Inhibition of *Listeria monocytogenes* growth and biofilm production by nisin and phenolic compounds.

by

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ABSTRACT

The synergistic antimicrobial and anti-biofilm activities of nisin and phenolic compounds against *Listeria monocytogenes* were investigated. Among the twenty six phenolic compounds tested in this study, butyl gallate, octyl gallate, lauryl gallate, propyl gallate, naringenin and quercetin exhibited the most potent antimicrobial activity in *L. monocytogenes* with minimal inhibitory concentration (MIC) lower than 512 µg/ml. Butyl gallate was the only phenolic compound that exhibited synergistic inhibitory activity against all tested strains of *L. monocytogenes* in combination with nisin. In addition, nisin showed anti-biofilm activity against *L. monocytogenes* ATCC 19115 at concentrations as low as 10 IU/ml. Naringenin, propyl gallate, octyl gallate and lauryl gallate at concentrations as low as 5 µg/ml increased the anti-biofilm activity of nisin at10 IU/ml. The enhanced anti-biofilm activity of nisin in combination with naringenin, propyl gallate, octyl gallate and lauryl gallate were also observed in different strains of *L. monocytogenes*. Fluorescent microscopic imaging demonstrated that biofilm formation by *L. monocytogenes* ATCC 19115 was inhibited in the presence of nisin alone, and with nisin and naringenin. These results suggest that phenolic compounds can be used as effective adjuvant to increase the ant-listerial and anti-biofilm activity of nisin.

PREFACE

This thesis is an original work by Kuan – Lin Li. No part of this thesis has been previously published.

DEDICATION

To my family, Wen Hsien Lee, Yueh Hsia Hsieh, Ya Hsian Li, Serena Kuangyi Chen and Luna.

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CHAPTER 1: INTRODUCTION

Classic foodborne hazards are defined as biological, chemical, physical or allergenic agents in food, which may relate to the conditions of food and have the potential to cause an adverse health effect (Manning, 2017). Biological hazards, such as bacteria, viruses, parasites and prions, may cause diseases in humans through the ingestion of contaminated foods (Apostolidis, Kwon, & Shetty, 2008; Kirk, et al., 2015). Food safety challenges associated with controlling these etiological agents in food are further complexed by the globalization of food supplies, resulting in the movement of pathogenic microorganisms across international borders (Arauz, Jozala, Mazzola, & Penna, 2009; Kirk, et al., 2015). Foodborne disease outbreaks related to domestic and imported foods can adversely influence international food trade and food security (Balciunas, et al., 2013; Bhatia & Bharti, 2015; Kirk, et al., 2015). Thus, government and industry leaders involved in food trade are responsible for the public health of the consumers when importing foods and the credibility of their food producing systems when exporting foods. As a result, governments and the food industry have invested significant resources to control and improve food safety in recognition of the economic and public health impacts that contaminated foods may have on the global community (Black, Kelly, & Fitzgerald, 2005; Kirk, et al., 2015).

Among a few different kinds of foodborne hazards, microbiological hazards are the second leading cause of food recalls in Canada between 2006 and 2013, which is next to undeclared allergens (Canadian Food Inspection Agency, 2015). However, in comparison to the rates of undeclared allergen recalls, which were variable over this time period, the rates of recalls related to microbiological agents increased (Canadian Food Inspection Agency, 2015). Hence, it

can be speculated that the reduction of microbiological foodborne hazards may alleviate public health and economic burdens of food safety in Canada.

1.1 Etiological Agents of Foodborne Illness

According to a 2015 World Health Organization (WHO) report (Kirk, et al., 2015), the mean estimate of global foodborne infection cases caused by 22 commonly reported foodborne bacteria, protozoans and viruses between 1990 and 2012 was 582 million per year. In a separate study conducted by the WHO, the estimate of global burden of foodborne disease caused by 31 hazards associated with various microbial and chemical hazards was 600 million illnesses (Arie , et al., 2015). The microbiological causes of global foodborne diseases are discussed below.

1.1.1 Viruses

The most common foodborne viruses are norovirus and hepatitis A (Marion & Erwin, 2004). Although uncommon, viruses, such as rotavirus, astrovirus and sapovirus, have been reported to be the cause of several foodborne illnesses in North America (Thomas, et al., 2013). Norovirus constituted the largest proportion of the foodborne illness (124 million out of 582 million illnesses) according to a WHO estimate (Belliot, Lopman, Ambert-Balay, & Pothier, 2014; Kirk, et al., 2015). Based on the annual estimate of domestically acquired foodborne illnesses from a known pathogen in the US, norovirus is the leading cause of foodborne illnesses (58%) in the country (i.e., 5.5 million out of 9.4 million illnesses) (Scallen, et al., 2011). Estimates of domestically acquired foodborne illnesses per year in Canada have also shown that norovirus accounts for 65.1% of foodborne illnesses with known etiological agents (Thomas, et al., 2013; Government of Canada, 2016a).

