cowan, 1999). Among all plant species, less than 10% are edible. Plant-source antimicrobial compounds can be divided in several major groups – phenolic and polyphenolic compounds, essential oils, alkaloids, plant peptides, and others (Cowan, 1999).

In addition to serving as food sources, plants are also used for medicinal purposes. The history of plant being used for medication is estimated to be more than 6,000 years according to archeological evidence (Stockwell, 1988).

Another potential advantage of plant-source antimicrobials is that many can be obtained from by-products of food industry. For example, fruit peel (Al-Zoreky, 2009), seeds (Perumalla and Hettiarachchy, 2011), or kernels (Engels et al., 2009a, 2009b, 2011) are all potential sources of natural antimicrobials.

2.1. Phenolic and polyphenolic compounds

Phenolic and polyphenolic compounds are ubiquitous in plants. They are formed through secondary metabolism and function in defending the plant against pathogens, pests, and herbivorous animals (Daglia, 2012). Phenolic and polyphenolic compounds are further classified as flavonoids, phenolic acids, hydrolysable tannins and condensed tannins, lignans, etc. These compounds have shown activity in antibacterial, antifungal, or antiviral assays (Cushnie and Lamb, 2005; Engels et al., 2011; Hatano et al., 2003; Tian et al., 2009b; Yi et al., 2010).

The antimicrobial mechanisms of phenolic compounds are yet to be described, but researchers are conducting studies on the subject. The antimicrobial mechanisms of

flavonoids are summarized and discussed by Cushnie and Lamb (2005). The inhibition of the synthesis of nucleic acids (Mori et al., 1987), inhibition of the cell membrane functions of microorganisms (Tsuchiya et al., 2000), and inhibition of the transportation of electrons in energy metabolism (Haraguchi et al., 1998) are suggested as possible antimicrobial mechanisms of flavonoids. It is unknown, however, whether flavonoids are bacteriostatic or bactericidal, or both (Cushnie and Lamb, 2005).

Many plants have been screened for antimicrobial phenolic compounds. Studies have been conducted addressing the antimicrobial activity of polyphenols extracted from tea (Friedman et al., 2007). Xi et al. (2012) reported that tea extracts inhibited *V. parahaemolyticus* as well as total psychrotrophic microflora, extending the shelf life of the raw oysters. Since tea is a relatively expensive, the prospect of using it in an industrial application is uncertain. There were also changes in the oyster's sensory profile.

Taguri et al. (2004) studied 10 different antimicrobial polyphenolic compounds derived from plant sources, including epigallocatechin gallate, tannic acid, punicalagin, procyanidins and other extracts from tea. The antimicrobial assays were conducted on *Staphylococcus*, *Salmonella*, and different *E. coli* and *Vibrio* strains. The results showed that most polyphenolic compounds, when present at certain concentrations (0.05-32 mg/ml), will inhibit selected bacteria. The antimicrobial activity of polyphenols may also be species-specific. Proestos et al. (2006) analyzed the antioxidant and antimicrobial activity of the polyphenols from plants. Gram-negative bacteria were more resistant to polyphenols.

The agar diffusion assay of phenolic compounds may be influenced by solubility of the compounds (Proestos et al. 2006). Ikigai et al. (1993) studied the antimicrobial mechanism of catechins derived from tea. The cell membrane was simulated using liposomes and the damage to the liposome was determined by measuring the leakage of intra-liposomal molecules (Ikigai et al., 1993). This did not explain how membrane is damaged but was evidence of the catechins' antimicrobial property.

2.2. Essential oils

Essential oils (EOs) are plant-source aromatic volatile oily liquids (Burt, 2004). The EOs can be obtained by several methods including expression, fermentation, extraction, etc., but are mostly obtained by steam distillation (Van de Braak and Leijten, 1999). Plants containing essential oils can also be used as antimicrobials (Solórzano-Santos et al., 2012). One of the earliest uses of EOs was reported from ancient Greece and Rome (Guenther, 1948). A lab-conducted antimicrobial test was carried out by De la Croix in 1881 (Boyle, 1955). Currently EOs are not only used in foods, perfumes and pharmaceuticals, but also used as natural preservatives because of their antioxidant and antimicrobial activity (Burt, 2004). Some herbal extracts, such as clove bud extract, are listed as generally recognized as safe (GRAS) substances by FDA. The composition of many EOs have been studied; and some of the EOs with commercial potentials are summarized by Bauer et al. (2008). Usually more than one volatile compound is present in an individual plant, and the composition is varied among different cultivars, harvesting seasons, or geographical locations (Arras and Grella, 1992; Marotti et al., 1994; Juliano et al., 2000).

The antibacterial effects of EOs derived from some common plants, including cinnamon, clove, rosemary, thyme, lemongrass, sage, and so on were tested *in vitro* and found to be promising (Smith-Palmer et al., 1998; Hammer et al., 1999; Pintore et al., 2002; Burt and Reinders, 2003). The minimum inhibitory concentrations of these EOs ranged from 0.2 to 10 μl/ml (Burt, 2004). The purified major components of the EOs were also tested in vitro on common foodborne pathogens. Kim et al. (1995) studied inhibitory effects of eugenol, citral, perillaldehyde, and carvacrol on *E. coli*, *L. monocytogenes* and *Salmonella* Typhimurium, respectively, and the minimum inhibitory concentratons (MICs) were typically 0.5 to 1 μl/ml. Cosentino et al. (1999) conducted similar research on carvacrol, terpineol and thymol on *E. coli, L. monocytogenes, S.* Typhimurium*, Staphylococcus aureus*, and *Bacillus cereus* and reported similar MICs. However, the protocols of antimicrobial assays for EOs' MIC may differ among researchers, thus direct comparisons of MICs may be difficult (Burt, 2004).

2.3. Alkaloids

Alkaloids have been recognized for thousands of years as a poison; however, the first alkaloid isolated for a medicinal purpose was opium, in the early 19th century (Roberts and Wink, 1998). Alkaloids are involved in secondary metabolism of plants when they are produced as a chemical defense (Roberts and Wink, 1998). Alkaloids are those compounds that are "cyclic containing nitrogen in a negative oxidation state which is of limited distribution in living organisms" (Pelletier, 1983). These compounds are usually toxic and may have a strong bitter taste, functioning as deterrents to animal and insects (Bernays, 1983).

Different individual alkaloids may have direct antimicrobial activity, or antibiotic enhancement properties (Cushnie et al. 2014). For example, Yan et al. (2008) discussed the antimicrobial properties of berberine. More research addressed extractions of individual alkaloids from medicinal plants (Navarro and Delgado, 1999). Domadia et al. (2008) suggested that the antibacterial activity of alkaloids is by enzyme binding and deactivation. Alkaloids may also find their use in synergies with classical antimicrobials (Mitchell et al., 2012).

2.4. Plant peptides

The first discovered antimicrobial peptide originating from plants is purothionin extracted from wheat flour (De Caleya et al., 1972). This compound inhibits Gram-positive and Gram-negative bacteria. These plant antimicrobial peptides are involved in the defense mechanism of the plant against pathogens (Pelegrini et al., 2011). The length of these plant-source antimicrobial peptides vary from 30 to 50 amino acid residues (Broekaert et al., 1997), and contain a high portion of cysteine and glycine, which form disulfide bridges to enhance the structural stability (Pelegrini et al., 2011). The antimicrobial actions may be caused by the amphiphilicity and cationic charge of the peptide (Yeaman and Yount, 2003), yet more research is needed to elucidate the selectivity and individual antimicrobial mechanism (Pelegrini et al., 2011).

3. Typical Medicinal Plants – Research and Application

3.1. Pomegranate peel (*Punica granatum* L.)

Pomegranate (*Punica granatum* L.) originates from the Middle East area and was first introduced into North America by Spanish settlers in the 18th century. The production of pomegranate around the world is approximately 1.5 million tons with about half coming from Iran (Fischer et al., 2011). According to the Food and Agriculture Organization (FAO, 2010) the market for pomegranate has increased during recent years. Pomegranate fruit and juice are reported to have abundant antioxidant contents (Gil et al., 2000) and associated health benefits (Aviram and Dornfeld, 2001). Pomegranate peel (PP), the by-product of juice manufacture, possesses stronger antioxidant activity (Guo et al., 2003; Cai et al.,2004; Singh et al.,2002; Li et al., 2006) and has been used as a traditional remedy to treat gastroenteritis, diarrhea, and parasites (Lansky et al., 2000; Longtin, 2003). Recent research shows PP's antimicrobial (Al-Zoreky, 2009; Fawole et al., 2012), antioxidant (Guo et al., 2003; Li et al., 2006), anticancer (Lansky and Newman, 2007), and antidiabetic bioactivities (Khalil, 2004).

The extracts of PP are a complex of polyphenolic compounds that can be categorized as hydrolysable tannins, hydroxybenzoic acids, hydroxycinamic acids and dihydroflavonols (Fischer et al., 2011). Most studies have addressed the antioxidant properties of PP (Singh et al., 2002; Guo et al., 2003; Li et al., 2006). Guo et al. (2003) studied the antioxidant activities of the peel, pulp and seed of 28 fruits using ferric reducing/antioxidant power (FRAP) assay and found the peel of white pomegranate has an exceptionally high ferric reducing equivalence. Li et al. (2006) went further studying the antioxidant activity between the peel and pulp of pomegranate.

The antimicrobial activity of PP on common pathogenic bacteria has been summarized by Al-Zoreky (2009). The antimicrobial assays show that the minimum inhibitory concentration (MIC) of PP aqueous methanol extracts on tested foodborne pathogens ranges from 0.25 to 4 mg/ml in culturing media (Al-Zoreky, 2009). Another representative research study shows MICs of PP against pathogens ranging from 0.2 to 0.78 mg/ml (Fawole et al., 2012).

Polyphenols from PP are reported to inhibit viruses such as influenza virus (Haidari et al., 2009 and Sundararajan et al., 2010). The antimicrobial mechanism is suggested to be by both direct damage to the structure of the virus and indirect inactivation through the inhibition of viral reproduction (Kotwal, 2008). Pomegranate polyphenols inhibited the RNA replication at 0.04 mg/ml and also have synergetic antiviral effect when applied with oseltamivir (Haidari et al. 2009).

3.2. Chinese gallnuts (*Rhus chinensis/Galla chinensis*)

Chinese gallnuts (CG) (or *wu bei zi* in Chinese language), the common name of the medicinal plant source material (*Galla chinensis*), is the gall formed on a species of sumac tree (*Rhus chinensis*). *Rhus chinensis* belongs to the family *Anacardiaceae* and genus *Rhus*. (Miller et al., 2001). When the *R. chinensis* is invaded by aphids, galls are formed on leaves. *Schlechtendalia chinensis* is specifically used for CG production (Djakpo and Yao, 2010), while invasion of other aphids may also produce galls with inferior quality.

Gallic acid (GA), methyl gallate, and gallotannins (hydrolysable tannins) are the major components of CG. The molecular structure of hydrolysable tannins include a

glucose center and 1-5 gallic acid units attached to the glucose center through glycosidic bonds. Gallic acid units attach to galloyl units to form depsides (Xiang et al. 2007). The first detail characterization CG composition was conducted by Nishizawa et al. (1982). The GA and GG contents were found to be related to extraction solvents.

Historically, CG has been widely used in TCM to treat various illnesses such as diarrhea, ulcer, skin infection, toxicosis, and oral diseases (Gao et al., 2000; Tian et al. 2009a, 2009b). Modern studies of CG show its antibacterial (Tian et al., 2009a and 2009b; Feng et al., 2012), antiviral (Wang et al., 2006; Gu et al., 2007), anticancer (Kuo et al., 2009), antioxidant (Cai et al., 2004), antidiabetic (Shim et al., 2003), anti-inflammatory (Kim et al., 2005), anti-cavity (Zou et al., 2008; Huang et al., 2012) and hepato-protective (Oh et al., 2002) activities.

The CG aqueous extracts and extracts in organic solvents demonstrated strong bactericidal and bacteriostatic activities in previous research (Wu-Yuan et al., 1988; Tian et al., 2009a and 2009b; Feng et al., 2012). Different solvents were used in extraction, including water, ethyl acetate, ethanol and ethyl either. The correlation between the antimicrobial activity and the polarity of extract was discussed by Tian et al. (2009a). Extract of CG with lower polarity (extracted with ethanol and ethyl acetate) showed stronger antimicrobial activity than those with higher polarity extracted with water. In Feng's research (2012), the inhibitory effect of 67 medicinal plants on plant pathogens was studied, and the CG extract demonstrated the strongest bacterial inhibition among all plant extracts tested.

The major components in CG, the hydrolysible tannins, such as tannic acid and ellagic acid are reported to have antimicrobial activities (Zhao et al., 1997, Smith et al.,

2005, Buzzini et al., 2008). The antimicrobial mechanisms are believed to be from two aspects – ion chelation (Sebat et al., 2001) and damages to cell wall and membrane structure and functionality, causing leakage of cytoplasm from membrane as well as enzyme dysfunction (Wu-Yuan et al, 1988).

Bacteria can also overcome the inhibition by tannins (Smith et al., 2005). Some specific strains of bacteria can develop better metal ion sequestration when exposed to tannins (Mila et al., 1996). Tannins can decrease the fluidity of the cell membrane (Smith et al., 2005). Some bacteria can alter the composition of cell membrane by increasing the unsaturated lipids in the membrane to compensate for this effect (Rozes et al., 1998). It is also suggested that the metabolism level of bacteria is increased so that the damaged membrane can be repaired or replaced; however, more evidence of this metabolic change is needed (Smith et al., 2005).

The CG's antibacterial and ion-chelating properties have been studied for protection of enamel (Zou, L. et al. 2008, Huang, X. L. et al. 2012). The chemical composition of water extract of CG was analyzed by Huang (2012) using a LC-TOF-MS method, and showed that most of the active compounds were gallic acid and galloyl glucose or oligo-galloyl glucose and their isomers. The research results demonstrated that the CG water extract significantly inhibited enamel demineralization. The precipitation of proteins and the chelation of ions caused by CG extracts played the most important role in demineralization prevention. Although antimicrobial tests were not conducted in this research, the mechanisms of enamel protection (i.e. protein precipitation and ion chelation) may be important for bacterial inhibition.

3.3. Baikal skullcap (*Scutellaria baicalensis* Georgi)

The root of the plant Baikal Skullcap (BS), named *huang qin* in Chinese, has been used as a treatment for bacterial and viral infection, inflammation, and as an alternative treatment for cancer (Lu et al., 2011). It is among the 50 fundamental herbs in traditional Chinese medicine (TCM) and is officially recorded in Chinese Pharmacopoeia (Li et al., 2004). Over 40 components have been identified from BS, and the major active compounds in BS are flavonoids (Horvath et al., 2005), a group of phenolic compounds commonly occur in the plant kingdom (Havsteen, 1983). The BS contains a complex of flavonoids (Horvath et al., 2005) reported to have antimicrobial properties (Harborne, 1999; Arweiler et al., 2011; Lu et al., 2011).

3.4. Forsythia fruit (*Forsythia suspensa* (Thunb.) Vahl)

Forsythia is a deciduous, ornamental, woody, and shrub that can be identified by its bright yellow flowers in early spring. *Forsythia suspensa* is native from China, with cultivars around the world. Forsythia fruit (FF) is a dry capsule with several winged seeds inside. It is used as herbal medicine and is among the 50 fundamental herbs in TCM.

The FF contains both phenolic compounds such as suspensaside (Guo et al., 2007) and essential oils such as rengyol (Endo et al., 2004). The discovery of essential oils contents in FF were initially discussed by Endo et al. (2004) in their research. Both the essential oils contents and the phenolic compounds have been shown to have antimicrobial activities (Jiao et al., 2012, Qu et al., 2008, 2012), although to what extent

the essential oils and/or polyphenols contribute to the total antimicrobial activity remains unclear.

Two major compounds found in FF, forsythiaside and forsythin, were studied for their antioxidant and antimicrobial activities (Qu et al. 2008). The antimicrobial study was conducted on both Gram-positive and Gram-negative bacteria. The results showed that both compounds inhibited selected bacterial strains.

4. Future Directions of Polyphenolic Antimicrobials in Seafood Preservation.

The polyphenolic compounds have been studied in seafood conservation because of their antioxidant and antimicrobial properties (Guillén and García, 2007). In seafood products, naturally occurring polyphenols act as preservatives similarly as synthetic phenolic compounds, since they share the aromatic ring structure and hydroxyl groups. However, since the plant-derived polyphenols can react with protein content in food, the antioxidant activity of them may be compromised (Shahidi and Naczk, 2004).

One major challenge of applying polyphenols in food products is the potential negative impact to sensory properties of the food product (Pérez-Mateos et al., 2001). The polyphenols may change the flavor, color and scent of the food product (Xi, Y. et al., 2012).

A perspective of polyphenol application demands more research, showing that polyphenols can make traditional antimicrobial more effective. Hatano et al. (2005) summarized their research on inhibition of polyphenol against methicillin-resistant *Staphylococcus aureus* and the synergies between polyphenols and antibiotics (Hatano

et al., 2003). Although the research is for clinical potentials, more synergetic research can be conducted on preservation.

Polyphenolic antimicrobials can be combined with other processing. Juneja et al. (2013) developed a predictive model for the effect of combined treatment of sodium chloride and apple polyphenols on the heat resistance of *L. monocytogenes*. The results demonstrated the synergy between polyphenols and salt in weakening the heat resistance of *L. monocytogenes*.

Plant-source phenolic compounds can assist with the smoking in the processing of fish and meat products to inhibit spoilage and pathogenic bacterial growth. For example, liquid smoke, the condensation of pyrolysis of woods, bark, nut shells and other smoke-generating material contains phenolic compounds, which can inhibit microorganisms, such as *Listeria, Salmonella, E. coli* and *Staphylococcus spp*., in smoked foods (Lingbeck, 2014).

Polyphenols may change chemical structure due to thermal processing. González et al. (2010) found that the thermal processing of food can also hydrolyze the hydrolysable tannins to release free gallic acid. This results in tannins' better antioxidant activity. There was no discussion addressing the antimicrobial activity change; however, since it was shown that penta-galloyl-O-D-glucose has the strongest antimicrobial activity (Tian et al., 2009b), it can be implied that due to the generation of penta-galloyl-O-D-glucose, the antimicrobial activity is also strengthened.

Combinations of natural antimicrobials have been discussed. Li et al. (2012) found the combination of a plant-source antimicrobial (polyphenols, essential oil) and an

animal-source antimicrobial (chitosan) can significantly extend the shelf life of fish products.

References

Abbott, S. L., Janda, J. M., Johnson, J. A., Farmer III, J. J. (2006) Vibrio and related organisms. Ch. 47 In: Murray, P. R., Baron, E. J., Jorgensen, J.H., Pfaller, M. A. & Landry, M.L. (eds.). *Manual of Clinical Microbiology: Volume 1*, (Ed. 9), 723-733.

Adams, M. R., & Moss, M. O. (2008). *Food Microbiology.* Cambridge, UK: RSC Publishing.

Alkan, D., Aydemir, L. Y., Arcan, I., Yavuzdurmaz, H., Atabay, H. I., Ceylan, C., & Yemenicioğlu, A. (2011). Development of flexible antimicrobial packaging materials against *Campylobacter jejuni* by incorporation of gallic acid into zein-based films. *Journal of Agricultural and Food Chemistry*, *59*(20), 11003-11010.

Alpas, H., Kalchayanand, N., Bozoglu, F., & Ray, B. (2000). Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. *International Journal of Food Microbiology*, *60*(1), 33-42.

Alsina, M., & Blanch, A. R. (1994). A set of keys for biochemical identification of environmental Vibrio species. *Journal of Applied Bacteriology*, *76*(1), 79-85.

Al-Zoreky, N. S. (2009). Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. *International Journal of Food Microbiology*, *134*(3), 244-248.

Arnold, S. H., & Brown, W. D. (1978). Histamine toxicity from fish products. *Advances in Food Research*, *24*, 113-154.

Arras, G., & Grella, G. E. (1992). Wild thyme, *Thymus capitatus*, essential oil seasonal changes and antimycotic activity. *Journal of Horticultural Science*, *67*(2), 197-202.

Arweiler, N. B., Pergola, G., Kuenz, J., Hellwig, E., Sculean, A., & Auschill, T. M. (2011). Clinical and antibacterial effect of an anti-inflammatory toothpaste formulation with *Scutellaria baicalensis* extract on experimental gingivitis. *Clinical Oral Investigations*, *15*(6), 909-913.

Aviram, M., & Dornfeld, L. (2001). Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. *Atherosclerosis*, *158*(1), 195-198.

Awuah, G. B., Ramaswamy, H. S., & Economides, A. (2007). Thermal processing and quality: Principles and overview. *Chemical Engineering and Processing: Process Intensification*, *46*(6), 584-602.

Bauer, K., Garbe, D., & Surburg, H. (2008). *Common Fragrance and Flavor Materials: Preparation, Properties and Uses*. John Wiley & Sons. 167-226

Boyle, W. (1955). Spices and essential oils as preservatives. *The American Perfumer and Essential Oil Review* 66, 25–28.

Broekaert, W. F., Cammue, B. P., De Bolle, M. F., Thevissen, K., De Samblanx, G. W., Osborn, R. W., & Nielson, K. (1997). Antimicrobial peptides from plants. *Critical Reviews in Plant Sciences*, *16*(3), 297-323.