Although the estimate of illnesses from foodborne-related norovirus infections is as high as one million cases per year in Canada, actual surveillance data suggests norovirus infections constitute only 11.2% of the total number of laboratory confirmed foodborne enteric illnesses (352 out of 3,138 illnesses) according to the Outbreak Summary Reporting System (OS) between 2008 and 2014 (Belanger , Tanguay, Hamel, & Phypers, 2015). In comparison, this value was significantly lower than the laboratory confirmed leading cause of *Salmonella*, which was about 65% (2,041 out of 3,138 illnesses) (Belanger , Tanguay, Hamel, & Phypers, 2015). Similar observations have been noted by the National Enteric Surveillance Program that includes both sporadic and travel-acquired illnesses (Government of Canada, 2016). The discrepancy between the reported and the estimated numbers of illnesses caused by foodborne norovirus in Canada may be due to under-reporting, under-diagnosis and the lack of laboratory confirmation. It may also be ascribed to the fact that clinical symptoms of norovirus infection are acute onset of diarrhea and vomiting that persist only for 2 to 3 days in healthy adults. Therefore, it is very unlikely that the patients seek medical consultations and request their stool samples for diagnostic confirmation (Belliot, Lopman, Ambert-Balay, & Pothier, 2014).

The global estimate of foodborne illnesses associated with hepatitis A was 14 million out of 582 million illnesses (0.02% of the total estimated illnesses) (Kirk, et al., 2015). The estimate for domestically acquired illnesses caused by foodborne hepatitis A is annually 1,566 out of 9.4 million illnesses (0.0002%) in the US and 271 out of 1.6 million illnesses in Canada (0.0002%) (Thomas, et al., 2013). Compared to the total estimated global health burdens, hepatitis A appears to be responsible for a significantly less serious health burden in North America.

Similar to hepatitis A in North America, domestically acquired foodborne illnesses caused by the uncommon foodborne viruses mentioned above also contribute minimally to the

total estimated number of cases per year. Astrovirus, rotavirus and sapovirus were estimated to account for 15,000 out of 9.4 million illnesses per year in the US (Scallen, et al., 2011). These same viruses are annually responsible for approximately 15,000 out of 1.6 million estimated foodborne illnesses each year in Canada (Thomas, et al., 2013).

1.1.2 Parasites

Parasites of importance to public health and food safety include protozoa (unicellular eukaryotes) and helminths (multicellular eukaryotes) (Ortega, 2013). Protozoa infections constitute a significant number of foodborne illnesses globally. Protozoan parasites, such as *Giardia* spp., *Entamoeba histolytica*, *Toxoplasma gondii*, and *Cryptosporidium* spp., are the most frequent parasites involved in foodborne illnesses according to a report from the WHO (Arie , et al., 2015). Helminths causing major public health problems include cestodes (tapeworms), trematodes (flukes), and nematodes (roundworms) (Ortega, 2013; Newell, et al., 2010). Whereas cestodes are estimated to cause 430,864 infection cases worldwide per year, *Taenia solium* and *Echinococcus multilocularis* are responsible for 370,710 and 8,375 infections, respectively, and also exhibit high foodborne mortality (7.6% and 93%, respectively) among the parasites investigated by the WHO (Arie , et al., 2015). Globally, lower income regions, such as Latin America and Central Africa, are often associated with poor sanitation and have higher incidence rates of foodborne illnesses caused by parasites than developed countries (Centers for Disease Control and Prevention, 2015).

Within the US, *T. gondii*, *Giardia intestinalis*, *Cryptosporidium* spp., and *Cyclospora cayetanensis* are the top four most common parasites implicated in domestically acquired foodborne illnesses, annually accounting for approximately 86,686, 76,840, 57,616, and 11,407

infection cases per year, respectively (Scallen, et al., 2011). In Canada, *T. gondii* is the most frequently detected parasite causing 9,132 domestically acquired foodborne illnesses per year and is and is followed by *Giardia* spp. (7,776 illnesses), *C. cayetanensis* (2,450 illnesses) and *Cryptosporidium* spp. (2,321 illnesses) (Belanger , Tanguay, Hamel, & Phypers, 2015). Given the total estimated number of domestically acquired foodborne illness in North America (9.4 million illnesses in the US and 1.6 million illnesses in Canada per year), the proportion of foodborne illnesses caused by parasites is relatively small.

1.1.3 Bacteria

Campylobacter spp., enterotoxigenic *Escherichia coli*, nontyphoidal *Salmonella enterica* serovars, and *Shigella* spp. are the top four pathogenic bacterial species that account for approximately 582 million infections worldwide per year (Kirk, et al., 2015). Among the bacterial causes of domestically acquired foodborne illnesses in the US in 2011, nontyphoidal *Salmonella*. *Clostridium perfringens*, *Campylobacter* spp. and *Staphylococcus aureus* were the primary pathogenic species responsible for 1 million illnesses (11%), 965,958 illnesses (10%), 845,024 illnesses (9%), and 241,148 illnesses (3%) per year (Scallen, et al., 2011), respectively. In Canada, *C. perfringens* is the leading bacterial agent causing 176,963 foodborne illnesses (11%), followed by *Campylobacter* spp. at 145,350 illnesses (8%), nontyphoidal *Salmonella* spp. at 87,510 illnesses (5%), and *S. aureus* at 25, 110 illnesses (2%) (Thomas, et al., 2013). Although *Salmonella* and *Campylobacter* spp. are suspected to be the major bacterial burdens of foodborne illnesses globally, it is worth mentioning that *C. perfringens* is an important issue in North America.

Based on the mortality rates of 22 commonly reported foodborne pathogens worldwide (Kirk, et al., 2015), *Listeria monocytogenes* exhibited the highest percentage case mortality rate at 22.4% (3,175 deaths out of 14,169 illnesses), followed by invasive nontyphoidal *S. enterica* (Typhi and Paratyphi serovariants) at 10% (29,391 deaths out of 284,972 illnesses) and *Clostridium botulinum* at 5% (24 deaths out of 475 illnesses from low mortality regions Europe, North America, and West Pacific Region) (Kirk, et al., 2015). In the US, the estimated leading annual causes of deaths due to domestically acquired foodborne illnesses from known pathogens in the US (1,351 total deaths) include nontyphoidal *Salmonella* spp. (378 deaths), *Listeria monocytogenes* (255 deaths) and *Campylobacter* spp. (76 deaths). The estimated mortality rate of domestically acquired foodborne illnesses caused by *L. monocytogenes* is 16%, which is significantly higher than the mortality rate (<1%) of nontyphoidal *Salmonella* (Scallen, et al., 2011). In Canada, it has been estimated that 105 deaths are attributed to microbial foodborne illness, and *L. monocytogenes* had caused the most deaths (35 deaths), followed by nontyphoidal *Salmonella* (17 deaths) and verotoxigenic *E. coli* O157 (8 deaths) between 2000 and 2010 (Thomas, et al., 2013). Similar to the US, *L. monocytogenes* exhibits higher mortality rates (20%; 35 deaths out of 178 illnesses per year) than nontyphoidal *Salmonella* (<1%, 17 deaths out of 87,510 illnesses) in Canada (Thomas, et al., 2013; Thomas, et al., 2015a). According to the Outbreak Summary Reporting System *L. monocytogenes* represented only 2.1% (67 out of 3,138) of total laboratory confirmed infection cases per year, but 22 out of the 67 cases resulted in death (32.8% mortality rate) according to the OS record between 2008 and 2014 (Belanger , Tanguay, Hamel, & Phypers, 2015). In comparison to the mortality rates of laboratory confirmed foodborne illnesses of *Salmonella* and pathogenic *Escherichia*, which are 0.2% and 1.5%,

respectively, *L. monocytogenes* has significantly higher laboratory confirmed mortality rates in Canada.

Although the number of infection cases is lower than other major foodborne pathogens, such as *Salmonella* and *Campylobacter*, the patient outcomes of human infections with *L. monocytogenes* are serious and may lead to death. The Center for Disease Control and Prevention (CDC) in the US estimates that 99% of laboratory confirmed *L. monocytogenes* cases are associated with foodborne outbreaks (Scallen, et al., 2011). Thus, *L. monocytogenes* is considered a serious threat to food safety and public health due to its high mortality rate and its potential to contaminate domestic food products.

1.2 Threat of *L. monocytogenes* to Public Health in North America

The public health impact of human listeriosis (*Listeria monocytogenes* infection) in North America is significant in terms of its high mortality rates and severe clinical symptoms. Listeriosis in North America is tightly associated with recent development in food processing, distribution, dietary trends, and human host factors (e.g., aging population, ethnicity and health status) (Schlech, III & Acheson, 2000). Furthermore, epidemiological studies indicate that several factors can increase the risk of acquiring listeriosis and cause life-threatening consequences in certain populations. Therefore, a better understanding of the clinical impacts, epidemiology and transmission/survival characteristics of *L. monocytogenes* will allow for the development of intervention methods for food safety associated with *L. monocytogenes*. The following sections briefly discuss the clinical symptoms of listeriosis, the epidemiology of listeriosis and transmission/survival mechanisms of *L. monocytogenes* from environmental sources on the farm to fork.

1.2.1 Clinical presentation of listeriosis

Human listeriosis is caused by the consumption of foods contaminated with *L. monocytogenes* (Farber & Peterkin, 1991). Typical infection by this pathogen in healthy adults can cause symptoms, such as fever, diarrhea and vomiting, similar to infections by other foodborne pathogens, such as *Salmonella* and *Campylobacter* (Charlier, et al., 2017). In cases of invasive listeriosis, the bacterium spreads beyond the gut (Charlier, et al., 2017). The clinical presentation of *L. monocytogenes* infection can be categorized into non-pregnancy-related listeriosis and pregnancy-related listeriosis. Symptoms of illnesses caused by *L. monocytogenes* infection are briefly described below.

1.2.1.1 Non-pregnancy related listeriosis

Non-pregnancy related listeriosis refers to cases involving older neonates (>28 days of life) and beyond (Allerberger & Wagner, 2010). Adult patients suffering from invasive listeriosis usually report symptoms of illness 1 to 4 weeks after ingesting *L. monocytogenes*-contaminated food. However, some may report symptoms 70 days later or on the same day of consuming the contaminated food (Charlier, et al., 2017).