Burt, S. A., & Reinders, R. D. (2003). Antibacterial activity of selected plant essential oils against *Escherichia coli* O157: H7. *Letters in applied microbiology*, *36*(3), 162-167.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods – a review. *International Journal of Food Microbiology*, *94*(3), 223-253.

Buzzini, P., Arapitsas, P., Goretti, M., Branda, E., Turchetti, B., Pinelli, P., ... & Romani, A. (2008). Antimicrobial and antiviral activity of hydrolysable tannins. *Mini Reviews in Medicinal Chemistry*, *8*(12), 1179-1187.

Cai, Y., Luo, Q., Sun, M., & Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences*, *74*(17), 2157-2184.

Centers for Disease Control and Prevention (CDC). (2010). Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food-10 states, 2009. *MMWR. Morbidity and Mortality Weekly Report*, *59*(14), 418-422.

Centers for Disease Control and Prevention. (2011a). Cholera and other *Vibrios* Illness Surveillence (COVIS) Annual Summary 2011. 1-10

Centers for Disease Control and Prevention. (2011b). Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe--United States, August-September 2011. *MMWR. Morbidity and Mortality Weekly Report*, *60*(39), 1357.

Centers for Disease Control and Prevention (CDC). (2014a) Surveillance for Foodborne Disease Outbreaks, United States, 2011, Annual Report. Atlanta, Georgia: US Department of Health and Human Services, CDC. 1-14.

Centers for Disease Control and Prevention (CDC). (2014b) Surveillance for Foodborne Disease Outbreaks, United States, 2012, Annual Report. Atlanta, Georgia: US Department of Health and Human Services, CDC. 1-14.

Considine, K. M., Kelly, A. L., Fitzgerald, G. F., Hill, C., & Sleator, R. D. (2008). High‐pressure processing-effects on microbial food safety and food quality. *FEMS Microbiology Letters*, *281*(1), 1-9.

Cosentino, S., Tuberoso, C. I. G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., & Palmas, F. (1999). In vitro antimicrobial activity and chemical composition of Sardinian thymus essential oils. *Letters in Applied Microbiology*, *29*(2), 130-135.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, *12*(4), 564-582.

Cushnie, T. P., & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, *26*(5), 343-356.

Cushnie, T. P., Cushnie, B., & Lamb, A. J. (2014). Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. *International Journal of Antimicrobial Agents,* in press:http://*dx.doi.org/10.1016/j.ijantimicag.2014.06.001*.

Daniels, N. A., MacKinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., ... & Slutsker, L. (2000). *Vibrio parahaemolyticus* infections in the United States, 1973–1998. *Journal of Infectious Diseases*, *181*(5), 1661-1666.

DePaola, A., & Kaysner, C. A. (2004). *Vibrio*. Bacteriological Analytical Manual online. US. Food and Drug Administration, Washington, DC. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm

De Caleya, R. F., Gonzalez-Pascual, B., García-Olmedo, F., & Carbonero, P. (1972). Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro. *Applied Microbiology*, *23*(5), 998-1000.

De Coninck, B., Cammue, B., & Thevissen, K. (2013). Modes of antifungal action and *in planta* functions of plant defensins and defensin-like peptides. *Fungal Biology Reviews*, *26*(4), 109-120.

Doumith, M., Jacquet, C., Gerner-Smidt, P., Graves, L. M., Loncarevic, S., Mathisen, T., Martin, P. (2005). Multicenter validation of a multiplex PCR assay for differentiating the major *Listeria monocytogenes* serovars 1/2a, 1/2b, 1/2c, and 4b: toward an international standard. *Journal of Food Protection*, *68*(12), 2648-2650.

Doyle, M. P., & Buchanan, R. (2011). Food Microbiology: Fundamentals and Frontiers (4th edition) ASM Press.

Eitenmiller, R. R., Orr, J. H., & Wallis, W. W. (1982). Histamine formation in fish: microbiological and biochemical conditions. *Chemistry and Biochemistry of Marine Food Products*, 39-50.

Endo, K., & Hikino, H. (1984). Structures of rengyol, rengyoxide, and rengyolone, new cyclohexylethane derivatives from Forsythia suspensa fruits. *Canadian Journal of Chemistry*, *62*(10), 2011-2014.

Engels, C., Knodler, M., Zhao, Y.-Y., Carle, R., Gänzle, M. G., & Schieber, A. (2009a). Antimicrobial activity of gallotannins isolated from mango (Mangifera indica L.) kernels. *Journal of Agricultural and Food Chemistry, 57*(17), 7712-7718.

Engels, C., Ganzle, M. G., & Schieber, A. (2009b). Fractionation of gallotannins from mango (Mangifera indica L.) kernels by high-speed counter-current chromatography and determination of their antibacterial activity. *Journal of Agricultural and Food Chemistry*, *58*(2), 775-780.

Engels, C., Schieber, A., & Gänzle, M. G. (2011). Studies on the inhibitory spectrum and mode of antimicrobial action of gallotannins from mango kernels (Mangifera indica L.). *Applied and Environmental Microbiology*.

Farkas, D. F., & Hoover, D. G. (2000). High pressure processing. *Journal of Food Science*, *65*(s8), 47-64.

Fawole, O. A., Makunga, N. P., & Opara, U. L. (2012). Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. *BMC Complementary and Alternative Medicine*, *12*(1), 200-210.

Feng, C. T., Su, H. J., Chen, C. T., Ho, W. C., Tsou, Y. R., & Chern, L. L. (2012). Inhibitory effects of chinese medicinal herbs on plant-pathogenic bacteria and identification of the active components from gallnuts of chinese sumac. *Plant Disease*, *96*(8), 1193-1197.

Fischer, U. A., Carle, R., & Kammerer, D. R. (2011). Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MS. *Food Chemistry*, *127*(2), 807-821.

Friedman, M. (2007). Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. *Molecular Nutrition & Food research*, *51*(1), 116-134.

Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M., & Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry*, *48*(10), 4581-4589.

González, M. J., Torres, J. L., & Medina, I. (2010). Impact of thermal processing on the activity of gallotannins and condensed tannins from *Hamamelis virginiana* used as functional ingredients in seafood. *Journal of Agricultural and Food Chemistry*, *58*(7), 4274-4283.

González-Lamothe, R., Mitchell, G., Gattuso, M., Diarra, M. S., Malouin, F., & Bouarab, K. (2009). Plant antimicrobial agents and their effects on plant and human pathogens. *International Journal of Molecular Sciences*, *10*(8), 3400-3419.

Gould, L. H., Walsh, K. A., Vieira, A. R., Herman, K., Williams, I. T., Hall, A. J., Cole, D. (2013). Surveillance for foodborne disease outbreaks – United States, 1998-2008. *MMWR Surveill Summ*, *62*(2), 1-34.

Gudmundsson, M., Hafsteinsson, H., Ohlsson, T., & Bengtsson, N. (2002). Minimal processing in practice: seafood. *Minimal Processing Technologies in the Food Industry*, 245-266.

Guenther, E. (1948). *The Essential Oils.* D. Van Nostrand, New York.

Guillén, G., & Montero García, P. (2007). Polyphenol uses in seafood conservation. American Journal of Food Technology, 2, 593-601.

Guo, C., Yang, J., Wei, J., Li, Y., Xu, J., & Jiang, Y. (2003). Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. *Nutrition Research*, *23*(12), 1719-1726.

Hackney, C. R., Ray, B., & Speck, M. L. (1980). Incidence of *Vibrio parahaemolyticus* in and The Microbiological Quality of Seafood in North Carolina. *Journal of Food Protection*, *43*(10), 769-773.

Haidari, M., Ali, M., Ward Casscells III, S., & Madjid, M. (2009). Pomegranate (*Punica granatum*) purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir. *Phytomedicine*, *16*(12), 1127-1136.

Hammer, K. A., Carson, C. F., & Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. *Journal of applied microbiology*, *86*(6), 985-990.

Haraguchi, H., Tanimoto, K., Tamura, Y., Mizutani, K., & Kinoshita, T. (1998). Mode of antibacterial action of retrochalcones from *Glycyrrhiza inflate*. *Phytochemistry*, *48*(1), 125-129.

Harborne, J. B., & Baxter, H. (1999). *The handbook of natural flavonoids. Volume 1 and Volume 2*. John Wiley & Sons.

Hatano, T., Kusuda, M., Hori, M., Shiota, S., Tsuchiya, T., & Yoshida, T. (2003). Theasinensin A, a Tea Polyphenol Formed from (-)-Epigallocatechin Gallate, Suppresses Antibiotic Resistance of Methicillin-Resistant< EM EMTYPE=. *Planta medica*, *69*(11), 984-989.

Hatano, T., Kusuda, M., Inada, K., Ogawa, T. O., Shiota, S., Tsuchiya, T., & Yoshida, T. (2005). Effects of tannins and related polyphenols on methicillin-resistant *Staphylococcus aureus*. *Phytochemistry*, *66*(17), 2047-2055.

Havsteen, B. (1983). Flavonoids, a class of natural products of high pharmacological potency. *Biochemical pharmacology*, *32*(7), 1141-1148.

Horvath, C. R., Martos, P. A., & Saxena, P. K. (2005). Identification and quantification of eight flavones in root and shoot tissues of the medicinal plant Huang-qin (*Scutellaria baicalensis* Georgi) using high-performance liquid chromatography with diode array and mass spectrometric detection. *Journal of Chromatography A*, *1062*(2), 199-207.

Huang, X. L., Liu, M. D., Li, J. Y., Zhou, X. D., & ten Cate, J. M. (2012). Chemical composition of *Galla chinensis* extract and the effect of its main component (s) on the prevention of enamel demineralization in vitro. *International Journal of Oral Science*, *4*(3), 146-151.

Hurtado, J. L., Montero, P., & Borderias, A. J. (2000). Extension of shelf life of chilled hake (*Merluccius capensis*) by high pressure/ Prolongación de la vida útil de merluza (*Merluccius capensis*) sometida a altas presiones conservada en refrigeración. *Food Science and Technology International*, *6*(3), 243-249.

Ikigai, H., Nakae, T., Hara, Y., & Shimamura, T. (1993). Bactericidal catechins damage the lipid bilayer. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1147*(1), 132-136.

Ingianni, A., Floris, M., Palomba, P., Madeddu, M. A., Quartuccio, M., & Pompei, R. (2001). Rapid detection of *Listeria monocytogenes* in foods, by a combination of PCR and DNA probe. *Molecular and Cellular Probes*, *15*(5), 275-280.

Jackson, K. A., Iwamoto, M., & Swerdlow, D. (2010). Pregnancy-associated listeriosis. *Epidemiology and Infection*, *138*(10), 1503-1509.

Jami, M., Ghanbari, M., Zunabovic, M., Domig, K. J., & Kneifel, W. (2014). *Listeria monocytogenes* in Aquatic Food Products – A Review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 798-813.

Jiao, J., Fu, Y. J., Zu, Y. G., Luo, M., Wang, W., Zhang, L., & Li, J. (2012). Enzyme-assisted microwave hydro-distillation essential oil from *Fructus forsythia*, chemical constituents, and its antimicrobial and antioxidant activities. *Food Chemistry*, *134*(1), 235-243.

Juliano, C., Mattana, A., & Usai, M. (2000). Composition and in vitro antimicrobial activity of the essential oil of Thymus herba-barona Loisel growing wild in Sardinia. *Journal of Essential Oil Research*, *12*(4), 516-522.

Juneja, V. K., Altuntaş, E. G., Ayhan, K., Hwang, C. A., Sheen, S., & Friedman, M. (2013). Predictive model for the reduction of heat resistance of *Listeria*

monocytogenes in ground beef by the combined effect of sodium chloride and apple polyphenols. *International Journal of Food Microbiology*, *164*(1), 54-59.

Khalil, E. A. (2004). Antidiabetic effect of an aqueous extract of pomegranate (*Punica granatum* L.) peels in normal and alloxan diabetic rats. *The Egyptian Journal of Hospital Medicine*, *16*(1), 92-99.

Kim, J., Marshall, M. R., & Wei, C. I. (1995). Antibacterial activity of some essential oil components against five foodborne pathogens. *Journal of Agricultural and Food Chemistry*, *43*(11), 2839-2845.

Kim, S. H., Park, H. H., Lee, S., Jun, C. D., Choi, B. J., Kim, S. Y., ... & Shin, T. Y. (2005). The anti-anaphylactic effect of the gall of *Rhus javanica* is mediated through inhibition of histamine release and inflammatory cytokine secretion. *International immunopharmacology*, *5*(13), 1820-1829.

Kingsley, D. H., Hoover, D. G., Papafragkou, E., & Richards, G. P. (2002). Inactivation of hepatitis A virus and a calicivirus by high hydrostatic pressure. *Journal of Food Protection*, *65*(10), 1605-1609.

Kotwal, G. J. (2008). Genetic diversity-independent neutralization of pandemic viruses (eg HIV), potentially pandemic (eg H5N1 strain of influenza) and carcinogenic (eg HBV and HCV) viruses and possible agents of bioterrorism (variola) by enveloped virus neutralizing compounds (EVNCs). *Vaccine*, *26*(24), 3055-3058.

Koutsoumanis, K. P., & Sofos, J. N. (2005). Effect of inoculum size on the combined temperature, pH and a_w limits for growth of *Listeria monocytogenes*. *International Journal of Food Microbiology*, *104*(1), 83-91.

Kuo, P. T., Lin, T. P., Liu, L. C., Huang, C. H., Lin, J. K., Kao, J. Y., & Way, T. D. (2009). Penta-O-galloyl-β-D-glucose suppresses prostate cancer bone metastasis by

transcriptionally repressing EGF-induced MMP-9 expression. *Journal of Agricultural and Food Chemistry*, *57*(8), 3331-3339.

Lansky, E., Shubert, S., & Neeman, I. (2000). Pharmacological and therapeutic properties of pomegranate. *Serie A. Séminaires Méditerranées*, *42*, 231-235.

Lansky, E. P., & Newman, R. A. (2007). *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *Journal of Ethnopharmacology*, *109*(2), 177-206.

Li, H. B., Jiang, Y., & Chen, F. (2004). Separation methods used for *Scutellaria baicalensis* active components. *Journal of Chromatography B*,*812*(1), 277-290.

Li, T., Hu, W., Li, J., Zhang, X., Zhu, J., & Li, X. (2012). Coating effects of tea polyphenol and rosemary extract combined with chitosan on the storage quality of large yellow croaker (*Pseudosciaena crocea*). *Food Control*, *25*(1), 101-106.

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J., & Cheng, S. (2006). Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chemistry*, *96*(2), 254-260.

Lingbeck, J. M., Cordero, P., O'Bryan, C. A., Johnson, M. G., Ricke, S. C., & Crandall, P. G. (2014). Functionality of liquid smoke as an all-natural antimicrobial in food preservation. *Meat Science*, *97*(2), 197-206.

Longtin, R. (2003). The pomegranate: nature’s power fruit?. *Journal of the National Cancer Institute*, *95*(5), 346-348.

Love, R. M. (1980). Histidine. *The chemical biology of fishes. Volume II, Advances 1968–1977, Academic Press.*

Lu, Y., Joerger, R., & Wu, C. (2011). Study of the chemical composition and antimicrobial activities of ethanolic extracts from roots of *Scutellaria baicalensis* Georgi. *Journal of Agricultural and Food Chemistry*, *59*(20), 10934-10942.

Lynch, M., Painter, J., Woodruff, R., Braden, C., & Centers for Disease Control and Prevention. (2006). Surveillance for foodborne-disease outbreaks--United States, 1998-2002. *Morbidity and mortality weekly report. Surveillance summaries (Washington, DC: 2002)*, *55*(10), 1.

Ma, C., Deng, X., Ke, C., He, D., Liang, Z., Li, W., Cui, Z. (2014). Epidemiology and etiology characteristics of foodborne outbreaks caused by *Vibrio parahaemolyticus* during 2008–2010 in Guangdong Province, China. *Foodborne pathogens and disease*, *11*(1), 21-29.

Maqsood, S., Benjakul, S., & Shahidi, F. (2013). Emerging role of phenolic compounds as natural food additives in fish and fish products. *Critical reviews in food science and Nutrition*, *53*(2), 162-179.

Marotti, M., Piccaglia, R., Giovanelli, E., Deans, S. G., & Eaglesham, E. (1994). Effects of planting time and mineral fertilization on peppermint (Mentha x piperita L.) essential oil composition and its biological activity. *Flavour and Fragrance Journal*, *9*(3), 125-129.

Mila, I., Scalbert, A., & Expert, D. (1996). Iron withholding by plant polyphenols and resistance to pathogens and rots. *Phytochemistry*, *42*(6), 1551-1555.

Miller, A. J., Young, D. A., & Wen, J. (2001). Phylogeny and biogeography of Rhus (Anacardiaceae) based on ITS sequence data. *International Journal of Plant Sciences*, *162*(6), 1401-1407.

Mitchell, G., Lafrance, M., Boulanger, S., Séguin, D. L., Guay, I., Gattuso, M., Malouin, F. (2012). Tomatidine acts in synergy with aminoglycoside antibiotics against multiresistant Staphylococcus aureus and prevents virulence gene expression. *Journal of Antimicrobial Chemotherapy*, *67*(3), 559-568.

Mori, A., Nishino, C., Enoki, N., & Tawata, S. (1987). Antibacterial activity and mode of action of plant flavonoids against *Proteus vulgaris* and *Staphylococcus aureus*. *Phytochemistry*, *26*(8), 2231-2234.

Morris, C., Brody, A. L., & Wicker, L. (2007). Non-thermal food processing/ preservation technologies: a review with packaging implications. *Packaging Technology and Science*, *20*(4), 275-286.

Murchie, L. W., Cruz-Romero, M., Kerry, J. P., Linton, M., Patterson, M. F., Smiddy, M., & Kelly, A. L. (2005). High pressure processing of shellfish: a review of microbiological and other quality aspects. *Innovative Food Science & Emerging Technologies*, *6*(3), 257-270.

Navarro, V., & Delgado, G. (1999). Two antimicrobial alkaloids from *Bocconia arborea*. *Journal of Ethnopharmacology*, *66*(2), 223-226.

Nishizawa, M., Yamagishi, T., Nonaka, G. I., & Nishioka, I. (1982). Tannins and related compounds. Part 5. Isolation and characterization of polygalloylglucoses from Chinese gallotannin. *Journal of the Chemical Society, Perkin Transactions 1*, 2963-2968.

Olsen, S. J., MacKinnon, L. C., Goulding, J. S., Bean, N. H., & Slutsker, L. (2000). Surveillance for foodborne-disease outbreaks – United States, 1993–1997. *MMWR CDC Surveill Summ*, *49*(1), 1-62.

Özçelik, B., Kartal, M., & Orhan, I. (2011). Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. *Pharmaceutical Biology*, *49*(4), 396-402.

O'Hara, C. M., Brenner, F. W., & Miller, J. M. (2000). Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. *Clinical microbiology reviews*, *13*(4), 534-546.

Pelegrini, P., Del Sarto, R. P., Silva, O. N., Franco, O. L., & Grossi-de-Sa, M. F. (2011). Antibacterial peptides from plants: what they are and how they probably work. *Biochemistry Research International*, 1-9.

Peña-Rodriguez, C., Martucci, J. F., Neira, L. M., Arbelaiz, A., Eceiza, A., & Ruseckaite, R. A. (2014). Functional properties and in vitro antioxidant and antibacterial effectiveness of pigskin gelatin films incorporated with hydrolysable chestnut tannin. *Food Science and Technology International*, 1082013214525429.

Perumalla, A. V. S., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts—Potential applications in food safety and quality. *Food Research International*, *44*(4), 827-839.

Pérez-Mateos, M., Boyd, L., & Lanier, T. (2004). Stability of omega-3 fatty acids in fortified surimi seafoods during chilled storage. *Journal of Agricultural and Food Chemistry*, *52*(26), 7944-7949.

Pintore, G., Usai, M., Bradesi, P., Juliano, C., Boatto, G., Tomi, F., Casanova, J. (2002). Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. oils from Sardinia and Corsica. *Flavour and Fragrance Journal*, *17*(1), 15-19.

Proestos, C., Boziaris, I. S., Nychas, G. J., & Komaitis, M. (2006). Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity. *Food Chemistry*, *95*(4), 664-671.

Qu, H., Zhang, Y., Wang, Y., Li, B., & Sun, W. (2008). Antioxidant and antibacterial activity of two compounds (forsythiaside and forsythin) isolated from *Forsythia suspensa*. *Journal of Pharmacy and Pharmacology*, *60*(2), 261-266.

Qu, H., Zhang, Y., Chai, X., & Sun, W. (2012). Isoforsythiaside, an antioxidant and antibacterial phenylethanoid glycoside isolated from *Forsythia suspensa*. *Bioorganic Chemistry*, *40*, 87-91.

Rozes, N., & Peres, C. (1998). Effects of phenolic compounds on the growth and the fatty acid composition of Lactobacillus plantarum. *Applied Microbiology and Biotechnology*, *49*(1), 108-111.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., Griffin, P. M. (2011). Foodborne illness acquired in the United States – major pathogens. *Emerg Infect Dis*, *17*(1). 7-15

Schlech 3rd, W. F., Lavigne, P. M., Bortolussi, R. A., Allen, A. C., Haldane, E. V., Wort, A. J., ... & Broome, C. V. (1983). Epidemic listeriosis – evidence for transmission by food. *The New England Journal of Medicine*, *308*(4), 203-206.

Sebat, J. L., Paszczynski, A. J., Cortese, M. S., & Crawford, R. L. (2001). Antimicrobial Properties of Pyridine-2, 6-Dithiocarboxylic Acid, a Metal Chelator Produced by Pseudomonas spp. *Applied and Environmental Microbiology*, *67*(9), 3934-3942.

Seeliger, H. P. R., & Höhne, K. (1979). Serotyping of *Listeria monocytogenes* and Related Species. *Methods in Microbiology* (*Vol. 13*), Academic Press.

Shahamat, M., Seaman, A., & Woodbine, M. (1980). Survival of *Listeria monocytogenes* in high salt concentrations. *Zentralblatt für Bakteriologie. 1. Abt. Originale A, Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie*, *246*(4), 506-511.

Silk, B. J., Date, K. A., Jackson, K. A., Pouillot, R., Holt, K. G., Graves, L. M., ... & Mahon, B. E. (2012). Invasive listeriosis in the Foodborne Diseases Active Surveillance Network (FoodNet), 2004–2009: further targeted prevention needed for higher-risk groups. *Clinical Infectious Diseases*, *54*(suppl 5), S396-S404.

Singh, R. P., Chidambara Murthy, K. N., & Jayaprakasha, G. K. (2002). Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. *Journal of Agricultural and Food Chemistry*, *50*(1), 81-86.

Smith, A. H., Zoetendal, E., & Mackie, R. I. (2005). Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microbial Ecology*, *50*(2), 197-205.

Solórzano-Santos, F., & Miranda-Novales, M. G. (2012). Essential oils from aromatic herbs as antimicrobial agents. *Current opinion in biotechnology*, *23*(2), 136-141.

Stockwell, C. (1988). Nature's Pharmacy. *Century Hutchinson Ltd.*, London. United Kingdom.

Stratton, J. E., & Taylor, S. L. (1991). Scombroid poisoning. In *Microbiology of Marine Food Products* (pp. 331-351). Springer US.

Sundararajan, A., Ganapathy, R., Huan, L., Dunlap, J. R., Webby, R. J., Kotwal, G. J., & Sangster, M. Y. (2010). Influenza virus variation in susceptibility to inactivation by pomegranate polyphenols is determined by envelope glycoproteins. *Antiviral Research*, *88*(1), 1-9.

Taguri, T., Tanaka, T., & Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biological and Pharmaceutical Bulletin*, *27*(12), 1965-1969.

Taylor, S. L. (1985). Histamine poisoning associated with fish, cheese and other food. World Health Organization, Geneva. 1-47

Taylor, S. L., Guthertz, L. S., Tillman, M. L. F., & Lieber, E. R. (1978). Histamine Production by Food-borne Bacterial Species. *Journal of Food Safety*, *1*(3), 173-187.

Tian, F., Li, B., Ji, B., Zhang, G., & Luo, Y. (2009a). Identification and structure–activity relationship of gallotannins separated from *Galla chinensis*. *LWT-Food Science and Technology*, *42*(7), 1289-1295.

Tian, F., Li, B., Ji, B., Yang, J., Zhang, G., Chen, Y., & Luo, Y. (2009b). Antioxidant and antimicrobial activities of consecutive extracts from *Galla chinensis*: The polarity affects the bioactivities. *Food chemistry*, *113*(1), 173-179.

Tsuchiya, H., & Iinuma, M. (2000). Reduction of membrane fluidity by antibacterial sophoraflavanone G isolated from *Sophora exigua*. *Phytomedicine*, *7*(2), 161-165.

US Food and Drug Administration. (2003). Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. *US Food and Drug Administration Center for Food Safety and Applied Nutrition, College Park, Md*.

US Food and Drug Administration. (2011). *Fish and Fishery Products Hazards and Controls Guidance – Fourth Edition (Chap. 7)*. 113-152

Voisin, E. A. (2002). *U.S. Patent No. 6,426,103*. Washington, DC: U.S. Patent and Trademark Office.

Wang, R. R., Gu, Q., Wang, Y. H., Zhang, X. M., Yang, L. M., Zhou, J., ... & Zheng, Y. T. (2008). Anti-HIV-1 activities of compounds isolated from the medicinal plant *Rhus chinensis*. *Journal of Ethnopharmacology*, *117*(2), 249-256.

Wu-Yuan, C. D., Chen, C. Y., & Wu, R. T. (1988). Gallotannins inhibit growth, water-insoluble glucan synthesis, and aggregation of *mutans streptococci*. *Journal of Dental Research*, *67*(1), 51-55.

Xi, D., Liu, C., & Su, Y. C. (2012). Effects of green tea extract on reducing *Vibrio parahaemolyticus* and increasing shelf life of oyster meats. *Food Control*, *25*(1), 368-373.

Xi, Y., Sullivan, G. A., Jackson, A. L., Zhou, G. H., & Sebranek, J. G. (2012). Effects of natural antimicrobials on inhibition of *Listeria monocytogenes* and on chemical, physical and sensory attributes of naturally-cured frankfurters. *Meat Science*, *90*(1), 130-138.

Xiang, P., Lin, Y., Lin, P., Xiang, C., Yang, Z., & Lu, Z. (2007). Effect of cationization reagents on the matrix‐assisted laser desorption/ionization time‐of‐flight mass spectrum of Chinese gallotannins. *Journal of Applied Polymer Science*, *105*(2), 859-864.

Yi, S. M., Zhu, J. L., Fu, L. L., & Li, J. R. (2010). Tea polyphenols inhibit *Pseudomonas aeruginosa* through damage to the cell membrane.*International Journal of Food Microbiology*, *144*(1), 111-117.

Yan, D., Jin, C., Xiao, X. H., & Dong, X. P. (2008). Antimicrobial properties of berberines alkaloids in *Coptis chinensis* Franch by microcalorimetry. *Journal of biochemical and biophysical methods*, *70*(6), 845-849.

Yeaman, M. R., & Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacological reviews*, *55*(1), 27-55.

Yuan, R., & Lin, Y. (2000). Traditional Chinese medicine: an approach to scientific proof and clinical validation. *Pharmacology & Therapeutics*, *86*(2), 191-198.

Zhang, X. & Li, Y. (2013). The antibacterial activity of plant polyphenols and its application in food preservation. *Journal of Food Safety and Quality*, *4*(3), 769-773.

Zhao, G., Chung, K. T., Milow, K., Wang, W., & Stevens Jr, S. E. (1997). Antibacterial properties of tannic acid and related compounds against the fish pathogen *Cytophaga columnaris*. *Journal of Aquatic Animal Health*, *9*(4), 309-313.

Zou, L., Zhang, L., Li, J., Hao, Y., Cheng, L., Li, W., & Zhou, X. (2008). Effect of *Galla chinensis* extract and chemical fractions on demineralization of bovine enamel *in vitro*. *Journal of Dentistry*, *36*(12), 999-1004.

CHAPTER 3:

EXTRACTION OF PLANT-SOURCE ANTIMICROBIAL COMPOUNDS AND ANTIBACTERIAL TESTS ON AGAR AND BROTH CULTURES OF *VIBRIO PARAHAEMOLYTICUS* AND *LISTERIA MONOCYTOGENES*

Abstract

This research was conducted to study antimicrobial effects of aqueous methanol extracts from several medicinal plants against several seafood related pathogenic bacteria. The antibacterial activity of four plants, pomegranate peel ("PP", *Punica Granatum* L.), Chinese gallnut ("CG", *Galla chinensis*), forsythia fruits ("FF", *Forsythia suspensa*) and baikal skullcap ("BS", *Scutellaria baicalensis*) were tested using the agar diffusion assay and the microdilution assay. Dry plant materials were ground and extracted with 70% methanol respectively. Five concentrations (1, 2, 5, 10, 20 mg/ml) of each extract were tested in agar diffusion assay, and the zones of inhibition were measured. The minimum inhibitory concentration (MIC) of CG and PP extracts on *V. parahaemolyticus* were 1 mg/ml and 2 mg/ml. Only CG inhibited *L. monocytogenes* when present at ≥ 5mg/ml. Five concentrations of each extract (0.008, 0.04, 0.2, 1.0, and 5.0 mg/ml) were applied to *V. parahaemolyticus*, *L. monocytogenes*, and *M. morganii* cultures which were then incubated at 37°C, and sampled after 8, 24, and 96 hours. Samples were spread plated, incubated and enumerated to determine the actual bacterial population. The results were compared with control groups to determine the inhibitory effects. The CG extracts at concentrations of ≥0.04 mg/ml and ≥1 mg/ml

significantly inhibited growth of *V. parahaemolyticus* and *L. monocytogenes*, respectively. At concentrations of ≥1 mg/ml and 5 mg/ml, the PP extracts significantly inhibited growth of *V. parahaemolyticus*, and *L. monocytogenes,* respectively. Both extracts reduced bacterial populations by up to 4 logs. No inhibition of pathogen growth was observed with FS and BS extracts except for BS at 5 mg/ml with *V. parahaemolyticus* in broth and 20 mg/ml with *L. monocytogenes* on agar. None of the plant extracts showed inhibition on *M. morganella*.

Introduction

Many plant-source materials have been used in traditional Chinese medicine (TCM) for many centuries in China and other Asian countries. In recent years, TCM has been gradually accepted by the western world. In the USA, TCM is studied and used as a complementary and alternative medicine (CAM) as well as a dietary supplement. By the year 2000, more than 35,000 plant-source materials were screened for potential medical use by the National Cancer Institute (NCI) and the United States Department of Agriculture (Yuan. et al, 2000). Scientific research on the use of TCM has increased with thousands of functional compounds being isolated from TCM (Zhou. et al, 2011). For example, Chinese gallnut (CG, *Rhus chinensis* Mill.), pomegranate peel (PP, *Punica Granatum* L.), forsythia fruit (FF, *Forsythia suspensa* (Thunb.) Vahl) and baikal skullcap root (BS, *Scutellaria baicalensis* Georgi.) are several of the most commonly used TCM. They are widely cultivated in various locations in China and various areas around the world, including North America. Research has been conducted to identify antimicrobial compounds contained in these plants. Phenolic compounds, such as essential oils, gallotannins and flavonoids, are the major compounds with antimicrobial activity found in these plants (Jiao et. al. 2012). The antimicrobial properties were verified using antibacterial assays (Tian et al. 2009; Kang et al. 2008; Fawole et al. 2012), and being tested on food products (Kanatt et al, 2010). However, little research has addressed the inhibitory effect of extracts TCM against important foodborne pathogens, such as *Listeria monocytogenes*, and *Vibrio parahaemolyticus,* which are major food safety concerns with seafood products. In addition, *Morganella morganii* is the organism primarily responsible for the formation of scombrotoxin (i.e. histamine) in

temperature-abused fish (Arnold, S., and Brown, D., 1978). The inhibition of *M. morganii* will help to control the formation of scombrotoxins.

The purpose of this research was to identify the potential inhibitory effects of plant extracts against *Listeria monocytogenes* in shrimp, *Vibrio parahaemolyticus* in oysters and *Morganella morganii* in tuna products. Antimicrobial assays were conducted on pure cultures of selected bacterial strains both on agar plates and in broth. When the extracts showed bacterial inhibition, further studies were conducted in food product matrices. The CG, PP, BS and FF contains multiple compounds, and specific information is still required to determine which specific compounds have the highest antimicrobial activities. Therefore, the plant extracts used in this research were crude extracts in aqueous methanol.

Materials and Methods

Plant materials

Dry Pomegranate peel (PP, *Punica Granatum* L.), Chinese gallnut (CG, *Rhus chinensis* Mill.), forsythia fruit (FF, *Forsythia suspensa* (Thunb.) Vahl) and baikal skullcap root (BS, *Scutellaria baicalensis* Georgi.) root were purchased from Mayway Inc. (CA, USA) and pharmacy of No. 263 Military Hospital (Beijing, China). In the following content of the chapter, CG, PP, FF, and BS from Mayway Inc. are abbreviated as CGM, PPM, FFM, BSM, respectively; CG, PP, FF and BS from No. 263 hospital were abbreviated as CGH, PPH, FFH, BSH, respectively. The HPLC grade methanol was purchased from Fisher Scientific (PA, USA) and a food processor (Osterizer^{TM}) was used to grind raw plant materials.

The dry, capsule-like CG was carefully cracked using a hammer. The remains of aphids found inside the galls were removed and the hull tissue was collected and ground. The crude ground gall shell was sifted through a 40-mesh sieve and the fine powder was collected. Five grams of sifted powder was mixed with 100 ml aqueous methanol solution (MeOH:H_2O = 70:30, v/v) and stirred with mild heating (30-40°C) for 3 hours. The mixture was separated by gravity filtration using #1 filter paper (Whatman®) and the filtrate was collected. The aliquot was standardized with 70% methanol to 100 ml and stored at -20°C for further use.

Large pieces of dry PP were separated by hand into approximately 1 x 1 cm pieces or smaller, and then ground and sifted through a 40-mesh sieve. The fine powder was extracted and collected using the same protocol as for the CG. The dry root and stem of BS was torn or cut into short pieces (<2 cm), ground and sifted through 40-mesh. Then the sifted powder was extracted and collected using the same protocols as the CG. Forsythia dry fruits were ground, sifted through 40-mesh, extracted and collected using the same protocols of CG stated above.

The concentration of the extract solutions were calculated as:

$$Concentration\ \left(\frac{mg}{ml}\right) = \frac{Mass\ of\ dry\ powdered\ plant\ (mg)}{Volume\ of\ solution\ in\ 70\%\ methanol(ml)}$$

Stock solutions (50 mg/ml) of those four plants were diluted with 70% methanol to 20 mg/ml, 10 mg/ml, 5 mg/ml, 2 mg/ml, 1 mg/ml, 0.4 mg/ml and 0.08 mg/ml, and used as treatment later in agar diffusion assays and microdilution assays.

Preparation of bacterial cultures

Listeria monocytogenes stock strains (isolated by local CDC) and (Scott A) were obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into 9.9 ml tryptic soy broth (TSB, BD Bacto™) and then incubated at 37°C for 24 hours. After the first incubation, the cultures were transferred into another TSB tube and incubated for 24 hours at 37°C. After the second incubation, the culture was T-streaked on Modified Oxford Agar (MOX, BD Difco™) and incubated for 24 hours at 37°C. A single clear colony with dark media surrounded was taken from the MOX plate and transferred into TSB tube and incubated for 24 hours at 37°C to reach the population of approximately 10^9 CFU/ml.

Vibrio parahaemolyticus stock strains (VP13) and (VP16) were obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into 9.9 ml TSB+NaCl (1.5% sodium chloride, the total NaCl concentration of the medium was 2%) tube and then incubated at 37°C for 24 hours. After the first incubation, the cultures were transferred into another TSB+NaCl tube and incubated for another 24 hours at 37°C. After the second incubation, the culture was T-streaked on thiosulfate-citrate-bile salts-sucrose agar (TCBS, BD Difco™) and incubated for 24 hours at 37°C. A single colony with dark green center was taken from the MOX plate and transferred into TSB+NaCl tube and incubated for 24 hours at 37°C to reach the population of approximately 10^9 CFU/ml.

Morganella morganii stock strain (25830) was obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into 9.9 ml TSB tube and then incubated at 37°C for 24 hours. After the first

incubation, the cultures were transferred into another TSB tube and incubated for another 24 hours at 37°C. After the second incubation, the culture was T-streaked on McConkey Agar (BD BBL™) and incubated for 24 hours at 37°C. A single colony that was not red was taken from the McConkey plate and transferred into TSB tube and incubated for 24 hours at 37°C to reach approximate population of 10^9 CFU/ml.

Minimum inhibitory concentration (MIC) test using agar diffusion assays

Agar diffusion assays were performed on Petri plates with tryptic soy agar (TSA, from BD). *L. monocytogenes*, *M. morganii*, and *V. parahaemolyticus* cultures were prepared as stated in Section 2.2. Whatman # 1 filter paper was cut into round paper disks with an average diameter of 7.2 mm, and autoclaved at 121°C for 15 minutes. Each TSA plate was divided into 7 areas. Bacteria cultures were inoculated onto the TSA plates using a sterile swab described by Williams (2013) with minor modifications. A sterile sampling swab was dipped into bacterial culture tube, then gently squeezed against the inner wall of the tube to remove excessive liquid. The whole TSA plate was covered by swabbing in one direction, then covered again by swabbing in a direction perpendicular to the first. After the swab inoculation, 7 sterile paper disks were placed on the agar. Ten microliters of 70% methanol, 5% commercial bleach (Clorox®, 6.0% sodium hydrochloride, the final concentration of NaClO is 0.3%, approx. 3 mg/ml), and extracts with concentrations of 20, 10, 5, 2, and 1 mg/ml were then pipetted onto the paper disks. The TSA plates with bacterial inoculation and extracts added were incubated at 37°C for 24 hours. The diameter of inhibitory zones was determined as the average of two perpendicular measurements.

MIC test using microdilution 96-well plate assay

Sterile 96*250 μl well plates were from BD biosciences. One hundred and twenty-five microliters of TSB was pipetted into each well. Bacteria cultures (100 μl) were added into 9.9 mL sterile 0.1% peptone (BD Bacto™) water. The diluted culture was homogenized on vortex and 10 μl (approx. 10^5 cfu) was inoculated into each well. Fifteen microliters of treatment were added to the assigned wells. Commercial bleach (15 μl, diluted into 5%, NaClO concentration was 28.5 mg/ml) was added into the assigned wells as a positive control, and 15 μl water was added into the assigned wells as a negative control. The treatments were extracts of CG, PP, BS or FF dissolved in 70% methanol, and extract concentrations in each well were 0, 0.008, 0.04, 0.2, 1, and 5 mg/ml, respectively. The experimental design is shown in Appendix A.

The whole 96-well plate was sealed to reduce moisture lost through evaporation and then incubated at 37 °C. The inoculum at starting time was diluted in 10^{-1} and 10^{-3} concentrations and spread plated on TSA. Samples were collected at 8 hours, 24 hours and 96 hours using the protocol described below. The well plate was manually shaken for 30 seconds to homogenize the culture in each well, then 15 μl sample of culture was transferred into a 10 ml 0.1% peptone water tube to make a diluted culture. The tube was mixed on a vortex mixer for 10 seconds, and 50 μl of the diluted culture was plated on duplicate TSA plates using a Spiral plater (Spiral Biotech Autoplate® 4000). The plates were incubated at 37 °C for 24 hours and colony forming units were counted and calculated following the instructions in Autoplate 4000 operation manual.

Statistical Analysis

The results were analyzed in GraphPad Prism Version 6.01. For both agar diffusion assay and microdilution assay, the results of treatments and controls were compared using two-way analysis of variance (ANOVA). Whenever the overall F test was significant, multiple comparisons were performed using Tukey's Studentized T-test (Zar, 2010) to find differences among treatments (Zar, 2010).

Results and Discussion

Results of agar diffusion assay

The results of agar diffusion assays are shown in Table 3.1 and 3.2. These data are based on triplicate samples. Seventy percent methanol was used as the negative control to demonstrate inhibition from the solvent. If the plant extract shows antibacterial activity, it will form a larger inhibitory zone compared with the inhibitory zone formed by the solvent itself. The results show that concentrations of 1 mg/ml (i.e. 1,000 ppm) and 2 mg/ml of CGM and CGH, respectively, showed significantly larger areas of inhibition on *V. parahaemolyticus*, compared with 70% methanol alone. These results indicate that CG is effective in inhibiting growth of *V. parahaemolyticus* in agar, and at higher concentrations of CG the inhibitory zones increased in diameter. Similarly, both PPM and PPH at concentrations of 5 mg/ml and higher, demonstrated strong inhibitions to *V. parahaemolyticus* by forming larger inhibitory zones compared with 70% methanol on Petri plates. Extracts from the other two plants, (i.e., FFM, FFH, BSM and BSH), did not form significantly larger inhibitory zones compared with 70% methanol, which indicates little to no antibacterial activities against *V. parahaemolyticus*. Against *L.*

monocytogenes, only CGM and CGH extracts showed significant inhibitions (i.e., CGM showed significant inhibition at concentrations of 10 mg/ml and 20 mg/ml, and CGH at 5 mg/ml and above). None of the plant extracts used in the assays were effective against *M. morganii* (Data not shown).

Seventy percent methanol inhibited the growth of *V. parahaemolyticus* on TSA plates, but did not show inhibitory effect on *L. monocytogenes*. Commercial bleach (5%) was used as a positive control to demonstrate the effectiveness of plant extracts compared with a traditional sanitizer. The bleach was diluted following the instruction, and the concentration of sodium hypochlorite was approximately 2.85 mg/ml (i.e. 2850 ppm). The diffusion assays against *L. monocytogenes* showed that CG extracts formed similar size inhibitory zones to chlorine. The *V. parahaemolyticus* was even more sensitive to CG and PP compared with chlorine.

The bacteria species used in this study showed different sensitivities to the crude extracts. The difference in sensitivities may be due to many factors, including different cell wall structures between Gram positive and Gram negative organisms (Montville and Matthews, 2005). Two strains of *V. parahaemolyticus* and two *L. monocytogenes* strains were tested in the assay. A primary agar diffusion assay was conducted and demonstrated that there is no significant difference between two tested strains of each species. Therefore, in major part of the study, the two strains of *V. parahaemolyticus* were mixed as a cocktail culture.