Vulnerable groups, such as older adults and immunocompromised individuals, infected by *L. monocytogenes* commonly show symptoms of invasive listeriosis (Doganay, 2003). The most common clinical manifestations of invasive listeriosis include, but are not limited to, bacteremia, sepsis and meningitis (Doganay, 2003). It is also possible for vulnerable patients infected by *L. monocytogenes* to develop localized infections, such as septic arthritis (infection

of the joints), osteomyelitis (infection of the bones), prosthetic graft infections, inner chest and abdominal infections or infections of the skin and eye (Allerberger & Wagner, 2010; Doganay, 2003). Although rare, healthy young individuals infected with *L. monocytogenes* can develop invasive listeriosis (Doganay, 2003). However, healthy adults will most likely experience self-limited acute gastroenteritis and fever after they are exposed to high dose of *Listeria* (Doganay, 2003).

1.2.1.2 Pregnancy related listeriosis

Pregnancy related listeriosis (perinatal listeriosis) refers to listeriosis involving pregnant women or neonates (< 28 days of life) (Allerberger & Wagner, 2010). Pregnant women with *L. monocytogenes* infection may have very subtle flu symptoms (Mayo Clinic, 2017). However, listeriosis during pregnancy may result in miscarriage, stillbirth, preterm labor, and sepsis or meningitis in the newborn (Allerberger & Wagner, 2010). A study based in England and Wales reported that the chance of live birth significantly increases if the mother shows listeriosis symptoms during the third trimester of pregnancy when compared to the second and first trimester (Awofisayo-Okuyelu, Amar, Ruggles, & Grant, 2015). Neonatal listeriosis can be classified to: i) early-onset listeriosis (within 6 days of birth) that is usually acquired through trans-placental transmission (transmission through placenta); ii) late-onset listeriosis (7-28 days after birth) due to exposure to *L. monocytogenes* during delivery; or iii) nosocomial listeriosis acquired from hospitals (Allerberger & Wagner, 2010). Neonatal listeriosis can result in physical retardation, and very severe cases can result in infantiseptica granulomatosis or bacteremia leading to death (Awofisayo-Okuyelu, Amar, Ruggles, & Grant, 2015; Allerberger & Wagner, 2010). Cases that presented late-onset neonatal listeriosis were 14 times more likely to

have symptoms associated with the central nervous system (i.e. meningitis) than bacteremia according to the England and Wales study (Awofisayo-Okuyelu, Amar, Ruggles, & Grant, 2015).

1.2.2 Epidemiology of *L. monocytogenes* in North America

The first major foodborne listeriosis outbreak was linked to coleslaw consumption in Canada in 1981 (Farber & Peterkin, 1991). The coleslaw outbreak resulted in 41 illnesses (34 perinatal and 7 adult cases) with 9 stillbirths and 28.6% adult mortality rate (Schelch, III, Lavigne, & Bortolussi, 1983). The outbreak that raised *L. monocytogenes* to a higher level of food safety concern in North America occurred in California 1985, and the outbreak was linked caused by Mexican-style soft cheese (142 illnesses with deaths of 30 fetuses/newborns and 18 adults) (Farber & Peterkin, 1991).

According to the epidemiological studies of past listeriosis outbreaks, people at the greatest risk of acquiring listeriosis are pregnant women and their newborns, senior adults aged ≥65, and people with compromised immune systems (Allerberger & Wagner, 2010). Within the US in 2014, 562 reported cases were non-pregnancy related listeriosis cases (median age 70) and 96 cases were reported to be pregnancy related (Centers for Disease Control and Prevention, 2015). Within Canada, there were 670 reported listeriosis cases between 1995 and 2004 (Clark, et al., 2010). Elderly adults (≥60 years) constitute more than 50% of the reported listeriosis cases with very few cases that were pregnancy-related listeriosis (Clark, et al., 2010). However, listeriosis outbreaks in Canada have high percentages of pregnancy related listeriosis cases. In 2008, an outbreak in Quebec caused by contaminated cheese resulted in 36 severe infections that included 13 pregnant women, resulting in five neonatal infections and three cases of perinatal

deaths (two intrauterine fetal demises and one early neonatal death) (Taillefer, Boucher, Laferriere, & Morin, 2010).

Although rare, listeriosis from nosocomial sources can occur (Mazengia, et al., 2017). Recently, in the US, there was a listeriosis outbreak linked to serving contaminated foods to hospital patients (Mazengia, et al., 2017; Gual, et al., 2013; Rietberg, et al., 2016). Hospital acquired listeriosis is extremely dangerous due to high proportion of immunocompromised patients residing on site. One recent hospital listeriosis outbreak in 2010 was linked to contaminated diced celery (Gual, et al., 2013). This outbreak resulted in five deaths out of the ten outbreak-related patients who were immunocompromised by more than one underlying condition or treatment (Gual, et al., 2013). The high mortality rate of the 2010 outbreak suggests that underlying health conditions have a significant influence on the risk of infection and disease outcome. This was also observed in a nationwide epidemiological retrospective study of reported human listeriosis cases in France between 2001 and 2008 (Goulet, et al., 2012). The French study reported that cases at age <65 years suffering from chronic lymphocytic leukemia (CLL) and liver cancer were ≥1000 and 748 fold greater, respectively, and these individuals were more likely to acquire non-pregnancy related listeriosis when compared with cases in the same age group that did not have underlying health issues (Goulet, et al., 2012). Furthermore, patients with either lung or pancreatic cancer that acquired non-pregnancy related listeriosis had the highest case fatality ratio at 40% (Goulet, et al., 2012).