Possible differences in inhibitory activity between plant batches were also analyzed. The CGM and CGH started to show significant inhibition against *V. parahaemolyticus* at 1 mg/ml and 2 mg/ml, respectively. They also had different MICs

against *L. monocytogenes*, at 10 mg/ml and 5 mg/ml, respectively. The difference in MICs may be from the different cultivars; also, it might be from different processing conditions or storage conditions of the plant materials. However, both batches inhibited both pathogens at higher concentrations.

Neither extract of FF and BS showed significant inhibition against either pathogen according to statistical analyses, but based on visual observations, higher concentrations (10 mg/ml and 20 mg/ml) of FF and BS formed weak inhibitory rings around the paper disks. To verify these potential inhibitions, FF and BS were both tested using microdilution assays discussed in the following section.

The MIC values of CG extracts in this research were higher than reported by others (Tian et al. 2009, Feng, et al. 2012). Unlike the studies by Tian and Feng, however, the plant-source compounds in this study were extracted in aqueous methanol and used in the solution form. Concentrations of plant extracts were calculated as:

$$Concentration\ (mg/ml) = \frac{Mass\ of\ dry\ powdered\ plant\ (mg)}{Volume\ of\ solution\ in\ 70\%\ methanol(ml)}$$

Direct comparisons among MIC results of different research are difficult, since the protocols vary among researchers (Burt, 2004). There are several reasons for using extracts in solution instead of pure extracts. First, it was more accurate to use extracts in the solution form for the antimicrobial tests. The stock extraction was standardized by using 100.0 ml aqueous methanol to extract 5.00 grams of powdered dry plant tissue, and the stock extracts were easily diluted and used in antimicrobial assays. The extracts were stored at -20 °C to minimize degradation. Second, it requires extra

processing steps to acquire pure extracts. The rotary evaporation and freeze drying processes may cause the loss of volatile and unstable compounds (such as essential oils in FF) (Endo and Hikino, 1984), thus were not conducted. Third, re-dissolving of pure extracts may be difficult. However, to assist the calculation of the concentration, freeze dried plant extracts were obtained and the yields were calculated, thus the concentration of the extracts based on dry plant material (as used in the study) can be converted to concentration based on freeze dried pure extracts if necessary, and vice versa.

Although both methanol and ethanol have been previously used in other research (Cai et al., 2004; Lu et al., 2011; Fawole et al., 2012), methanol was chosen as the solvent in this study, because of its minimal antimicrobial activity among all alcohols (Tilley and Schaffer, 1926), the better extraction ability, the lower cost, and the better compatibility with HPLC analysis.

Previously, CG has been reported to inhibit human and plant pathogenic bacteria (Tian et al., 2009; Feng et al., 2012); the hydrolysable tannins, which are the major active components in CG (Nishizawa et al., 1982), have been reported to inhibit *L. monocytogenes*; however, few research addressed on inhibition of *V. parahaemolyticus.* This study showed that as a Gram-negative bacterium, the *V. parahaemolyticus* is even more sensitive to tannins than some Gram-positive microorganisms, which does not happen to typical Gram-negative bacteria (Proestos et al. 2006).

Results of microdilution assays

The results of the microdilution assay against *V. parahaemolyticus* are shown in Figure 3.1. The results from two batches of each plant were combined and expressed. Generally, treatments of CG, PP and FS at concentrations of 0.04 mg/ml, 1 mg/ml and 5 mg/ml, respectively, successfully inhibited the growth of *V. parahaemolyticus*. When one of these treatments was used the population of *V. parahaemolyticus* remained below detection, at about 10^4 cfu/ml. The bacterial numbers in the methanol or water controls were approximately 10^7 to 10^8 cfu/ml.

The results of microdilution assay against *L. monocytogenes* are shown in Figure 3.2. The bacterial cultures were completely inhibited at CG concentrations of 1 mg/ml (and 5 mg/ml, not shown) after 24 hours. For the PP treatment, *L. monocytogenes* was still present at 10^5 and 10^6 cfu/ml with a PP concentration of 5 mg/ml. The treatments using BS and FF extracts did not inhibit bacterial growth thus were not included in figure. *L. monocytogenes* numbers in the methanol or water controls were 10^8 cfu/ml or higher.

The microdilution assays with *M. morganii* did not demonstrate any significant inhibition (data not shown). The results were somehow confirmative to the results in previous agar diffusion assay. On TSA plate, the CG and PP extracts can form a transparent ring areas around the paper disks; however, when examined closely, smaller size colonies can still form within the ring area, thus the inhibition cannot be reported. It can be implied that the bacterial growth of *M. morganii* might have been retarded at very beginning (transparent ring areas were formed around extracts), but given enough nutrients (TSA), *M. morganii* can overcome the adverse impact from plant

extracts. The smaller colonies within the transparent ring area indicated a slower growth.

To achieve more accurate concentrations of extracts. In the assays with both *V. parahaemolyticus* and *L. monocytogenes*, the CG, PP, and FS showed lower MICs than in the agar diffusion assays. The reason is that the plant extracts may be more evenly distributed throughout the broth cultures compared with the agar plates (Proestos et al. 2006).

Seventy percent methanol and water were used as two negative controls in the microdilution assays. The purpose was to analyze the possible antimicrobial effects from the solvent methanol. The CG, PP, BS and FF extracts were all dissolved in 70% methanol; when added as treatments, the methanol content might also inhibit bacterial growth, although the methanol is considered as an organic solvent with weak antimicrobial activity (Tilley and Schaffer, 1926). The concentration of methanol in the final culture was kept comparatively low. In all plant extract treatment groups and the methanol control group, the final methanol content in the broth was:

$$\frac{15\ \mu l\ \times 70\%}{(15+10+125)\mu l} = 7\%$$

According to the results of ANOVA and multiple comparisons, the bacterial populations in the PP and CG treatment groups were significantly different from the water control; however, there were no significant differences between the bacterial growth of the methanol control group and that of water control group. Therefore, the inhibition of 7% methanol was negligible. The overall inhibitory effects of the plant extracts were mostly from the plant contents, not the solvent methanol.

The pH of plant extracts were tested and the pH of plant extracts varied from 5.3-5.6. This was not the optimal pH range for *V. parahaemolyticus*; however, the lowest pH for Vibrio growth in nutrition culture is reported to be 4.5 (Adams and Moss, 2008)

Bioscreen C^{TM} optical density (OD) measurement was used to estimate the bacterial growth. However, the OD value was not a good indicator of bacterial growth, as some of the plant extracts, such as CG and PP, formed precipitates and caused cloudiness when added to TSB media. This increased the OD value without actual bacterial growth. According to Nishizawa, et al. (1982), the major compounds in CG are hydrolysable tannins, which denatured proteins and caused precipitation. The ability of CG to denature proteins may explain some of the antimicrobial mechanisms as the proteins contained in bacterial cell walls and other functional proteins such as enzymes may lose their biological functions.

In terms of different microorganisms, *M. morganii* was the least sensitive to crude plant extracts. In addition, *L. monocytogenes* showed higher resistance to the crude plant extracts than did *V. parahaemolyticus*. Most of the plant extracts were not completely lethal to these bacteria, but the bacterial growth were lower compared to controls. Thus, addition of plant extracts may not be effective by themselves, but may have application as part of the “hurdle technology” (Alasalvar, et al. 2011).

During the extraction process and antimicrobial assays, any differences between the different batches of each plants (CG, PP, FF and BS) were monitored. Batch “M” (purchased from Mayway) had a stronger/darker color compared with batch “H” (purchased from a hospital in China). In the antimicrobial tests, batch “M” showed slightly stronger inhibitory effects compared with batch “H”. This may be due to several

factors. First, the growing and processing conditions of the plants may be different, and the cultivars may be different as well. Second, differences may be due to oxidation/deterioration during production or packaging of the plants. The batch “M” plants were packaged in sealed, two layer plastic bags while the batch “H” came in loose packages without sealing.

Summary

Chinese gall (CG) extracts had the strongest inhibitory activity against both *V. parahaemolyticus* and *L. monocytogenes*. Pomegranate peel (PP) at higher concentrations demonstrated inhibition against those two bacteria. Forsythia fruit (FF) and Baikal skullcap (BS) did not show significant inhibitions. Crude extract from Chinese gall and pomegranate peel were used in additional tests in cooked shrimp and raw tuna.

References

Adams, M. R., & Moss, M. O. (2008). *Food microbiology.* Cambridge, UK: RSC Publishing

Alasalvar, C., Miyashita, K., Shahidi, F., & Wanasundara, U. (Eds.). (2011).*Handbook of Seafood Quality, Safety and Health Applications*. John Wiley & Sons.

Arnold, S. H., & Brown, W. D. (1978). Histamine (?) toxicity from fish products. *Advances in Food Research*, *24*, 113-154.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*(3), 223-253.

Cai, Y., Luo, Q., Sun, M., & Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences*, *74*(17), 2157-2184.

Endo, K., & Hikino, H. (1984). Structures of rengyol, rengyoxide, and rengyolone, new cyclohexylethane derivatives from Forsythia suspensa fruits. *Canadian Journal of Chemistry*, *62*(10), 2011-2014.

Fawole, O. A., Makunga, N. P., & Opara, U. L. (2012). Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. *BMC Complementary and Alternative Medicine*, *12*(1), 200-210.

Feng, C. T., Su, H. J., Chen, C. T., Ho, W. C., Tsou, Y. R., & Chern, L. L. (2012). Inhibitory effects of Chinese medicinal herbs on plant-pathogenic bacteria and identification of the active components from gallnuts of Chinese sumac. *Plant Disease*, *96*(8), 1193-1197.

Jiao, J., Fu, Y. J., Zu, Y. G., Luo, M., Wang, W., Zhang, L., & Li, J. (2012). Enzyme-assisted microwave hydro-distillation essential oil from *Fructus forsythia*, chemical constituents, and its antimicrobial and antioxidant activities. *Food Chemistry*, *134*(1), 235-243.

Kanatt, S. R., Chander, R., & Sharma, A. (2010). Antioxidant and antimicrobial activity of pomegranate peel extract improves the shelf life of chicken products. *International Journal of Food Science & Technology*, *45*(2), 216-222.

Kang, M. S., Oh, J. S., Kang, I. C., Hong, S. J., & Choi, C. H. (2008). Inhibitory effect of methyl gallate and gallic acid on oral bacteria. *The Journal of Microbiology*, *46*(6), 744-750.

Lu, Y., Joerger, R., & Wu, C. (2011). Study of the chemical composition and antimicrobial activities of ethanolic extracts from roots of Scutellaria baicalensis Georgi. *Journal of Agricultural and Food Chemistry*, *59*(20), 10934-10942.

Montville, T. J., & Matthews, K. R. (2005). *Food microbiology: an introduction (Chap 2)*. ASM Press.

Nishizawa, M., Yamagishi, T., Nonaka, G. I., & Nishioka, I. (1982). Tannins and related compounds. Part 5. Isolation and characterization of polygalloylglucoses from Chinese gallotannin. *Journal of the Chemical Society, Perkin Transactions 1*, 2963-2968.

Proestos, C., Boziaris, I. S., Nychas, G. J., & Komaitis, M. (2006). Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity. *Food Chemistry*, *95*(4), 664-671.

Tian, F., Li, B., Ji, B., Yang, J., Zhang, G., Chen, Y., & Luo, Y. (2009). Antioxidant and antimicrobial activities of consecutive extracts from *Galla chinensis*: The polarity affects the bioactivities. *Food Chemistry*, *113*(1), 173-179.

Tilley, F. W., & Schaffer, J. M. (1926). Relation between the chemical constitution and germicidal activity of the monohydric alcohols and phenols. *Journal of Bacteriology*, *12*(5), 303.

Williams, R. (2013) *Food Microbiology Laboratory Manual*, Virginia Tech

Yuan, R., & Lin, Y. (2000). Traditional Chinese medicine: an approach to scientific proof and clinical validation. *Pharmacology & therapeutics*, *86*(2), 191-198.

Zar, J. H. (2010). *Biostatistical Analysis*: Prentice Hall.

Zhou, J., Xie, G., & Yan, X. (2011). *Encyclopedia of Traditional Chinese Medicines-Molecular Structures, Pharmacological Activities, Natural Sources and Applications: Vol. 6: Indexes* (Vol. 6). Springer.

Table 3.1. Diameters of inhibitory zones (mm) formed by plant extracts against *V. parahaemolyticus* cultures on agar, after 24 hours incubation at 37±1°C

Agar Diffusion Assay Results

Plant Extract/ Treatment	MeOH	1mg/ml	2mg/ml	5mg/ml	10mg/ml	20mg/ml	NaClO, 3 mg/ml
CGM	7.8±0.4	9.7±0.8	12.3±0.8	16.1±0.3	17.8±0.7	19.5±0.8	8.2±0.2
CGH	8.2±0.2	8.3±0.3	10.7±0.8	14.0±0.8	16.4±0.8	18.2±0.7	8.4±0.3
PPM	8.4±0.1	8.3±0.2	9.5±0.5	13.3±0.4	15.7±0.4	17.1±0.2	9.1±0.4
PPH	8.1±0.1	8.1±0.5	8.2±0.5	10.3±0.4	12.7±0.7	15.2±0.0	8.1±0.1
BSM	8.5±0.3	7.9±0.5	8.3±0.3	8.2±0.1	7.8±0.3	8.1±0.1	9.5±0.4
BSH	8.4±0.6	8.8±0.2	7.6±0.2	7.3±0.1	8.2±0.5	8.7±0.3	9.5±0.3
FFM	8.4±0.2	8.7±0.2	7.6±0.4	7.9±0.4	7.5±0.2	7.5±0.2	8.3±0.2
FFH	8.1±0.4	8.4±0.3	8.9±0.3	8.2±0.2	7.7±0.2	7.5±0.2	9.1±0.2

The diameter of inhibitory zone was expressed as mean ± standard deviation (SD). The underlined values are significantly larger ($P<0.05$) than the control (i.e. diameter of MeOH treatment), based on two-way analysis of variance and Tukey's multiple comparison. The paper disks used had a diameter of 7.2mm.

CGM: Chinese gall from Mayway Inc. (CA, USA); CGH: Chinese gall from a hospital (Beijing, China); PPM: pomegranate peel from Mayway Inc. (CA, USA); PPH: pomegranate peel from a hospital (Beijing, China); BSM: Baikal skullcap root from Mayway Inc. (CA, USA); BSH: Baikal skullcap root from a hospital (Beijing, China); FFM: forsythia fruit from Mayway Inc. (CA, USA); FFH: forsythia fruit from a hospital (Beijing, China)

Table 3.2. Diameters of inhibitory zones (mm) formed by plant extracts against *L. monocytogenes* cultures on agar, after 24 hours incubation at 37±1°C

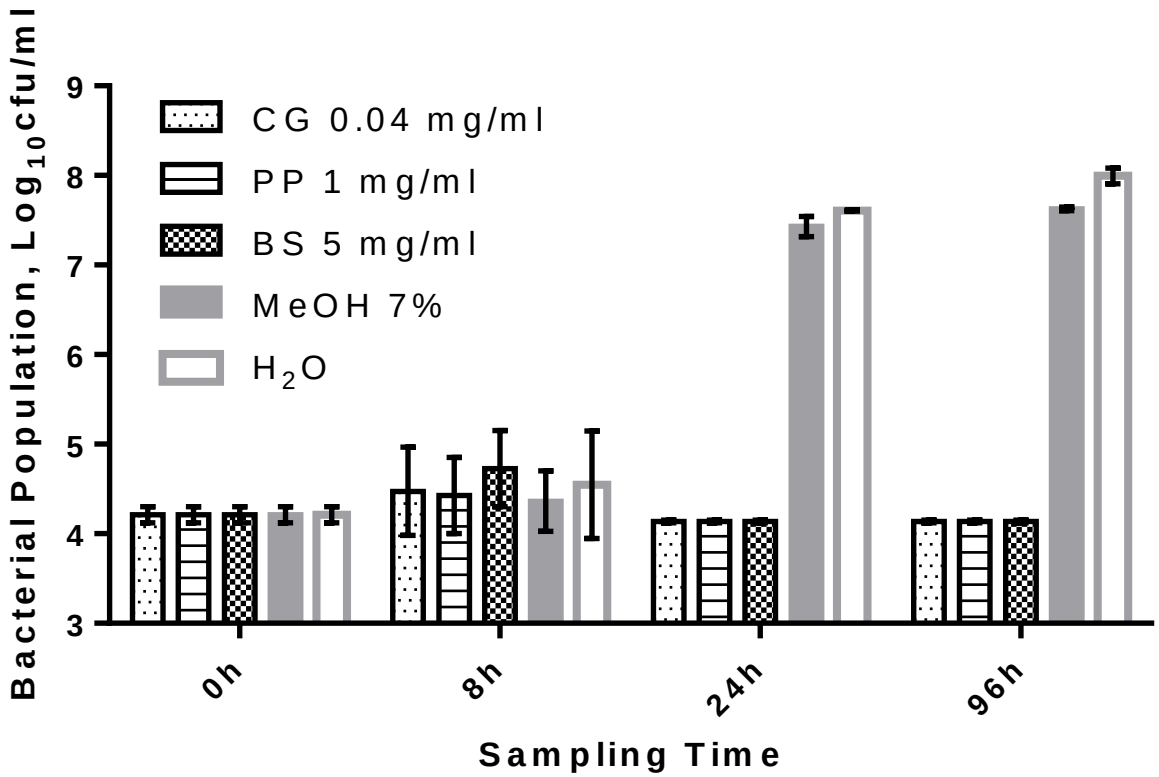
Agar Diffusion Assay Results, PE against

Plant Extracts/ Treatment	MeOH	1mg/ml	2mg/ml	5mg/ml	10mg/ml	20mg/ml	NaClO, 3 mg/ml
CGM	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	8.3±0.6	9.2±0.4	9.0±0.3
CGH	7.2±0.0	7.2±0.0	8.0±0.3	9.3±0.5	9.3±0.1	9.6±0.2	10.2±0.6
PPM	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.3±0.1	9.1±0.3
PPH	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.6±0.2	10.6±0.6
BSM	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	8.3±0.3	10.4±0.6
BSH	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.3±0.1	8.0±0.5	12.6±0.0
FFM	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.6±0.2	7.2±0.0	10.8±0.7
FFH	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.5±0.2	7.8±0.1	10.4±0.1

The underlined values are significantly larger ($P<0.05$) than the control (i.e. diameter of MeOH treatment), based on two-way analysis of variance and Tukey's multiple comparison. The paper disks used had a diameter of 7.2mm.

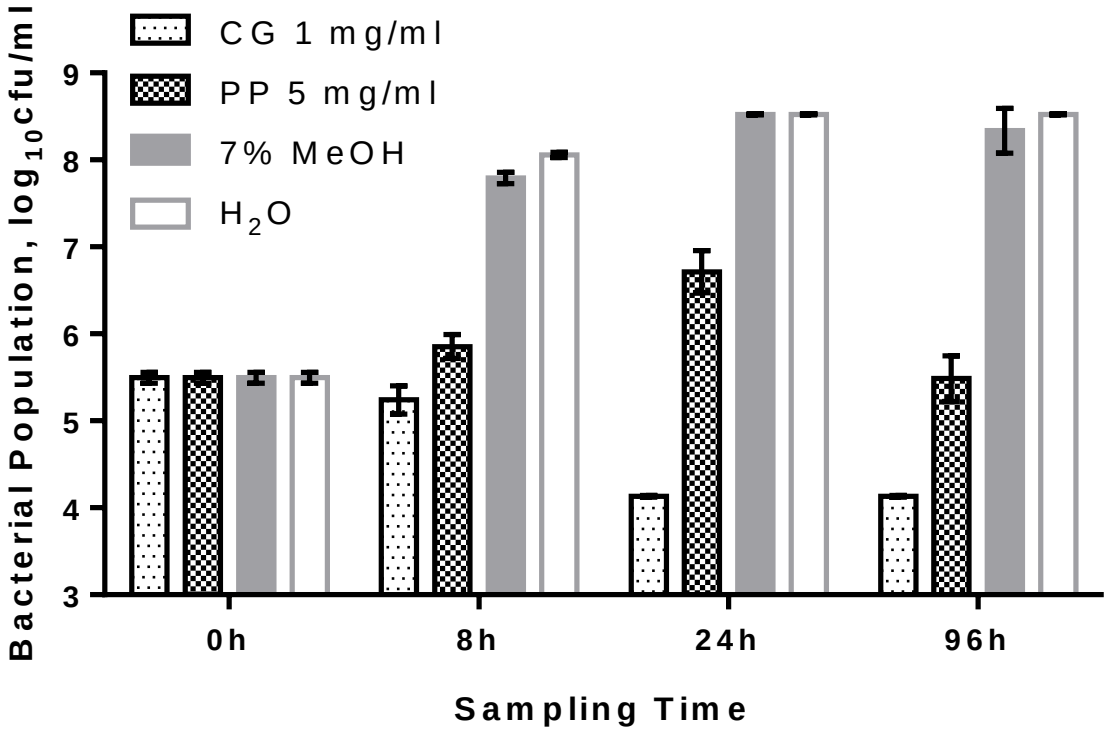
CGM: Chinese gall from Mayway Inc. (CA, USA); CGH: Chinese gall from a hospital (Beijing, China); PPM: pomegranate peel from Mayway Inc. (CA, USA); PPH: pomegranate peel from a hospital (Beijing, China); BSM: Baikal skullcap root from Mayway Inc. (CA, USA); BSH: Baikal skullcap root from a hospital (Beijing, China); FFM: forsythia fruit from Mayway Inc. (CA, USA); FFH: forsythia fruit from a hospital (Beijing, China)

Figure 3.1. Population (cfu/ml) of *V. parahaemolyticus* in tryptic soy broth (TSB) with plant extract treatments, after 0 hour, 8 hours, 24 hours and 96 hours incubation at 37±1°C



Columns showed bacteria growths (cfu/ml) in samples. The initial populations (0 h) of bacteria cultures were calculated from the plate count of the inoculum. At both 24h and 96h, Chinese gall (CG), pomegranate peel (PP), and Baikal skullcap root (BS) treatments significantly reduced the bacterial growth ($P< 0.0001$). Error bars represent standard deviation (SD)

Figure 3.2. Population (cfu/ml) of *L. monocytogenes* in broth with plant extract treatments, after 0, 8, 24 and 96 hours incubation at 37±1°C



Columns showed bacteria growths (cfu/ml) in samples. The initial populations (0 h) of bacteria cultures were calculated from the plate count of the inoculum. Starting from 8 hours, Chinese gall (CG) and pomegranate peel (PP) treatments significantly reduced the bacterial growth ($P < 0.0001$). Error bars represent standard deviation (SD)

CHAPTER 4

ANTIMICROBIAL TEST OF GALLA CHINENSIS EXTRACTS WITH VIBRIO PARAHAEMOLYTICUS AND LISTERIA MONOCYTOGENES ON COOKED SHRIMP AND RAW TUNA

Abstract

This research studied antimicrobial effects of aqueous methanol extracts from pomegranate peel ("PP", *Punica Granatum* L.) and Chinese gallnut ("CG", *Galla chinensis*) against *Vibrio parahaemolyticus* and *Listeria monocytogenes* on seafood products. Cooked shrimp and raw tuna slurry were treated with 70% methanol extracts of PP and CG, respectively. The concentration of soaking solution for shrimp is 5 mg/ml, and the concentration of extract in tuna was 1.7 mg/ml. The antimicrobial assay on *V. parahaemolyticus* was conducted at 12°C, and the assay in tuna was conducted on both 4°C and 12°C.

Both CG and PP extracts significantly inhibited growth of *V. parahaemolyticus* on shrimp and tuna respectively. The inhibition from CG was stronger than PP. Only CG significantly inhibited *L. monocytogenes*. Overall the *V. parahaemolyticus* was more susceptible to both plant extracts than *L. monocytogenes*. Both plant extracts showed stronger antimicrobial activity in the assay conducted on shrimp. The reason may be that the binding between plant extracts and fish broth/slurry weakened the antibacterial activity of plant extracts. In both assays, neither extract showed a complete inhibition of

bacteria, thus the plant extracts can work as a food preservative, but are not sufficient to be lethal against *V. parahaemolyticus or L. monocytogenes.*

Introduction

Vibrio parahaemolyticus and *Listeria monocytogenes* are two important foodborne pathogenic bacteria. According to Centers of Disease Control and Prevention (CDC, 2011), the number of foodborne illnesses caused by *V. parahaemolyticus* is increasing. In 2013, the highest number of cases was observed since 1996. *L. monocytogenes* was responsible for the third largest number of foodborne deaths in the USA (CDC, 2010). According to CDC's foodborne outbreak online database, outbreaks caused by *V. parahaemolyticus* are specifically associated with consumption of raw shellfish and crustacean; outbreaks caused by *L. monocytogenes* are associated with ready-to-eat (RTE) food products. In both situations, pre-consumption heat treatment is either absent or not sufficient. Instead, plant extracts have the potentials to be used as natural preservatives that can improve food safety.

Previous research discussed the antimicrobial activities of CG and PP extracts (Al-Zoreky, 2009; Tian et al., 2009; Feng et al., 2012). Some research further addressed the potential application of plant-source polyphenols in food preservation (Taguri et al., 2004; Guillén and García, 2007; AL-Zoreky, 2009; González et al., 2010; Xi et al., 2012). However, there has been little research specifically addressing the application of CG's or its major components, gallotannins' application in seafood product. Research described in this chapter was the second part of the study. Based on the antimicrobial tests described in the previous chapter, CG and PP were two plants that inhibited the growth of *V. parahaemolyticus* and *L. monocytogenes*. Thus, only these two plant extracts were tested. Cooked shrimp and raw tuna were used for the food matrices. Cooked RTE shrimp was used to simulate contamination from post-processing and

consumer's handling. Raw tuna was used to simulate contamination of a RTE food that is without heat treatments (e.g., sushi and sashimi). Unlike other studies which applied polyphenols after inoculation (Al-Zorely, 2009; Xi et al, 2012), a pre-inoculation strategy was employed in this study. The study hypothesis is that the extracts applied to RTE food products can significantly reduce the growth of the inoculated pathogens.

Materials and Methods

Plant materials

Pomegranate peel (PP, *Punica Granatum* L.) and Chinese gallnut (CG, *Rhus chinensis* Mill.) were purchased from Mayway Inc. (CA, USA) and pharmacy of No. 263 Military Hospital (Beijing, China) respectively, in the form of dry materials. The HPLC grade methanol was purchased from Fisher Scientific (PA, USA) and a food processor (Osterizer™) was used to grind raw plant materials. The dry, capsule-like CG was carefully cracked using a hammer. The remains of aphids found inside the galls were removed and the shell part was collected and ground. The crude ground gall shell was sifted through a 40-mesh sieve and the fine powder was collected. Five grams of sifted powder was mixed with 100 ml aqueous methanol solution (MeOH:H_2O = 70:30, v/v) and stirred with mild heating (30-40°C) for 3 hours. The mixture was separated by gravity filtration using #1 filter paper (Whatman®) and the filtrate was collected. The aliquot was standardized with 70% methanol to 100 ml and stored at -20°C for further use.

Large pieces of dry PP were separated by hand into approximately 1 cm x 1 cm pieces or smaller, and then ground and sifted through a 40-mesh sieve. The fine powder was extracted and collected using the same protocol as for the CG.

The concentration of the extract solutions were calculated as:

$$Concentration\ \left(\frac{mg}{ml}\right) = \frac{Mass\ of\ dry\ powdered\ plant\ (mg)}{Volume\ of\ solution\ in\ 70\%\ methanol(ml)}$$

Stock solutions (50 mg/ml) of those four plants were stored at -20 °C for later dilutions and uses in antimicrobial tests in shrimp and tuna.

Preparation of bacterial cultures

Listeria monocytogenes stock strain (isolated by local CDC) was obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into 9.9 ml tryptic soy broth (TSB, BD Bacto™) and then incubated at 37°C for 24 hours. After the first incubation, the cultures were transferred into another TSB tube and incubated for another 24 hours at 37°C. After the second incubation, the culture was T-streaked on Modified Oxford Agar (MOX, BD Difco™) and incubated for 24 hours at 37°C. A single clear colony with dark media surrounded was taken from the MOX plate and transferred into TSB tube and incubated for 24 hours at 37°C to reach the population of approximately 10^9 CFU/ml.

Vibrio parahaemolyticus stock strain (VP16) was obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into 9.9 ml TSB+NaCl (1.5% sodium chloride, the total NaCl concentration of

the medium was 2%) tube and then incubated at 37°C for 24 hours. After the first incubation, the cultures were transferred into another TSB+NaCl tube and incubated for another 24 hours at 37°C. After the second incubation, the culture was T-streaked on Thiosulfate-citrate-bile salts-sucrose agar (TCBS, BD Difco™) and incubated for 24 hours at 37°C. A single colony with dark green center was taken from the MOX plate and transferred into TSB+NaCl tube and incubated for 24 hours at 37°C to reach the population of approximately 10^9 CFU/ml.

Antimicrobial test on RTE food model – fully cooked shrimp

Frozen, cooked shrimps were purchased from Kroger grocery store (Kroger®, tail-on, peeled and deveined, 71-90/lbs, lot # R502803, best by 04/06/2015). Fifty milliliter of each stock solution (50 mg/ml) of CG and PP was diluted to 5 mg/ml using sterile de-ironized water. Shrimps were thawed at 4 °C for 12 hours, and divided into 4 groups. Each group of shrimp was then sterilely transferred into diluted CG extract (5 mg/ml), diluted PP extract (5 mg/ml), 7% methanol, or sterile water. The shrimp were soaked and shaken for 5 minutes. After the soaking treatment, the shrimp (5.5-7.5 g) were drained and aseptically transferred into a stomacher bag. Bacterial cultures of *L. monocytogenes* and *V. parahaemolyticus* were diluted to approximately 10^5 and 10^7 CFU/ml, then 100 μl of *L. monocytogenes* or *V. parahaemolyticus* were inoculated into each bag. The shrimp inoculated with *V. parahaemolyticus* were incubated at 12 °C for 10 days. On Day 1, 4, 7 and 10, two bags were removed. Sterile peptone salt water (0.1% with 2% NaCl) was added to make a 20 g mixtures which was then homogenized using a stomacher (AES™). One hundred microliters of the mixture was removed and

plated on TCBS agar. The plates were incubated at 37 °C for 24 hours and counted for colony forming units. The shrimp inoculated with *L. monocytogenes* were divided into two groups, and incubated at 4°C and 12 °C respectively, for 10 days. On Day 1, 4, 7 and 10, two samples were removed. Sterile peptone water (0.1%) was added to make a 20 g mixture and then the mixture was homogenized in the stomacher. Fifty microliters of the homogenized sample were plated on MOX agar using a spiral plater (Spiral Biotech Autoplate® 4000). The plates were incubated at 37 °C for 24 hours and counted for colony forming units.

Antimicrobial test on raw seafood model – frozen tuna

Frozen raw tuna steaks were purchased from a local grocery store in Hampton, VA (Berkley&Jensen®, wild caught ahi tuna steaks raw, lot code 24690-PO20091, best by 10/01 2014). The tuna steaks were thawed at 4°C for 12 hours and then cut into small pieces (approx. 10 mm*10 mm*5 mm or smaller) and approximately 20 grams were sterilely weighed in a stomach bag. Methanol (7%), sterile water, CG and PP extracts (5 mg/ml in 7% methanol) were prepared respectively and 10 ml of either of them were added into one bag. The stomacher bag with tuna pieces and crude extracts were homogenized in the stomacher, and divided into two groups for inoculation with *V. parahaemolyticus* and *L. monocytogenes*. The cultures of *V. parahaemolyticus* were diluted to approximately 10^7 CFU/ml, and 100 μl of diluted culture were inoculated into each bag. The bags were manually massaged for 5 min and then incubated at 12 °C for 10 days. On Day 1, 4, 7 and 10, the bags of tuna slurry were taken out and massaged for 5 minutes, then 100 μl was plated on TCBS agar. The plates were incubated at 37

°C for 24 hours and counted for colony forming units. The cultures of *L. monocytogenes* were diluted to approximately 10^5 CFU/ml and 100 μl was inoculated in each bag. The bags were manually massaged for 5 min and further divided into two groups, then incubated at 4°C and 12 °C respectively, for 10 days. On Day 1, 4, 7 and 10, the bags of tuna/treatment mixture were removed and massaged for 5 min and 50 μl was removed and then plated on MOX agar using spiral plater. The plates later were incubated at 37 °C for 24 hrs and counted for colony forming units.

Statistical Analysis

The results were analyzed in GraphPad Prism Version 6.01. The results were compared using two-way ANOVA. Whenever the overall F value was significant, multiple comparisons were performed using Tukey's Studentized T-test to find differences among treatments (Zar, 2010).

Results and Discussions

Antimicrobial test on shrimp against *V. parahaemolyticus*

The results of the test on shrimp and *V. parahaemolyticus* are shown in Figure 4.1. The planned incubation temperature was 12 °C, while according to measurements the actual incubation temperature was 14±2 °C. This was set to simulate the situation of a mild temperature abuse, which may be more likely to happen in the real world, and have been previously discussed (Hudson and Mott, 1993). The plate counts, indicate that inhibitions of bacterial growth by CG began on Day 1, and continued to Day 4. On day 4, the bacterial growth in CG treated samples was below detectable levels, and growth

inhibition occurred during the entire incubation process. The inhibition of bacterial growth by PP extracts reached its maximum on Day 4, but the population of *V. parahaemolyticus* recovered afterwards, and fluctuated between 10^4 and 10^5 cfu/g. In contrast, the bacterial growth in the control group increased from 10^4 to over 10^8 cfu/g. The methanol control treatment involved dipping the shrimp in 7% methanol prior to incubation. Although the concentration (7%) was the same as in the microdilution assay, the growth of *V. parahaemolyticus* was different in the two tests. In microdilution study, under optimum conditions (TSB+NaCl 37C) *V. parahaemolyticus* numbers increased throught the study. In the shrimp inoculation study, growth of *V. parahaemolyticus* was suppressed due to the methanol during the first several days. On Day 4, no bacterial growth was detected in the methanol treated shrimp. The bacterial growth gradually increased beginning on day 7, and was similar to growth in the water control group by day 10. In summary, the CG extract (5 mg/ml) treatment decreased the growth of *V. parahaemolyticus* on cooked shrimp by 4 logs during a 10-day incubation period. The PP treatments did not completely inhibit *V. parahaemolyticus* growth, but bacterial growth was reduced by 3 logs over a 10-day period.

Antimicrobial test on tuna and *V. parahaemolyticus*

The results of the tuna test are shown in Figure 3.2. The prior study using cooked shrimp test was to use the crude plant extracts as a soaking treatment. For the study using tuna, the tuna was homogenized and the crude plant extracts were mixed into the homogenized tuna. This enabled the exact concentration of crude plant extracts mixed with the ground tuna to be determined. On day 4, the raw tuna was spoiled and the

spoilage microflora formed numerous yellowish green colonies on TCBS plate. Thus, plate count numbers of *V. parahaemolyticus* could not be determined. The inhibitory activity of CG against *V. parahaemolyticus* was still observed during the first 3 days as bacterial growth in tuna with CG treatments increased by less than 1 log. In contrast, the bacterial numbers in the control group increased by more than 3 logs from 10^4 to more than 10^7 cfu/g. The PP crude at 1.7 mg/ml in raw tuna did show a 1 log inhibition against *V. parahaemolyticus.* The methanol control group also showed a slight inhibition of bacterial growth.

Although the same concentration (5 mg/ml) of plant extracts were used in both the shrimp and tuna tests, the concentration of actual residue of treatments in tuna test might be different than those used in the shrimp study. In the shrimp study, whole shrimps were soaked in the crude extract solution. In the tuna study, the plant extracts were mixed into the homogenized tuna. Although the total residue of plant extracts in the shrimp might be lower than that in tuna, the plant extracts in shrimp study were more likely to be found on surface of the shrimp surface where direct contact with bacteria occurred. Therefore, the bacteria were exposed to a higher concentration of crude plant extract in shrimp compared with the tuna study. This may explain why the crude plant extracts demonstrated a stronger bacterial inhibition on the shrimp. The concentration of the crude plant extract in the homogenized tuna was less but still higher compared with the MIC study.

The inhibitory effects of the crude plant extracts on *L. monocytogenes* and *V. parahaemolyticus* were lower in tuna compared to the shrimp. Although this may be due to the different concentrations of the crude extracts, a more likely explaination may be

due to the differences in the food matrices. The raw tuna reacted with the crude plant extract forming cloudiness. The cooked shrimp was less reactive with plant extract. This treatment-food reaction (cloudiness) may have significantly reduced the efficacy of the crude plant extracts.

Antimicrobial test on shrimp against *L. monocytogenes*

The results of the test are shown in Figure 4-3. The inhibitory effects of CG and PP were tested both at 4 °C (Figure 3.3-a) and 12±1°C (Figure 3.3-b), simulating the situation of refrigerated storage (thawing) and mild temperature abuse, respectively. The initial bacterial population in the samples was 10^3 cfu/g level. During the 10 day incubation, *L. monocytogenes* in the 4 °C group increased by 3 logs in the control samples. In the CG treated samples, the inhibition started on Day 7. On Day 10, the CG treatment reduced bacterial growth by 2 logs, compared to the control. The PP treatment had a weaker inhibition on *L. monocytogenes* growth, and by day 10, bacterial numbers were 1 log less compared with the controls. The methanol control treatment reduced bacterial growth but the difference not significant. In the 12 °C group, the overall growth of *L. monocytogenes* was greater than at 4°C. Significant inhibition from CG extract began on Day 4, and continued until Day 10. On Day 10, the bacterial growth with the CG treatment was 2 logs lower than the control group. The PP treatment did not have any significant inhibition at 12°C.

Only CG and PP were used in the study on fish or shrimp, since the agar diffusion and microdilution studies demonstrated that the FF and BS extracts demonstrated weak

inhibitions against the bacterial growths. The *L. monocytogenes* study was also conducted at refrigeration temperatures, since *Listeria* can survive and grow at 4°C.

The studies were conducted at refrigerated temperature (4°C) and at slightly higher temperature (12°C). The inhibitory effect of the CG extracts was shown after a comparatively long incubation period (approximately 7 days). These results indicate that the CG treatment was unable to completely inhibit *L. monocytogenes*, but it did postpone the exponential phase of bacterial growth. The CG treatment may be able to reduce the risk of listeria in accidental situations when thawing or transportation span is longer than usual. The CG treatment may have application for RTE foods such as salad, deli meat, sushi and sashimi which are recommended to be kept at refrigerated temperatures,

The antimicrobial test on tuna against *L. monocytogenes*

The results are shown in Figure 4-4. During the 7-day incubation, the bacterial population in the tuna samples increased rapidly (3-4 logs), despite the addition of the crude plant extracts. Samples with added CG or PP extracts had significant lower bacterial populations compared with than control; but the differences were less than 1 log.

Along with the plant extract treatments, the change of sensory properties of the shrimp and tuna may be a concern (Pérez-Mateos et al., 2001). As discussed in section 3.2, raw tuna contains undenatured protein which can interfere with the activity of the plant extracts compared with a cooked product (Kawamoto et al. 1997). In this study, the tuna meat was whitened by CG and PP extracts after the contact.

Antimicrobial tests of plant extracts in culture media and on food matrices were conducted at different temperature. According to the results, *L. monocytogenes* were more resistant to plant extracts at lower temperature (4°C or 12°C) than at higher temperature (37°C). This might be from the different media used; it might be also from the difference in cell membrane permeability at different temperature (Nikaido, 2003).

Estimated standard plant counts (ESPC) were reported instead of standard plant counts (SPC), since a spiral plater was used. Even when the agar plate was too numerous to count (TNTC), (>>250 cfu/plate), the number of colonies were calculated using the formula provided. Therefore, fewer dilutions were required, and since the actual number of cfu seldom fell in the 25-250 range, it was not appropriate to be reported as SPC (Williams, 2013). The studies were conducted using inoculations higher than those found in the real world. This was to ensure the successful growth and sampling of the bacteria.

Summary

The use of crude CG extract can significantly reduce the population of *V. parahaemolyticus* and *L. monocytogenes* in both shrimp and tuna, but the inhibitory effects were less than those found on TSA plates or TSB tubes. Storage temperature must be controlled as the prerequisite of the application of the extracts. The application of plant extracts in conjunction with control of storage temperatures can control the growth of pathogens on seafood products. The use of plant extracts may also act as a “hurdle” to reduce the risk from potential product temperature abuse.

References

Al-Zoreky, N. S. (2009). Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. *International Journal of Food Microbiology*, *134*(3), 244-248.

Centers for Disease Control and Prevention. (2011). Cholera and Other Vibrios Illness Surveillence (COVIS) annual summary 2011.

Feng, C. T., Su, H. J., Chen, C. T., Ho, W. C., Tsou, Y. R., & Chern, L. L. (2012). Inhibitory effects of Chinese medicinal herbs on plant-pathogenic bacteria and identification of the active components from gallnuts of Chinese sumac. *Plant Disease*, *96*(8), 1193-1197.

González, M. J., Torres, J. L., & Medina, I. (2010). Impact of thermal processing on the activity of gallotannins and condensed tannins from Hamamelis virginiana used as functional ingredients in seafood. *Journal of Agricultural and Food Chemistry*, *58*(7), 4274-4283.

Guillén, G., & Montero García, P. (2007). Polyphenol uses in seafood conservation. *American Journal of Food Technology*, *2*, 593-601.

Juneja, V. K., Altuntaş, E. G., Ayhan, K., Hwang, C. A., Sheen, S., & Friedman, M. (2013). Predictive model for the reduction of heat resistance of *Listeria monocytogenes* in ground beef by the combined effect of sodium chloride and apple polyphenols. *International Journal of Food Microbiology*, *164*(1), 54-59.

Kawamoto, H., Mizutani, K., & Nakatsubo, F. (1997). Binding nature and denaturation of protein during interaction with galloylglucose. *Phytochemistry*, *46*(3), 473-478.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews*, *67*(4), 593-656.

Pérez-Mateos, M., Boyd, L., Allen, J. C., & Lanier, T. C. (2001, August). Omega-3 fortification of surimi seafood. In *46th Atlantic Fisheries Technology Conference (AFT)* (pp. 26-29).