L. monocytogenes serotypes 1/2a, 1/2b and 4b are the most common causes of human listeriosis, and the serotype 4b is most commonly associated with foodborne outbreaks of listeriosis according to the CDC in the US (Centers for Disease Control and Prevention, 2016). Within Canada, between 1995 and 2004, serotypes 1/2a (most frequently isolated), 1/2b and 4b

were the most frequent and common *L. monocytogenes* serotypes isolated from specimens (e.g., blood, stool, cerebrospinal fluid and brain tissues from human listeriosis cases) in invasive listeriosis cases (Clark, et al., 2010). These strains were also frequently isolated from recent outbreaks (2008 – 2015), that involved foods, such as ready-to-eat (RTE) meats and soft cheeses (Mexican style cheese in particular), and less frequently from fruits and vegetables (i.e. cantaloupe, mung bean sprouts or stone fruits) (Cartwright, et al., 2013; Buchanan, Leon, Hayman, Jackson, & Whiting, 2017). Although there is no report that suggests a certain *L. monocytogenes* serotype has an increased prevalence in certain types of food, the majority of *L. monocytogenes* isolates from food products tend to belong in serotype 4b and 1/2a (Khan, et al., 2016). This may explain why serotype 4b and 1/2a are typically isolated from invasive listeriosis cases.

Epidemiological data suggest that older adults are the most vulnerable population group to acquire invasive listeriosis in North America (Centers for Disease Control and Prevention, 2016). This can be due to their weakened immune systems and the likelihood of having one or more underlying health issues. Pregnant women are also vulnerable to invasive listeriosis with devastating patient outcomes. However, the incidence of pregnancy-related listeriosis in North America is lower when compared to invasive listeriosis in elderly adults. Thus, future strategies to reduce public health burdens of human listeriosis in North America should be targeted towards the elderly that may have one or more underlying health issues.

1.2.3 Sources and contamination routes of *L. monocytogenes* in the food system

L. monocytogenes is ubiquitous and can be introduced into the food system through many routes. This bacterium may first infect livestock from environmental sources, and then may get

introduced into processing facilities and persist in retail/food processing environment. Thus, farm, food processing and the retail sector in our food production system are critical controls points for preventing *L. monocytogenes* contamination in foods.

1.2.3.1 Environmental and farm sources

L. monocytogenes is ubiquitous and can be isolated from a wide range of environmental sources. Rich natural sources include soil, manure, farm slurry, sludge, silage, animal feed, water and feces of mammals and birds (Fenlon, 1985). Use of feces from infected animals as a fertilizer can contaminate raw foods, such as vegetables (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014). In fact, the levels of *L. monocytogenes* from sewage sludge sprayed on to agricultural land remain unchanged for at least 8 weeks (Watkins & Sleath, 1981). The bacteria can also be isolated from walls, floors, drains, decaying vegetation, rivers, pasture herbage, factory effluents, farms, and other environments (Fiesleler, Doyscher, Loessner, & Schuppler, 2014).

Livestock, especially ruminants, are a common farm reservoir for *L. monocytogenes* (Dhama, et al., 2015; Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014). According to a 23-year long survey (from year 1972 to 1994) based in Denmark examining the prevalence of *L. monocytogenes* in cow herds, milk samples of the 0.2 to 4.2% herds were positive for *L. monocytogenes* (Jensen, Aarestrup, Jensen, & Wegener, 1996). Furthermore, during the same time of the survey study, 79% of the bovine mastitis isolates and 48% of the human clinical isolates of *L. monocytogenes* had overlapping ribotypes, suggesting an association between human listeriosis and contaminated milk from dairy cows that harbour *L. monocytogenes* (Jensen, Aarestrup, Jensen, & Wegener, 1996).

1.2.3.2 Food processing environment

Contaminated raw foods, animals or humans are vehicles for contamination of surfaces in food processing facilities (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014). *L. monocytogenes* introduced into food processing facilities may persist in hard to clean places and result in cross-contamination of foods (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014). Within seafood production, this bacterium was isolated more frequently from the facility surfaces (i.e. drains and equipment) for fish slaughter and smoking than from raw fish (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014; Vogel, Huss, Ojeniyi, Ahrens, & Gram, 2001). Furthermore, according to a Danish study, 7.3% to 17.4% of finished turkey products tested positive for *L. monocytogenes* after processing; however, all turkeys were tested negative for *L. monocytogenes* prior to slaughter (Ojeniyi, Christensen, & Bisgaard, 2000). The results suggest that the potential risk for *L. monocytogenes* contamination may persist in the food processing environment once introduced. In fact, a US outbreak of listeriosis in 2000 that involved contaminated deli turkey meats in multiple states was associated with a *L. monocytogenes* strain that persisted in the processing facility for more than 10 years (Hurd, et al., 2000; Kathariou, 2002). Persistence of *L. monocytogenes* in the food processing environment is highly linked to bacterial ability to adapt to the environmental conditions of a processing facility (Khan, et al., 2016). *L. monocytogenes* strains can persist in a variety of processing plants, such as for meat, fish, dairy and RTE meats, from several months to several years (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014). Factors involved in strain adaptation in a processing plant include strain modification (alteration of genetic and physiological traits), biofilm formation, and inefficient contamination control procedures (Martin, et al., 2014). These factors will be discussed in detail in later sections.