Taguri, T., Tanaka, T., & Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biological and Pharmaceutical Bulletin*, *27*(12), 1965-1969.

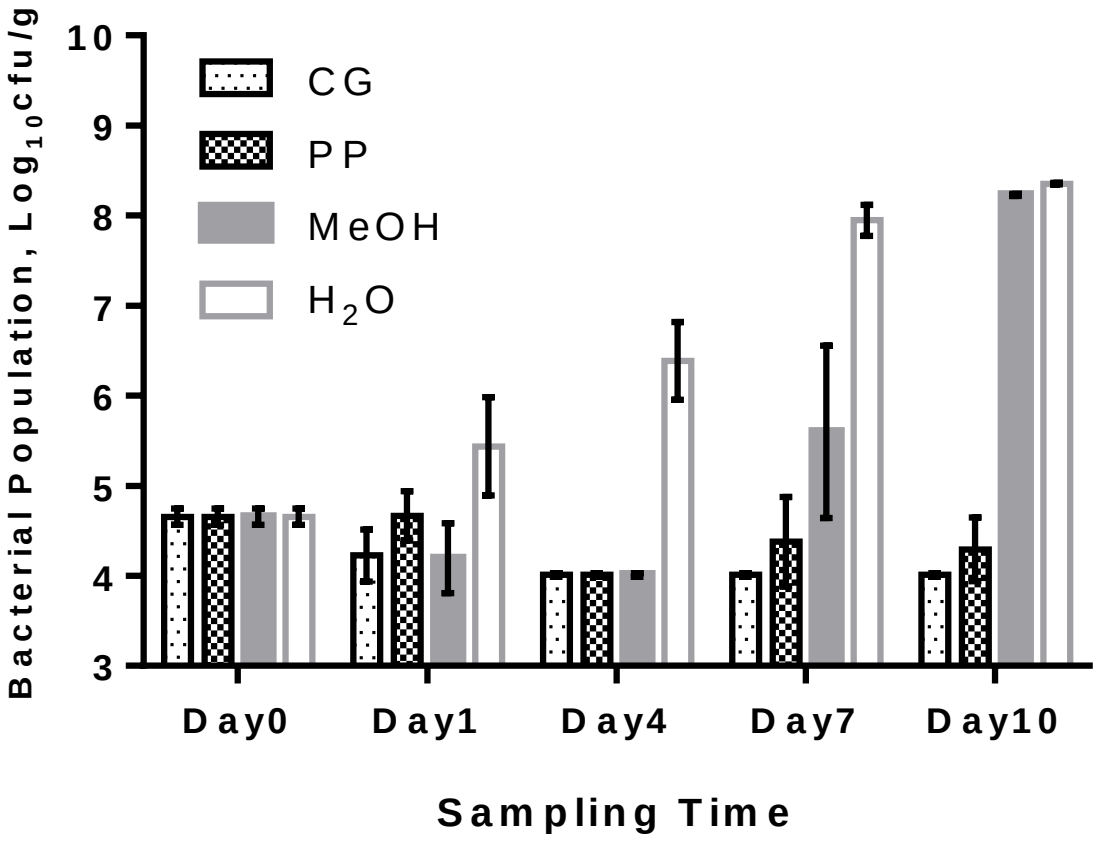
Tian, F., Li, B., Ji, B., Yang, J., Zhang, G., Chen, Y., & Luo, Y. (2009). Antioxidant and antimicrobial activities of consecutive extracts from *Galla chinensis*: The polarity affects the bioactivities. *Food Chemistry*, *113*(1), 173-179.

Williams, R. (2013) *Food Microbiology Laboratory Manual*, Virginia Tech

Xi, D., Liu, C., & Su, Y. C. (2012). Effects of green tea extract on reducing *Vibrio parahaemolyticus* and increasing shelf life of oyster meats. *Food Control*, *25*(1), 368-373.

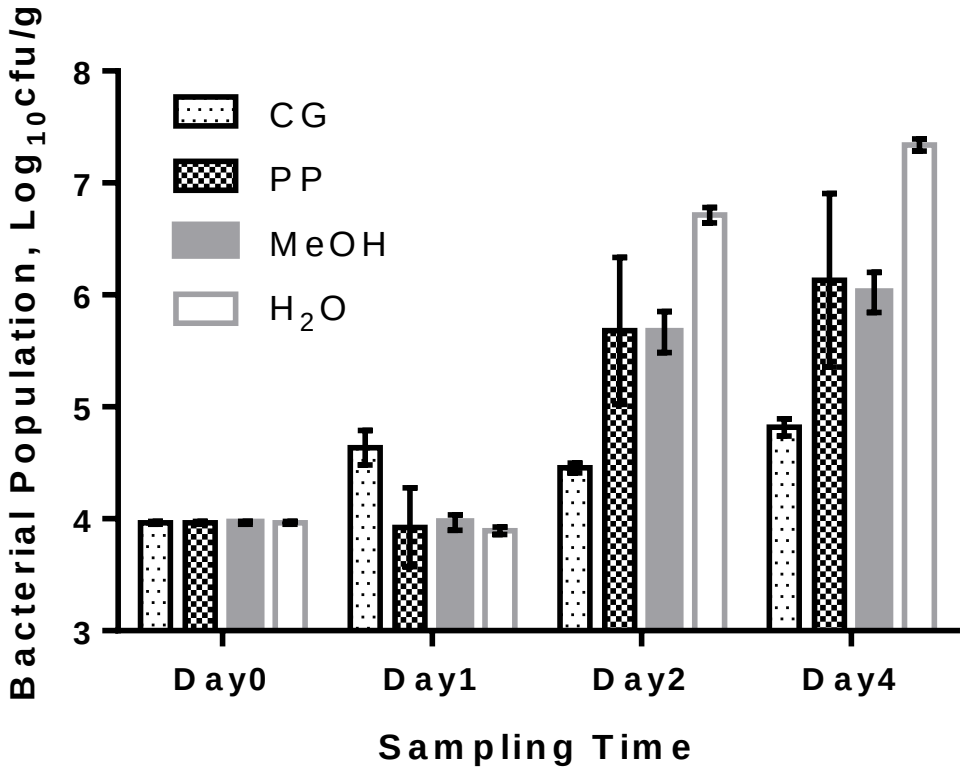
Zar, J. H. (2010). *Biostatistical Analysis*: Prentice Hall.

Figure 4.1. Population (cfu/ml) of *V. parahaemolyticus* on cooked shrimp with treatments of Chinese gall (CG) extract, pomegranate peel (PP) extract, 7% methanol and water, after 0 day, 1 day, 4 days, 7 days and 10 days incubation at 12±1°C



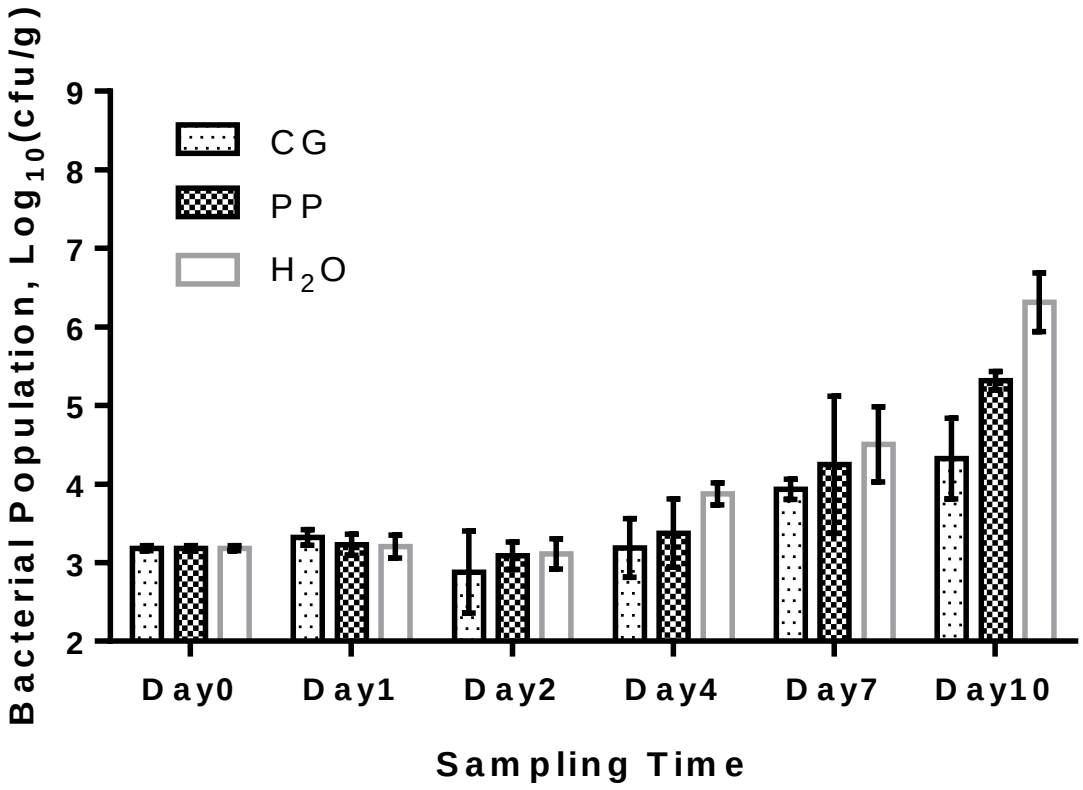
The columns showed bacteria growths (cfu/g) in samples. The concentration of plant extracts used as soaking agent was 5 mg/ml in 7% methanol. The initial populations (Day 0) of bacteria cultures were calculated from the plate count of the inoculum. On Day 4, 7, and 10, Chinese gall (CG) and pomegranate peel (PP) significantly inhibited the growth of *V. parahaemolyticus* on shrimp ($P<0.01$) based on two-way analysis of variance. Error bars represent the standard deviation (SD).

Figure 4.2. Population (cfu/ml) of *V. parahaemolyticus* on raw tuna with treatments of Chinese gall extract, pomegranate peel extract, methanol and water, after 0 day, 1 day, and 4 days incubation at 14±2°C



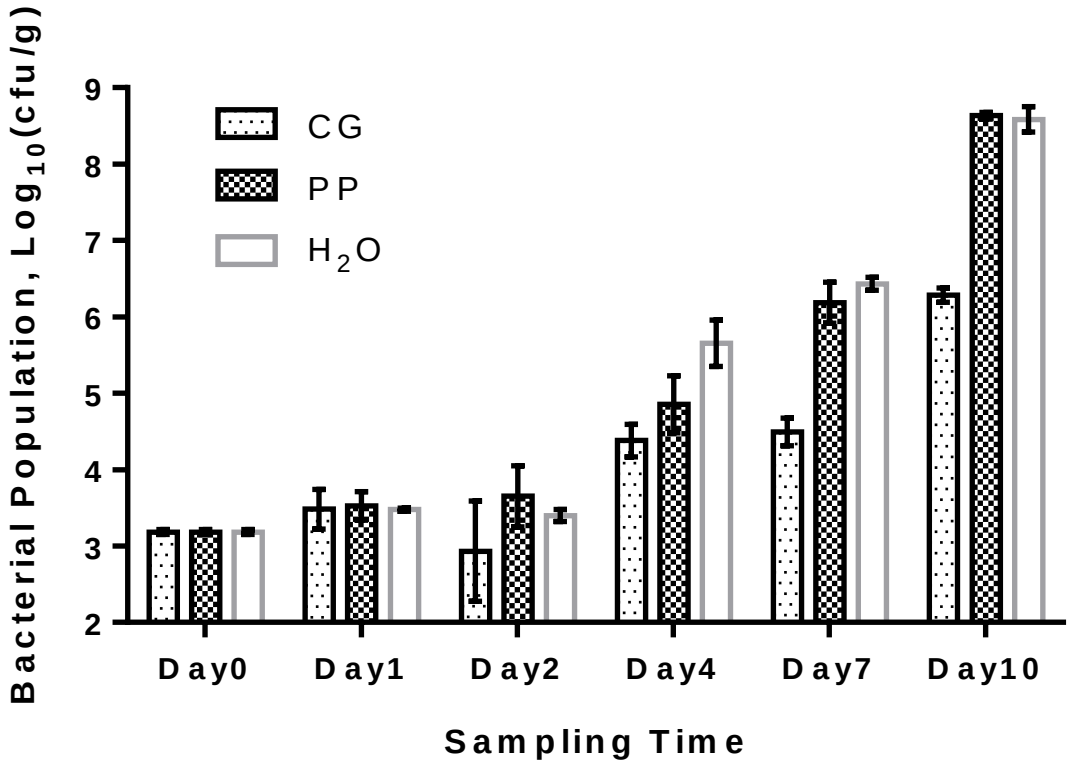
The columns showed bacteria growths (cfu/g) in samples. The concentration of plant extracts in tuna was 1.7 mg/g. The initial populations (Day 0) of bacteria cultures were calculated from the plate count of the inoculum. On Day 2, and 4, Chinese gall (CG) and pomegranate peel (PP) significantly inhibited the growth of *V. parahaemolyticus* ($P<0.05$) based on two-way analysis of variance. After Day 4, the growth of *V. parahaemolyticus* cannot be quantified due to the boost of natural microflora on tuna. Error bars represent the standard deviation (SD).

Figure 4.3. Population (cfu/ml) of *L. monocytogenes* on cooked shrimp with treatments of Chinese gall extract, pomegranate peel extract, methanol and water, after 0 day, 1 day, 2 days, 4 days, 7 days and 10 days incubation at 4±1°C



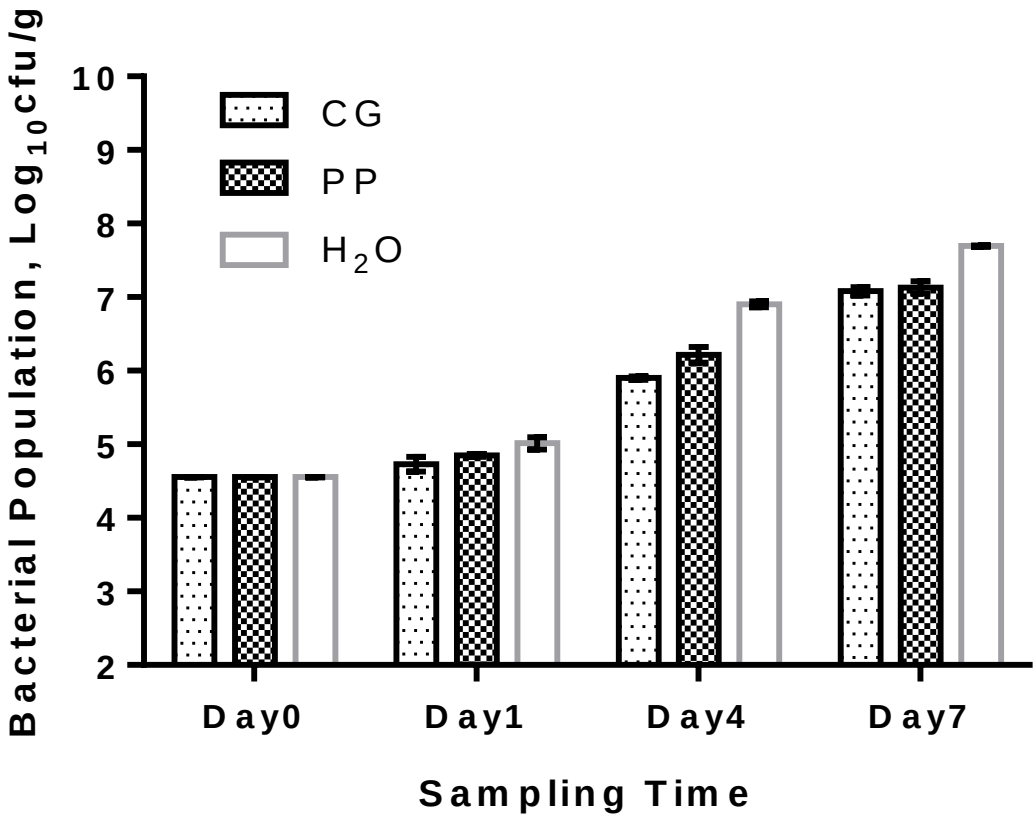
The columns showed bacteria growths (cfu/g) in samples. The concentration of plant extracts used as soaking agent was 5 mg/ml in 7% methanol. The initial populations (Day 0) of bacteria cultures were calculated from the plate count of the inoculum. Neither of Chinese gall (CG) or pomegranate peel (PP) showed significant inhibition on the growth of *L. monocytogenes* on shrimp ($P>0.05$) until Day 10, based on two-way analysis of variance. Error bars represent the standard deviation (SD).

Figure 4.4. Population (cfu/ml) of *L. monocytogenes* on cooked shrimp with treatments of Chinese gall extract, pomegranate peel extract, methanol and water, after 0 day, 1 day, 2 days, 4 days, 7 days and 10 days incubation at 12±1°C



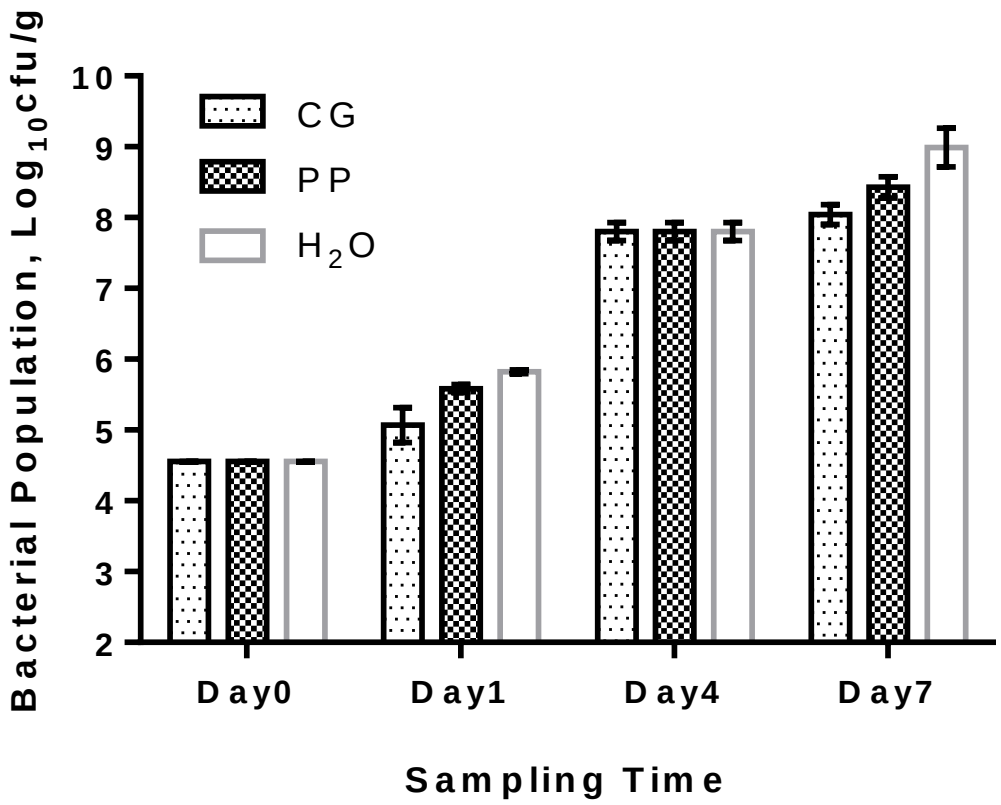
The columns showed bacteria growths (cfu/g) in samples. The concentration of plant extracts used as soaking agent was 5 mg/ml in 7% methanol. The initial populations (Day 0) of bacteria cultures were calculated from the plate count of the inoculum. Starting from Day 2, Chinese gall (CG) showed significant inhibition on the growth of *L. monocytogenes* on shrimp ($P<0.05$). Pomegranate peel (PP) did not show significant inhibition on the growth of *L. monocytogenes* on shrimp ($P>0.05$) except for Day 4, based on two-way analysis of variance. Error bars represent the standard deviation (SD).

Figure 4.5. Population (cfu/ml) of *L. monocytogenes* on raw tuna with treatments of Chinese gall extract, pomegranate peel extract, methanol and water, after 0 day, 1 day, 4 days, and 7 days incubation at 4±1°C



The columns showed bacteria growths (cfu/g) in samples. The concentration of plant extracts in tuna was 1.7 mg/g. The initial populations (Day 0) of bacteria cultures were calculated from the plate count of the inoculum. On Day 4 and 7, both Chinese gall (CG) and pomegranate peel (PP) significantly inhibited the growth of *L. monocytogenes* on tuna ($P<0.05$) based on two-way analysis of variance. Error bars represent the standard deviation (SD).

Figure 4.6. Population (cfu/ml) of *L. monocytogenes* on raw tuna with treatments of Chinese gall extract, pomegranate peel extract, methanol and water, after 0 day, 1 day, 4 days, and 7 days incubation at 12±1°C



The columns showed bacteria growths (cfu/g) in samples. The concentration of plant extracts in tuna was 1.7 mg/g. The initial populations (Day 0) of bacteria cultures were calculated from the plate count of the inoculum. On Day 1 and 7, both Chinese gall (CG) and pomegranate peel (PP) significantly inhibited the growth of *L. monocytogenes* on tuna ($P<0.05$) based on two-way analysis of variance. Error bars represent the standard deviation (SD).

CHAPTER 5:

FRACTIONATION AND ANALYSIS OF *GALLA CHINENSIS* AND ANTIMICROBIAL ASSAYS OF ITS FRACTIONS

Abstract

The goal of this study was to analyze the major components of Chinese Gall (CG, *Galla chinensis*) extracts that are responsible for the antimicrobial activity against *Listeria monocytogenes*, *Vibrio parahaemolyticus*, and *Morganella morganii*. The crude extract of CG in 70% methanol was fractionated using reversed-phase HPLC. Seven fractions (CG1-CG7) of the UV-absorbing compounds were collected based on the major peaks at 280nm. The compounds were analyzed using LC-MS and identified as gallic acid, digallic acid, methyl gallate, and gallotannins (oligo-galloyl-D-glucose, nGG, n=1~10). Fractions of CG were freeze dried and standardized to 10 mg/ml in 70% methanol, and tested for their antimicrobial activities using both agar diffusion assay and microdilution assay.

The fractions of CG2+CG3, CG4, CG5, and CG6 showed significant inhibition to *L. monocytogenes* in tryptic soy broth (TSB), but the inhibitions on tryptic soy agar (TSA) were not observed. All fractions showed significant inhibition to *V. parahaemolyticus* in TSB and CG4, CG5, and CG6 showed significant inhibition on TSA. The growth of *M. morganii* on TSA was also inhibited by CG4, CG5, and CG6; however, in TSB there was no inhibition of *M. morganii* from any CG fraction.

The antimicrobial activity of fractions may be influenced by the molecular weight or polarity of the components. Among all components, the 3GG to 8GG have higher antimicrobial activities than free gallic acid, methyl gallate and digallic acid.

Introduction

Chinese Gallnut ("CG", *Galla chinensis*) is the gall of sumac trees. It has been appreciated for its antimicrobial and anti-inflammation activities and used as a traditional medicine in China and other Asian countries for centuries (Djakpo and Yao, 2010). Previous research shows that the CG contains abundant gallotannins (Nishizawa et al. 1982; Tian et al., 2009b), a series of polyphenols that have been reported to have antimicrobial and antioxidant activities (Scalbert, 1991; Akiyama et al., 2001; Buzzini et al., 2008; Anguilar-Galvez et al., 2014). The studies of antimicrobial activity of the major components in CG showed that the gallotannins can inhibit the growth of several foodborne pathogens and plant pathogens (Taguri et al., 2004; Tian et al., 2009a, 2009b; Engels et al., 2011; Feng et al., 2012); however, there has been few research addressing the inhibition of the major compound(s) in CG against *Vibrio parahaemolyticus* or *Listeria monocytogenes.* The relationship between galloylation of tannins and antimicrobial activity was briefly discussed by Tian et al. (2009b), yet the assays were not specifically to *V. parahaemolyticus* or *L. monocytogenes*.

The previous steps of this study (Chapter 3 and 4) showed that the CG crude extract in 70% methanol as a whole treatment can significantly inhibition some pathogenic bacteria species both in culture media and in seafood matrices. The results from this study showed that *V. parahaemolyticus* and *L. monocytogenes* reacted to tannins differently than typical Gram-positive and Gram-negative bacteria, when compared with previous research (Taguri et al., 2004; Tian et al., 2009b; Maqsood et al., 2013). Therefore, the specific anti-vibrio and anti-listeria activities of tannins are still unclear.

In this chapter, the study addressed identifying the major compounds of the CG extract, as well as the antimicrobial activity of individual compounds against *V. parahaemolyticus* and *L. monocytogenes*. The CG crude extract was fractionated using HPLC methods and analyzed by LC-MS. All the fractions collected were standardized into the same concentration and were tested using agar diffusion assays and microdilution assays described in Chapter 3 and 4.

Materials and Methods

Preparation of plant extract

Chinese gallnut (CG, *Rhus chinensis* Mill.) were purchased from Mayway Inc. (CA, USA) and pharmacy of No. 263 Military Hospital (Beijing, China) respectively, in the form of dry materials. HPLC grade methanol was purchased from Fisher Scientific (PA, USA) and a food processor (Osterizer™) was used to grind raw plant materials.

The dry, capsule-like CG were carefully cracked using a hammer. The remains of aphids found inside the galls were removed and the shell part was collected and ground. The crude ground gall shell was sifted through a 40-mesh sieve and the fine powder was collected. Five grams of sifted powder was mixed with 100 ml aqueous methanol solution (MeOH:H_2O = 70:30, v/v) and stirred with mild heating (30-40°C) for 3 hours. The mixture was separated by gravity filtration using #1 filter paper (Whatman®) and the filtrate was collected. The aliquot was standardized with 70% methanol to 100 ml and stored at -20°C for further use.

Large pieces of dry PP were separated by hand into approximately 1 cm x 1 cm pieces or smaller, and then ground and sifted through a 40-mesh sieve. The fine powder was extracted and collected using the same protocol as for the CG.

The concentration of the extract solutions were calculated as:

$$Concentration\ \left(\frac{mg}{ml}\right) = \frac{Mass\ of\ dry\ powdered\ plant\ (mg)}{Volume\ of\ solution\ in\ 70\%\ methanol(ml)}$$

Stock solutions (50 mg/ml) of those four plants were stored at -20 °C for later dilutions and uses in antimicrobial tests in shrimp and tuna.

HPLC-DAD analysis of CG extract

The HPLC method was based on Tian et al. (2009b) with modifications. An HP Agilent™ 1100 series HPLC with Diode-Array Detector (DAD) was used for the study (G1379A Degasser, G1313A ALS, G1311A Quat Pump, G1316A Column Oven, and G1315A DAD). An Agilent™ Eclipse XDB-C18 reversed-phase column (250mm*4.6mm, particle size 5 μm) with a guard column (model) was used for the analysis. The gradient elution used was as follows: Solvent A: 0.1% formic acid in acetonitrile; Solvent B: 0.1% formic acid in water; 0-20 min, 0%-15% A; 20-35 min, 15%-90% A; 35-40 min, 90% A; 40-41 min, 90%-0% A; 41-46 min, 0% A. The flow rate was 1.0 ml/min, and the injection was 5-25 μl. The absorbance was detected at 280 nm.

LC-MS analysis of CG extract

The LC-MS analysis was conducted on a Waters™ Acquity H-class separation module. The same Agilent™ Eclipse XDB-C18 reversed-phase column used for HPLC analysis described in Section 2.2 was used. The elution performed was described as follow: Solution A: 0.1% formic acid in acetonitrile; Solvent B: 0.1% formic acid in water, 0-30 min, 0%-20% A; 30-45 min, 20%-90% A; 45-50 min, 90% A; 50-51 min, 90%-0% A; 51-56 min, 0% A. MS analysis of column effluent was performed by (−)-electrospray ionization (ESI) on a Waters Acquity TQD (triple quadrupole) mass spectrometer equipped with a Z-spray electrospray interface. The ESI capillary voltage was –4.00 kV. The source and desolvation temperatures were 150°C and 400°C, respectively. The desolvation gas and cone gasses were N_2 at flow rates of 480 L/min and 20 L/min, respectively. The scanned m/z was 150-1500. Data acquisition was carried out with MassLynx software (version 4.1, Waters).

Preparation of CG fractions

The crude CG extract was obtained from 2.1. The fractionation condition was the same as described in 2.2, with the injection volume of 25 μl. The fractionation procedure was repeated 40 times. Fractions (CG1 – CG7) were collected as follows: CG1: 4.0-6.5 min; CG2: 11.0-14.0 min; CG3: 14.0-15.5 min; CG4: 17.0-20.5 min; CG5: 23.5-27.5 min; CG6: 28.0-31.5 min; CG7: 32.0-34.0 min. The fractions were evaporated under 15 psi vacuum at 45 °C using a rotary evaporator (Make/model) to remove the acetonitrile and formic acid, and then were freeze dried at -40 °C for 72 hours until all moisture was removed. The residues of the fractions were weighed and re-dissolved in 70% methanol and standardized to 10 mg/ml.

Preparation of bacteria cultures

A *Listeria monocytogenes* stock strain (isolated by local CDC) was obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into 9.9 ml tryptic soy broth (TSB, BD Bacto™) and then incubated at 37°C for 24 hours. After the first incubation, the cultures were transferred into another TSB tube and incubated for 24 hours at 37°C. After the second incubation, the culture was T-streaked on Modified Oxford Agar (MOX, BD Difco™) and incubated for 24 hours at 37°C. A single clear colony with dark media surrounded was taken from the MOX plate and transferred into TSB tube and incubated for 24 hours at 37°C to reach the population of approximately 10^9 CFU/ml.

Vibrio parahaemolyticus stock strain (VP16) was obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and transferred into 9.9 ml TSB+NaCl (1.5% sodium chloride, the total NaCl concentration of the medium was 2%) tube and then incubated at 37°C for 24 hours. After the first incubation, the cultures were transferred into another TSB+NaCl tube and incubated for another 24 hours at 37°C. After the second incubation, the culture was T-streaked on Thiosulfate-citrate-bile salts-sucrose agar (TCBS, BD Difco™) and incubated for 24 hours at 37°C. A single colony with dark green center was taken from the MOX plate and transferred into TSB+NaCl tube and incubated for 24 hours at 37°C to reach the population of approximately 10^9 CFU/ml.

Morganella morganii stock strain (25830) was obtained from the Department of Food Science and Technology, Virginia Tech. Frozen capsule cultures were thawed and

transferred into 9.9 ml TSB tube and then incubated at 37°C for 24 hours. After the first incubation, the cultures were transferred into another TSB tube and incubated for another 24 hours at 37°C. After the second incubation, the culture was T-streaked on McConkey Agar (BD BBL™) and incubated for 24 hours at 37°C. A single colony that was not red was taken from the McConkey plate and transferred into TSB tube and incubated for 24 hours at 37°C to reach approximate population of 10^9 CFU/ml.

Antimicrobial assays of CG fractions.

The antimicrobial activity of the fractions was studied using the agar diffusion assays and microdilution assays described in Chapter 3 with modifications.

Agar diffusion assay

Whatman # 1 filter papers were cut into round paper disks with an average diameter of 7.2 mm, and autoclaved at 121°C for 15 minutes. Each TSA plate was divided into 7 areas. Bacteria cultures were inoculated onto the TSA plates using a sterile swab as described by Williams (2013) with minor modifications. A sterile sampling swab was dipped into bacterial culture tube, then gently squeezed against the inner wall of the tube to remove excessive liquid. The whole TSA plate was inoculated by swabbing in one direction, then covered again by swabbing in the perpendicular direction to the first. After the swab inoculation, 7 sterile paper disks were placed on the agar. Ten microliters of 70% methanol, 5% commercial bleach (Clorox®, 6.0% sodium hypochlorite, the final concentration of NaClO is 0.3%, i.e. 3 mg/ml), and extracts with concentrations of 20, 10, 5, 2, and 1 mg/ml were then pipetted onto the paper disks.

The TSA plates with bacterial inoculation and extracts added were incubated at 37°C for 24 hours. The diameter of inhibitory zones was determined by averaging two perpendicular measurements.

Microdilution assay

Sterile 96*250 μl well plates were obtained from BD biosciences. The experimental design is shown in Appendix B. One hundred and twenty-five microliters of TSB was pipetted into each well. Bacteria cultures (100 μl) were added into 9.9mL sterile 0.1% peptone (BD Bacto™) water. Each of the diluted culture was homogenized on vortex and 10 μl (approx. 10^5 cfu) was inoculated into wells. For each bacterium, two rows of wells (24 wells in total) were inoculated. Fifteen microliters of unfractionated CG, CG1, CG2+CG3, CG4, CG5, CG6 and CG7 were added to the assigned wells respectively. The concentration of these treatments was 10 mg/ml. Commercial bleach (15 μl, diluted into 5%, NaClO concentration was 3 mg/ml) was added into the assigned wells as a positive control, and 15 μl water was added into the assigned wells as a negative control.

The whole 96-well plate was sealed to reduce moisture lost through evaporation and then incubated at 37 °C for 24 hours. The inoculum at starting time was diluted in 10^{-1} and 10^{-3} concentrations and spread plated on TSA. Each of the culture wells was sampled at 24 hours using the protocol described below. The well plate was gently shaken by hand for 30 seconds to homogenize the culture in each well, then 15 μl sample of culture was transferred into a 10 ml 0.1% peptone water tube to make a diluted culture. The tube was mixed using a vortex mixer for 10 seconds, 50 μl diluted

culture was plated on duplicate TSA plates using the Spiral plater (Spiral Biotech Autoplate® 4000). The plates were incubated at 37 °C for 24 hours and colony forming units were counted and calculated following the instructions in Autoplate 4000® operation manual.

Statistical analysis

The results were analyzed in GraphPad Prism Version 6.01. For both agar diffusion assay and microdilution assay, the results of treatments and controls were compared using two-way analysis of variance (ANOVA). Whenever the overall F test was significant, multiple comparisons were performed using Tukey's Studentized T-test (Zar, 2010) to find differences among treatments(Zar, 2010).

Results and Discussion

HPLC analysis and fractionation

The chromatogram of CG fractionation is shown in Figure 5.1. The CG crude extract showed several major peaks when scanned at 280nm, which were divided into seven fractions and collected respectively. The method used was modified from Tian's research (2009b) but with major changes. The purpose of the modifications was to acquire better separation of peaks, while the elution time was not the primary consideration.

Fractions collected from the HPLC elution were collected, evaporated and freeze dried. Dry contents were weighted into 1.5 ml Eppendorf™ centrifuge tubes. Although

the gap areas between two peaks were not collected, the yield was higher than 90%, indicating that the CG mainly consisted of UV-absorbing compounds.

Compared with previous research, this study obtained a fast HPLC methods to fractionate gallotannins with good separations of major components, and the method is more effective for lower galloylated tannins, mostly 5GG and below. The separation methods can be prolonged to acquire better resolutions of 5GG – 8GG with a flatter gradient, but the elution gradient used was also modified for fractionation. The fractionation of gallotannins from plant sources has also been conducted using different chromatographic methods, including column chromatography (Tian et al. 2009a) and high-speed counter-current chromatography (HSCCC) (Engels et al., 2010). However, the HPLC method used in this study has fewer requirements and can be modified easily.

LC-MS results

A representative chromatogram of the LC-MS is shown in Figure 5.2. The HPLC method was based on the same method in fractionation, but the elution time was longer. This was to obtain better resolution of the UV-absorbing compounds in the CG crude extract. The molecular weight of the major peaks formed by UV-absorbing compounds are shown in Table 5.1. The mono-galloyl-D-glucose (1GG) and free gallic acid were the major compounds in the fraction CG1. Digallic acid, methyl gallate, and 2GG were the major compounds found in CG2. Digallic acid was the most abundant in CG3. From CG4 to CG5, the major compounds were 3GG and 4GG. The fraction CG5 and CG6 had various gallotannins from 5GG (tannic acid), 6GG, 7GG and 8GG. The last fraction

collected, the CG7, contains compounds with molecular weight higher than 1500, which was beyond the detection range of the MS analysis. However, gallotannins with higher molecular weight compose only a small portion of the whole extract.

According to the MS identification, the major compounds in the CG extract were ranged from 0GG (gallic acid, methyl gallate, digallic acid) to 10 GG, which confirms previous research from Tian et al. (2009b). However, the composition of CG extract is dependent to extraction solvent. Compared with the report from Huang et al. (2012), the aqueous extract contained majorly free gallic acid and 1GG-3GG, while gallotannins with higher galloylation appear in organic solvents such as methanol, ethanol, and ethyl acetate (Tian et al., 2009b). The difference in CG extract composition can be explained by the polarity of the gallotannins – the higher galloylation, the smaller polarity.

Antimicrobial tests results

The results of the agar diffusion assay of CG fractions are shown in Table 5.2. Without fractionation, the crude extract of CG significantly inhibited all three pathogens at 10 mg/ml. The Fraction # 4-6 significantly inhibited *V. parahaemolyticus* and *M. morganii*. However, none of the fractions showed significant inhibition on *L. monocytogenes*.

The results were similar to the study in Chapter 3; however, a part of the results deserve further discussion. First, the fractions of the CG crude extract did not form inhibitory zones around filter paper disks on *L. monocytogenes* cultures. This indicated the loss of the antimicrobial activity during the fractionation. The compromise may be from the degradation of compounds in CG during the processing; however, it is more

likely from the concentration difference – the fractions of CG, once dried from freeze drying, cannot totally re-dissolved in 70% methanol. In the agar diffusion assay, the treatments were mostly suspensions, instead of solutions, in 70% methanol. Therefore, the actual concentrations of some fractions of CG were lower than 10 mg/ml. Second, the CG crude extract as well as some fractions can form inhibitory zones against *M. morganii*. Similar results were obtained in previous research described in Chapter 3, that the CG treatment can form clear inhibitory zones around paper disks but cannot inhibit the growth of *M. morganii* in TSB culture. This indicates that *M. morganii* may have been suppressed by the CG treatment but recovered afterwards. Third, from the results on *V. parahaemolyticus* and *M. morganii* cultures, the most active antimicrobial fraction was CG4, composed mostly by 3GG. Previous research reported the gallotannins with higher galloylation have the stronger antimicrobial activity (Tian et al., 2009b), but in this research, 3GG shows the strongest antimicrobial effect against *V. parahaemolyticus* and *M. morganii*.

The results of microdilution assay of the unfractionated CG extract and fractions of CG are shown in Figure 5.3. The unfractionated crude extract of CG significantly inhibited *V. parahaemolyticus* and *L. monocytogenes*. All 6 fractions of CG (CG2 and CG3 were tested combined) inhibited *V. parahaemolyticus*, and fractions CG2/3, CG4, CG5 and CG6 significantly reduced the population of *L. monocytogenes* comparing to the control. Neither the unfractionated CG extract nor any fraction reduced the population of *M. morganii*. The results were consistent with the agar diffusion except for *M. morganii*. The most possible explanation may be that the *M. morganii* was firstly

suppressed by the CG extracts or fractions but then successfully overcame the unfavorable conditions.

The relative abundance of each tannin component may impact the total antimicrobial activity of an individual fraction of the CG extract. In preliminary tests, each whole fraction of CG was re-dissolved in the same volume of 70% methanol. Generally, the results showed that the fractions with larger amount of tannin contents (shown as larger area in HPLC chromatogram in Figure 5.1.). To exclude this impact by relative abundance, all 7 fractions were collected, weighed, and standardized into 10 mg/ml solutions.

The fractions of CG extract caused precipitation of the protein in the culture media. The protein-combining ability has also been reported by others (Zhao et al., 1997; He and Yao, 2006; Engels et al., 2011). It can be implied that the gallotannins can deprive the protein components in the culture media which may starve the bacteria inoculated; also, the interaction between gallotannins and the protein of the bacteria may contribute to the antimicrobial activity.

Gallotannins as a typical group of hydrolysable tannins have been isolated and studied from other plant sources. Engels et al. (2009) isolated from mango kernels. They tested the antimicrobial activity of purified penta-O-galloylglucose (5GG), hexa-O-galloylglucose (6GG) and hepta-O-galloylglucose (7GG), against selected stains of *Bacillus spp.*, *E. coli*, *Staphylococcus spp*. and *Listeria monocytogenes*. Their results showed that gallotannins inhibited *L. monocytogenes*, but the inhibition was from a higher concentration (Engels et al., 2009). The difference might be from different settings in the antimicrobial tests, including media, incubation and enumeration

methods. It is indicated that certain composition in media may increase the resistance of the bacteria against gallotannins (Engels et al., 2011a), however, more detailed mechanisms are yet available.

Summary

In summary, the major components of the CG crude aqueous methanol extract are gallotannins. Some fractions of CG containing 3GG – 8GG showed significant inhibitions on *V. parahaemolyticus*, *M. morganii* and *L. monocytogenes*.

References

Aguilar-Galvez, A., Noratto, G., Chambi, F., Debaste, F., & Campos, D. (2014). Potential of tara (Caesalpinia spinosa) gallotannins and hydrolysates as natural antibacterial compounds. *Food Chemistry, 156*(0), 301-304.

Akiyama, H., Fujii, K., Yamasaki, O., Oono, T., & Iwatsuki, K. (2001). Antibacterial action of several tannins against Staphylococcus aureus. *Journal of Antimicrobial Chemotherapy*, *48*(4), 487-491.

Buzzini, P., Arapitsas, P., Goretti, M., Branda, E., Turchetti, B., Pinelli, P., Romani, A. (2008). Antimicrobial and antiviral activity of hydrolysable tannins. *Mini reviews* in Medicinal Chemistry, 8(12), 1179-1187.

Djakpo, O., & Yao, W. (2010). *Rhus chinensis* and *Galla chinensis*--folklore to modern evidence: review. *Phytother Res*, *24*(12), 1739-1747.