1.2.3.3 Retail environment

Within the US, *L. monocytogenes* is more frequently isolated from non-food contact surfaces (i.e. floors, drains and sinks) than food contact surfaces within a retail establishment (Hoelzer, et al., 2011). According to a study that was based in New York City, 2011, the percentage of retail establishments' samples that tested positive for *L. monocytogenes* from food/food contact surfaces and non-food contact surfaces were 3.6% and 17.0%, respectively (Hoelzer, et al., 2011). Furthermore, according to expert opinions that obtained from a structured expert elicitation process on *L. monocytogenes* about risks associated with cross-contamination in the retail environment, the majority of food safety and industrial experts believed that *L. monocytogenes* can transmit onto food through direct contact from the slicer blade guard, deli preparation sinks and clothing (Hoelzer, et al., 2012). The experts also believed that hands or gloves are a cross contamination vehicle for transmitting *L. monocytogenes* onto deli case handles, products and food-contact surfaces (Hoelzer, et al., 2012).

Risk assessment of RTE meats in the US, completed by the Food and Drug Administration and the Food Safety and Inspection Service in 2003, determined that deli meats in the retail environment pose the greatest risk for listeriosis (Food Safety and Inspection Service, United States Department of Agriculture, Office of Public Health Science, Risk Assessment Division, 2010). According to the US Food Safety and Inspection Service in a comparative risk assessment report for *L. monocytogenes* in RTE meats and poultry deli meats, 83% of the cases associated with deli meats are attributed to those sliced and packaged at retail facilities (Food Safety and Inspection Service, United States Department of Agriculture, Office of Public Health Science, Risk Assessment Division, 2010). Only 17% was attributed to pre-packaged deli meats

(Food Safety and Inspection Service, United States Department of Agriculture, Office of Public Health Science, Risk Assessment Division, 2010). This suggests that slicing of deli meat in a retail environment is a major source of post processing contamination in RTE meat. Hence, the control of *L. monocytogenes* contamination in deli processing equipment and surfaces is critical to reducing the risk of listeriosis.

1.3 *L. monocytogenes* Physiology and Food Safety

The ability of *L. monocytogenes* to contaminate food products is related to the remarkable capacity of this microorganism to survive in various biotic and abiotic environments. Understanding how *L. monocytogenes* survives in these environments is critically important for the food industry in order to improve current food safety strategies against this ubiquitously present organism. Some of the physiological properties of *L. monocytogenes* that are important in the food processing environments are its ability to grow at low temperature as well as survive or grow in acidic and high osmotic stress environment, their psychrotrophic nature, and their ability to produce biofilms (Buchanan, Leon, Hayman, Jackson, & Whiting, 2017).

L. monocytogenes belongs in the genus of *Listeria*, which also includes *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi* (Collins, et al., 1991; Kathariou, 2002). These microbes are Gram-positive, non-spore forming, rod-shaped bacteria. *L. monocytogenes* is motile when the temperature of the environment is below 37ºC (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014; Vivant, Garmyn, & Piveteau, 2013). The bacterium is a facultative anaerobe with the ability to survive intracellularly (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014; Vivant, Garmyn, & Piveteau, 2013). *L. monocytogenes* can typically grow at temperatures between 2ºC and 45ºC (the optimal growth temperatures is 37ºC), pHs between 4.4 and 9.6, and

water activity at $a_w \geq 0.92$ (Ghandi & Chikindas, 2007; Al-Holy, Al-Nabulsi, Osaili, Ayyash, & Shaker, 2012; Amrouche, Noll, Wang, Huang, & Chikindas, 2010; Apostolidis, Kwon, & Shetty, 2008; Noordhout, et al., 2014). Typical food conditions that do not support the growth of this bacterium include pH < 4.4, $a_w < 0.92$, a combination of pH < 5.0 with $a_w < 0.94$, or NaCl content > 16%, or temperatures < -18 ºC (freezing) (Arauz, Jozala, Mazzola, & Penna, 2009; Balciunas, et al., 2013).

1.3.1 Psychrotrophic nature of *L. monocytogenes*

The ability of *L. monocytogenes* to survive and grow at refrigeration temperature between 2ºC to 4ºC is a major factor that makes this pathogen hard to control in food (Ghandi & Chikindas, 2007). *L. monocytogenes* can grow at 4ºC in dairy products (skim, whole and chocolate milk, and whipped cream), albeit the doubling time can be very long (between 28.5 to 46 hours) (Rosenow & Marth, 1987). Furthermore, the storage of foods, such as RTE meats, dairy products and seafood, in refrigeration temperatures can inhibit the growth of competing microorganisms, while *L. monocytogenes* is able to grow to a substantial number after the contamination (Chan & Wiedmann, 2008). It should be noted that *L. monocytogenes* can also grow extremely slow at -1.5ºC in vacuum-packed sliced roast beef, and -0.4ºC in chicken broth and pasteurized milk (Lado & Yousef, 2007). However, the generation time of *L. monocytogenes* in vacuum-packed sliced roast beef was 100 hours, and >100 hours for chicken broth and pasteurized milk (Lado & Yousef, 2007).