Engels, C., Knodler, M., Zhao, Y.-Y., Carle, R., Gänzle, M. G., & Schieber, A. (2009). Antimicrobial activity of gallotannins isolated from mango (Mangifera indica L.) kernels. *Journal of Agricultural and Food Chemistry, 57*(17), 7712-7718

Engels, C., Ganzle, M. G., & Schieber, A. (2009). Fractionation of gallotannins from mango (Mangifera indica L.) kernels by high-speed counter-current chromatography and determination of their antibacterial activity. *Journal of Agricultural and Food Chemistry, 58*(2), 775-780

Engels, C., Schieber, A., & Ganzle, M. G. (2011). Inhibitory spectra and modes of antimicrobial action of gallotannins from mango kernels (Mangifera indica L.). *Appl Environ Microbiol, 77*(7), 2215-2223. doi: 10.1128/AEM.02521-10

Engels, C., Gänzle, M. G., & Schieber, A. (2012). Fast LC–MS analysis of gallotannins from mango (*Mangifera indica* L.) kernels and effects of methanolysis on their

antibacterial activity and iron binding capacity. *Food Research International*, 45(1), 422-426.

Huang, X. L., Liu, M. D., Li, J. Y., Zhou, X. D., & ten Cate, J. M. (2012). Chemical composition of *Galla chinensis* extract and the effect of its main component(s) on the prevention of enamel demineralization in vitro. *International Journal of Oral Science, 4*(3), 146-151.

He, Q., Shi, B., & Yao, K. (2006). Interactions of gallotannins with proteins, amino acids, phospholipids and sugars. *Food Chemistry,* 95(2), 250-254.

Maqsood, S., Benjakul, S., & Shahidi, F. (2013). Emerging role of phenolic compounds as natural food additives in fish and fish products. *Critical Reviews in Food Science and Nutrition*, *53*(2), 162-179.

Nishizawa, M., Yamagishi, T., Nonaka, G. I., & Nishioka, I. (1982). Tannins and related compounds. Part 5. Isolation and characterization of polygalloylglucoses from Chinese gallotannin. *Journal of the Chemical Society, Perkin Transactions 1*, 2963-2968.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry*, *30*(12), 3875-3883.

Taguri, T., Tanaka, T., & Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biological and Pharmaceutical Bulletin*, *27*(12), 1965-1969.

Tian, F., Li, B., Ji, B., Yang, J., Zhang, G., Chen, Y., & Luo, Y. (2009a). Antioxidant and antimicrobial activities of consecutive extracts from *Galla chinensis*: The polarity affects the bioactivities. *Food Chemistry*, *113*(1), 173-179.

Tian, F., Li, B., Ji, B., Zhang, G., & Luo, Y. (2009b). Identification and structure–activity relationship of gallotannins separated from *Galla chinensis*. *LWT-Food Science and Technology*, *42*(7), 1289-1295.

Williams, R. (2013) *Food Microbiology Laboratory Manual*, Virginia Tech

Zar, J. H. (2010). Biostatistical Analysis: Prentice Hall.

Zhao, G., Chung, K.-T., Milow, K., Wang, W., & Stevens, S. E. (1997). Antibacterial properties of tannic acid and related compounds against the fish pathogen *Cytophaga columnaris*. *Journal of Aquatic Animal Health*, *9*(4), 309-313

Table 5.1. Major compounds in CG crude extract in 70% methanol, according to LC-MS analysis.

Observed Peaks	Fraction	Retention time	Observed (m-1)/z	Theoretical m/z	Possible Compound
1	**CG1**	7.88	331, 663	332	1GG
2	**CG1**	8.45	169, 339	170	Gallic acid
3	**CG2**	18.37.	183, 321	184, 322,	Methyl gallate, digallic acid
4	**CG2**	19.27	483	484	2GG
5	**CG3**	20.17	321	322	Digallic acid
6	**CG4**	24.44	635	636	3GG
7	**CG5**	28.98	393, 635, 787	636, 788	3GG, 4GG
8	**CG5**	30.54	393, 787	788	4GG
9	**CG6**	33.27	469, 939	940	5GG
10	**CG6**	34.17	469, 545, 939, 1091	940, 1092	5GG, 6GG
11	**CG6**	34.61	469, 545, 621, 939, 1243,	940, 1092, 1244	5GG, 6GG, 7GG
12	**CG6**	35.05	487, 545, 621, 939, 1092, 1243, 1395	940, 1092, 1244, 1396	5GG, 6GG, 7GG, 8GG
13	**CG7**		>1500	1548, 1700, 1852	N/A

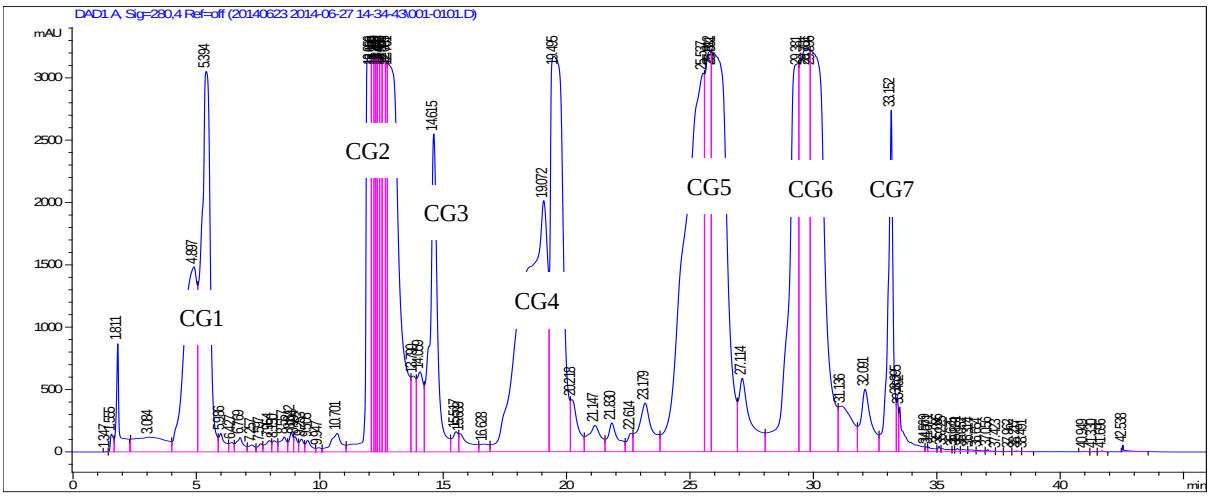
CG1-CG7 refer to the 7 HPLC fractions of CG crude extract; Observed peaks 1-13 are labeled on Figure 5.2. nGG: n-galloyl-D-glucose; The detection range of molecular weight was 150-1500. m/z and (m – 1)/z are the mass-to-charge ratio.

Table 5.2. Diameters of inhibitory zones (mm) formed by CG crude extract and fractions against *V. parahaemolyticus*, *M. morganii* and *L. monocytogenes* cultures on agar, after 24 hours incubation at 37±1°C

	Treatment								
Bacterium	MeOH, 70%	CGM 10 mg/ml	CG1 10 mg/ml	CG2+CG3 10 mg/ml	CG4 10 mg/ml	CG5 10 mg/ml	CG6 10 mg/ml	CG7 10 mg/ml	NaClO 3 mg/ml
VP	7.9±0.4	18.4±0.4	8.6±0.4	8.8±0.2	17.3±0.4	15.1±0.3	12.7±0.5	8.3±0.3	9.6±0.2
MM	7.2±0.0	12.7±1.0	7.2±0.1	7.2±0.0	15.8±0.6	13.4±0.5	11.9±0.5	7.2±0.0	9.1±0.6
LM	7.1±0.0	8.7±0.3	7.1±0.0	7.1±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	8.5±0.2

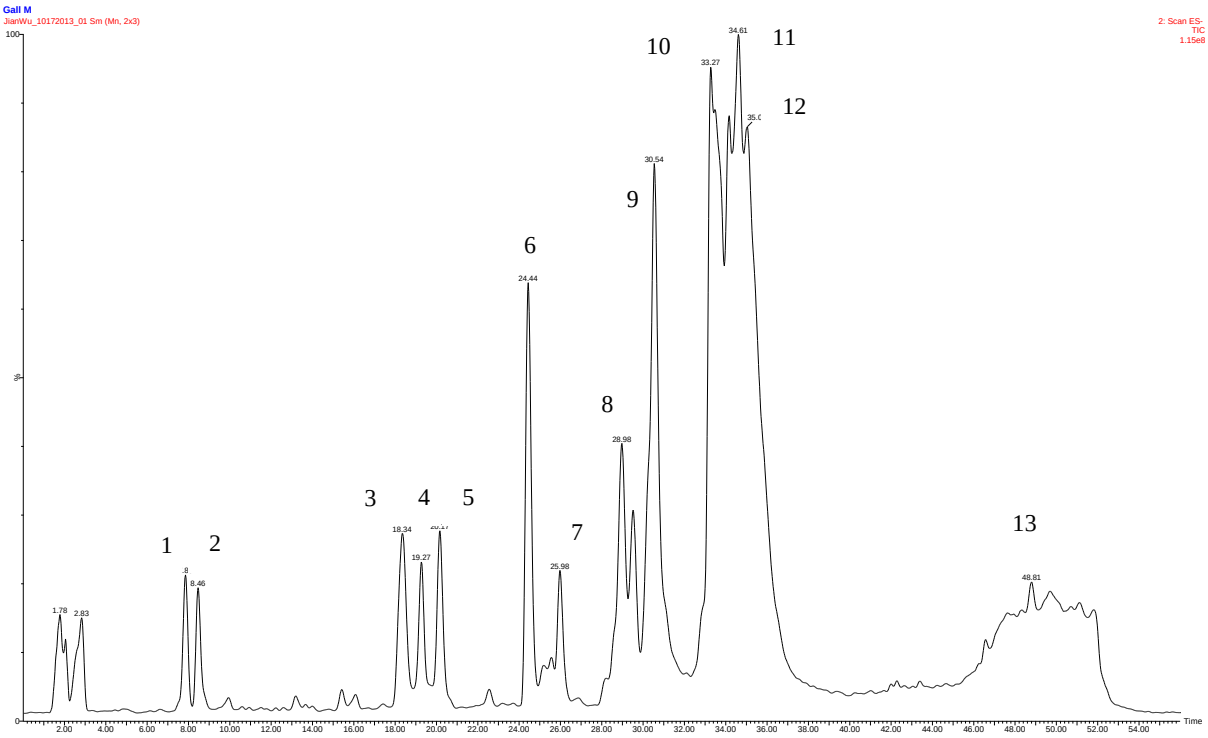
The values (mm) of diameters of inhibitory zones were based on triplicated samples, and each sample with 2 perpendicular measurements. Crude extract of Chinese gall purchased from Mayway Inc. (CGM) and its fractions (CG1-CG7) were dissolved in 70% methanol with concentration at 10 mg/ml. VP: *Vibrio parahaemolyticus*; MM: *Morganella morganii*; LM: *Listeria monocytogenes*. The underlined values are significantly different ($P<0.05$) from control (i.e. MeOH treatment) in each row, according to two-way analysis of variance with Tukey's multiple comparisons.

Figure 5.1. Chromatogram of the HPLC fractionation of CG extract in 70% methanol



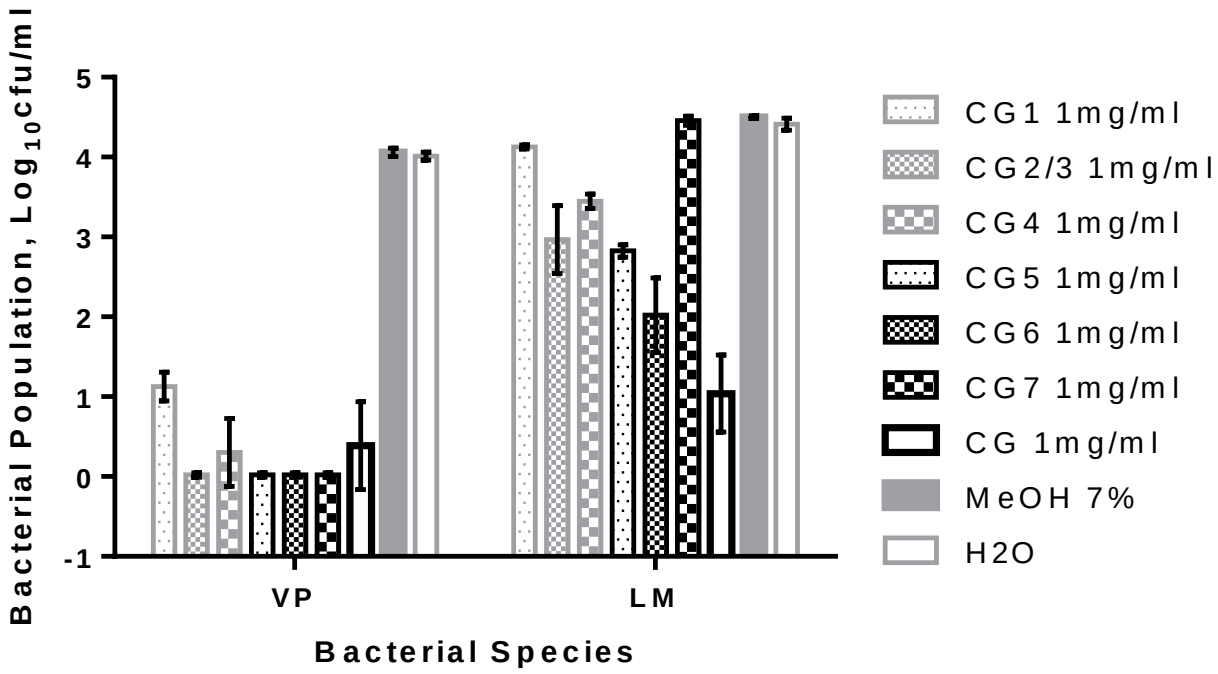
CG1 – CG 7 represent the 7 collected fractions. The collection of fractions was based on retention time as: CG1: 4.0-6.5 min; CG2: 11.0-14.0 min; CG3: 14.0-15.5 min; CG4: 17.0-20.5 min; CG5: 23.5-27.5 min; CG6: 28.0-31.5 min; CG7: 32.0-34.0 min

Figure 5.2. LC-MS analysis of CG extract in 70% methanol



The peaks 1-12 were identified by MS. The (m-1)/z values are 1: 331 (1GG); 2: 169 (gallic acid); 3: 183 (methyl gallate); 4: 483 (2GG); 5: 321 (digallic acid); 6: 635 (3GG); 7: 787 (4GG); 8: 787 (4GG); 9: 939 (5GG); 10: 939 (5GG) and 1091 (6GG); 11: 939 (5GG), 1091 (6GG) and 1243 (7GG); 12: 939 (5GG), 1091 (6GG),1243 (7GG) and 1395 (8GG)

Figure 5.3. Population (cfu/sample) of *V. parahaemolyticus* and *L. monocytogenes* in tryptic soy broth (TSB) with treatments of CG crude extract, CG HPLC fractions, 70% methanol, and sterile water



Column showed the bacterial growths in samples. The error bars stand for standard error of the mean (SEM). CG: The crude Chinese gall extract; CG1-CG7: the 7 HPLC fractions of CG.

CHAPTER 6:

CONCLUSIONS AND FUTURE DIRECTIONS

The purposes of this research were to better understand the antimicrobial properties of several commonly used medicinal plants and their potential application in reduction of seafood-borne illnesses caused by pathogenic bacteria including *Vibrio parahaemolyticus* and *Listeria monocytogenes*, and histamine-forming bacterium *Morganella morganii*. Materials from four medicinal plants, including Chinese gallnut (CG, *Rhus chinensis* Mill.), pomegranate peel (PP, *Punica Granatum* L.), forsythia fruit (FF, *Forsythia suspensa* (Thunb.) Vahl) and baikal skullcap root (BS, *Scutellaria baicalensis* Georgi.), were studied in this research. The extracts were tested for their inhibitory activities against *V. parahaemolyticus*, *L. monocytogenes*, and *M. morganii* on tryptic soy agar plate, in tryptic soy broth, and in tuna and shrimp respectively.

The results of the *in vitro* study demonstrated that the CG and PP successfully inhibited the growth of *V. parahaemolyticus* and *L. monocytogenes* in culture media. When comparing the two bacteria species, *V. parahaemolyticus*, as a Gram-negative microorganism, was more susceptible to plant extracts than *L. monocytogenes*, which is Gram-positive. The result was uncommon comparing to previous research, thus the mechanism of inhibition deserves further study and discussion.

The CG crude extract was analyzed and fractioned by HPLC and LC-MS. From the LC-MS analysis, the CG contains a series of hydrolysable tannins that contribute to the antimicrobial activity. The antimicrobial assay of HPLC fractions of CG crude extract

showed that gallotannins with a moderate galloylation (3GG – 8GG) is the strongest antimicrobial compound against tested bacterial species. The results of the study on shrimp and tuna matrices revealed that the CG and PP still inhibited *V. parahaemolyticus*, while the bacterial growth of *L. monocytogenes* was suppressed or delayed. All these results together indicated that the CG and PP may contribute to control possible vibriosis and listeriosis caused by consumption of seafood products. The histamine-forming *M. morganii*, on the other hand, was not totally inhibited by any of the selected plants. When comparing the results of antimicrobial assays on TSA and in TSB, the growth of *M. morganii* was delayed firstly, but later the growth resumed. However, it is still possible that the CG and PP can delay the formation of histamine in fish thus extending the shelf life of the fish products.

The current research can be supplemented and extended in several ways. First, more research can be conducted on the inhibitory effect of plant-source material on *M. morganii,* especially on the inhibition of the histidine decarboxylation. For example, the histamine concentration in plant extract treated tuna can be monitored and compared with controls. Second, research can be conducted on synergies of several preservation strategies, including natural antimicrobial additives, antimicrobial film made of natural materials, temperature control, heat treatment, modified atmosphere packaging, high static pressure processing and so on. Third, the antimicrobial plant-source materials can be tested on more food matrices including fresh produce, fruit, juice, and other ready-to-eat food products. Fourth, plant-source materials may contain flavor compounds that can interfere with the food product. Therefore, the related sensory study is also important. In conclusion, extracts from medicinal plants CG and PP can inhibited

pathogenic bacteria *V. parahaemolyticus* and *L. monocytogenes*, and reduce the risk of foodborne illnesses caused by these pathogens.

APPENDIX A: Experimental design of antimicrobial test of plant extracts against *V. parahaemolyticus*, *L. monocytogenes*, or *M. morganii* on 96-well plates (extract concentrations expressed as mg/ml)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CGM 5	CGM 1	CGM 0.2	CGM 0.04	CGM 0.008	Water	Water	CGH 5	CGH 1	CGH 0.2	CGH 0.04	CGH 0.008
B	CGM 5	CGM 1	CGM 0.2	CGM 0.04	CGM 0.008	Water	Water	CGH 5	CGH 1	CGH 0.2	CGH 0.04	CGH 0.008
C	PPM 5	PPM 1	PPM 0.2	PPM 0.04	PPM 0.008	MeOH	MeOH	PPH 5	PPH 1	PPH 0.2	PPH 0.04	PPH 0.008
D	PPM 5	PPM 1	PPM 0.2	PPM 0.04	PPM 0.008	MeOH	MeOH	PPH 5	PPH 1	PPH 0.2	PPH 0.04	PPH 0.008
E	FFM 5	FFM 1	FFM 0.2	FFM 0.04	FFM 0.008	No Trtmt	No Trtmt	FFH 5	FFH 1	FFH 0.2	FFH 0.04	FFH 0.008
F	FFM 5	FFM 1	FFM 0.2	FFM 0.04	FFM 0.008	No Trtmt	No Trtmt	FFH 5	FFH 1	FFH 0.2	FFH 0.04	FFH 0.008
G	BSM 5	BSM 1	BSM 0.2	BSM 0.04	BSM 0.008	Bleach	Bleach	BSH 5	BSH 1	BSH 0.2	BSH 0.04	BSH 0.008
H	BSM 5	BSM 1	BSM 0.2	BSM 0.04	BSM 0.008	Bleach	Bleach	BSH 5	BSH 1	BSH 0.2	BSH 0.04	BSH 0.008

The design of experiment in a 96-well plate are illustrated in the table above. The content in a single well includes 125 μl TSB media, 10 μl bacteria culture (*V. parahaemolyticus, L. monocytogenes or M. morganii*), and 15 μl plant extracts, or 15 μl sterile water, or 15 μl 70% methanol, or 15 μl diluted commercial bleach with approximately 3 mg/ml sodium hypochlorite (NaClO). The wells labeled as “No Trtmt” were not included.

APPENDIX B: Autoplate® 4000 plate count protocol and enumeration constants (from Autoplate® 4000 User's Manual)

1. Begin by counting the outer segment (number 1 for 150mm plates, number 8 for 100 mm plates) in either quadrant A or B. Each segment contains two spirals, except for number 13, which has 3 spirals. Count inward until at least 20 colonies are counted. It may be necessary to count inward to multiple segments to count at least 20 colonies. Complete the counting of the final segment. Note: do not count colonies that appear outside of the grid marking.

2. Count the same segment(s) on the opposite side of the plate (in the same letter quadrant as the first count; i.e., either A or B).

3. Add up the number of counted colonies from both sides (should equal at least 40 unless counting entire plate).

4. If segments 8-13 on one side contain less than 20 colonies, count the entire plate.

5. Divide by the volume constant found for the last segment counted or for the entire plate. Note: table values are for both sides.

6. Multiply by 1000 for cfu/ml.

7. Multiply by any dilution factor.

Table of constants for segment pairs (100 mm plate, 50 μl disposal setting, as used in the study):

Segment	Constant
8	1.214
9	2.968
10	5.500
11	9.157
12	14.482
13	25.015
plate	50.030