Prevention of *L. monocytogenes* growth in contaminated foods under refrigeration temperature can significantly reduce the risk of listeriosis (Chan & Wiedmann, 2008). The risk of listeriosis due to post processing contamination in retail environments has been demonstrated

by the potential of *L. monocytogenes* growth on sliced soft cheese surfaces at 7ºC (retail refrigeration cabinet temperature) that can reach between 1.8 to 4.0 log CFU per gram cheese within 14 days depending on the type of soft cheese (Lahou & Uyttendaele, 2017). To reduce the risk of listeriosis contamination, therefore, supplementary control measures are needed to prevent the growth of *L. monocytogenes* in sliced RTE meats, cheese and seafood in retail environments after post processing in addition to refrigeration.

1.3.2 Ability to survive in acidic environment

The food industry uses acidic pH as one of the major preservation methods to prevent the growth of spoilage and pathogenic foodborne pathogens. However, *L. monocytogenes* may survive low pH (pH > 4.4) in food. Adaptation of *L. monocytogenes* in mild acidic pH was reported to confer resistance to severe acidic conditions (Gahan, O'Driscoll, & Hill, 1996; Ghandi & Chikindas, 2007). Two studies demonstrated that acid-adapted *L. monocytogenes* (2 hours of exposure to pH 5.2) and acid tolerant *L. monocytogenes* mutants (isolated cells that survived prolonged exposure to pH 3.5) survived better in commercial yogurts and cottage cheese made in the laboratory (Gahan, O'Driscoll, & Hill, 1996; O'Driscoll, Gahan, & Hill, 1996). Furthermore, acid-adapted *L. monocytogenes* was reported to have developed resistance to heat-shock (52ºC), osmotic shock (25-30% NaCl) and alcohol stress (Phan-Thanh, Mahouin, & Alige, 2000). Proteins that showed increase in quantity under non-lethal acidic pH and lethal acidic pH were GroEL, ATP synthase and various transcriptional regulators (Phan-Thanh & Mahouin, 1999). Some of these proteins (especially GroEL) were also shown to have increased in quantity when *L. monocytogenes* was grown in cold temperature (10 ºC) (Liu, Graham, Bigelow, Morse, & Wilkinson, 2002), suggesting acid may confer cross-resistance to other types

of stress. This can have significant influence on the safety of foods that rely on heat, osmotic stress, cold temperature, and an acidic pH to prevent *L. monocytogenes* growth.

1.3.3 Ability to survive under osmotic stress

The ability of *L. monocytogenes* to grow at $a_w \geq 0.92$ makes it difficult to control this pathogen in foods that are meant to have a long shelf life. This is problematic because using salt or sugar to lower water activity as a food preservation technique is common in the food industry. For example, the bacterium can survive (but does not grow) in fermented hard salami with a_w between 0.79 and 0.86 at 4 ºC for >84 days (Johnson, Doyle, Casens, & Schoeni, 1988). Thus, a food product contaminated with *L. monocytogenes* with a_w as low as 0.86 may cross contaminate other foods or introduce the bacterium into retail environment.

Exposure to sub-lethal osmotic stress can confer cross-protection to other lethal stresses. According to Faleiro et al. (2003), *L. monocytogenes* isolated from cheese showed enhanced survival in salt stress (20% w/v NaCl) after adaptation to acid at pH 5.5 for at least 2 hours (Faleiro, Andrew, & Power, 2003). The same study also observed that *L. monocytogenes* incubated in 3.5% (w/v) NaCl can survive acid shock of 3.5 pH (Faleiro, Andrew, & Power, 2003). Therefore, decisions on processing and preservation of food with a high risk of contamination with *L. monocytogenes* should consider the possibility of conferring cross-protection to this pathogenic bacterium.

1.3.4 *L. monocytogenes* biofilm formation

A biofilm is a microbial community composed of either single or multiple species of microorganisms that attaches and grows on a surface (O'Toole, Kaplan, & Kolter, 2000). Bacteria in established biofilm communities are less susceptible to sanitation treatments that are popular in the food industry (Lunden, Autio, Markkula, Hellstrom, & Korkeala, 2003; Meyer, 2006). Furthermore, the extracellular polymeric materials produced by the cells to form the biofilm community can foster cell interactions in terms of nutrients and genetic materials that results in enhancement of their ability to survive (Buchanan, Leon, Hayman, Jackson, & Whiting, 2017).

The ability of *L. monocytogenes* to form a biofilm in a food processing environment is dependent on strain type, time, temperature, and surface of the food processing environment. According to a study of Kadam and et al. (2013), involving many strains (143 strains) of *L. monocytogenes*, biofilms formed at various degrees depending on the growth medium and temperature. In a different study, 32 strains of *L. monocytogenes* isolated from food processing environments, milk, and vegetables formed biofilms in at least one of the temperatures and the surface materials tested, but the degree of biofilm formation was heavily influenced by both temperature and surface type (Bonsaglia, et al., 2014). Therefore, it is very likely that *L. monocytogenes* will form biofilms in a food processing environment, and thus, an approach aimed at preventing/inhibiting biofilm production is important for the control of *L. monocytogenes* contamination in food processing facilities.

The ability of *L. monocytogenes* to form biofilms is one of the major reasons for the persistence of this bacterium in various food-related environments (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014). Conveyer belts (made of polyvinyl chloride and polyurethane)

and stainless-steel surfaces of processing equipment are often contaminated by *L. monocytogenes* even after sanitation (Midelet & Carpentier, 2002). One study reported that *L. monocytogenes* attaches more strongly to polymers than other tested microorganisms (*Staphylococcus sciuri*, *Pseudomonas putida,* and *Comamonas* spp.), and the concentration of pathogens in a biofilm had the strongest influence on the total number of CFU transferred from abiotic surfaces to beef (Midelet & Carpentier, 2002). Therefore, proliferation of biofilm communities may increase the risk of food contamination with *L. monocytogenes* and dissemination of this bacterium to other food associated environments.

L. monocytogenes can irreversibly adhere to surfaces of processing facilities and form a biofilm in response to environmental stress (Srey, Jahid, & Ha, 2013). Once adhered to a surface, this bacterium can become highly resistant to stress conditions (e.g., desiccation and sanitization) and persists on the attached surface after cleaning (Borucki, Peppin, White, Loge, & Call, 2003). Persistence of *Listeria* biofilms on food processing surfaces can potentially propagate the cycle of biofilm dissemination and result in continuous contamination of food products (Holah, Bird, & Hall, 2004; Holah, Taylor, Dawson, & Hall, 2002; Lunden, Miettinen, Autio, & Korkeala, 2000). For example, a *Listeria* outbreak at a hospital in the US, 2015, was linked to a contaminated milkshake machine (Mazengia, et al., 2017). The contaminated milkshake machine was used in 2014 when another listeriosis outbreak was linked to a contaminated pasteurized dairy ice cream product at the same hospital (Mazengia, et al., 2017). Thus, limiting the development of *L. monocytogenes* biofilms and controlling the growth of this pathogen are important to reduce the risk of persistence and continuous recontamination of food and food-contacting surfaces.

Currently, the food industry relies on hygiene control to limit production and dispersion of *L. monocytogenes* biofilms (Swaminathan & Gerner-Smidt, 2007). Special attention should be given to places that are hard to reach when cleaning. The reason is because biofilm formation tends to occur in sites difficult to clean and can gather food residues and water (Khan, et al., 2016). However, cleaning processes often require high temperature, pressure, and large volume of water (Srey, Jahid, & Ha, 2013). The specifics on sanitation and its limitations will be discussed later.

1.4 Common Control Methods for *L. monocytogenes* in Food Industry

It is difficult to completely eradicate *L. monocytogenes* due to its ubiquitous nature in farm, processing and retail environment. There are several methods to reduce the risk of contamination and growth of *L. monocytogenes* in all stages of food production. However, as mentioned briefly, there are significant draw backs in current standard methods used by the industry today.

1.4.1 Pasteurization

Pasteurization is a common technique in the food industry to reduce the number of spoilage and pathogenic bacteria in a food product. Pasteurization temperature ranges from 60ºC to 80ºC and held at that temperature for certain amount of time depending of the food product (i.e. 15 seconds at 72ºC to pasteurize milk) (Khan, et al., 2016; US Food and Drug Administration, 2015). *L. monocytogenes* cells can be irreversibly damaged and die after exposure to temperature above 56 ºC (Lado & Yousef, 2007). There is a linear relationship

between thermal treatment time and the log count of survivor of *L. monocytogenes* in different foods. According to Lado and Yousef (2007), pooled data of 411 studies revealed that as the heat treatment approached 74ºC the log count survivors decreased in inoculated meat, dairy, seafood, fruit, juice and vegetable products.

However, *L. monocytogenes* may have increased heat resistance depending on the strain and components of food (Aryani, Zwietering, & den Besten, 2016; Lado & Yousef, 2007). For example, high salt concentrations increase the heat tolerance of the pathogen, and the fat fraction of goat milk was shown to protect *L. monocytogenes* against heat (68 ºC for 15 seconds) (Humphrey, Richardson, Statton, & Rowbury, 1993; MacDonald & Sutherland, 1993). Furthermore, according to Aryani et al. (2016), *L. monocytogenes* strain L6 was more resistant to 65ºC thermal treatment when it was thermal-treated in laboratory growth media than when it was thermal treated in milk. Interestingly, all strains of *L. monocytogenes* tested in the study had 3 to 9 times higher heat resistance if they were thermal-treated in ham than in laboratory growth media (Aryani, Zwietering, & den Besten, 2016).

Although pasteurization is a cost-effective method for reducing the risk of listeriosis, some foods may protect *L. monocytogenes* if the products are thermal-treated at lower temperature to maintain its sensory qualities. Moreover, the disadvantages of pasteurization also include high initial capital investment, change in sensory property of the pasteurized food products, and the risk of post-processing contamination.

1.4.2 Preservatives

The food industry often uses salts, acids, nitrite, and smoke to control bacterial growth in food (Lado & Yousef, 2007). Although the reduction of water activity by increasing osmolarity and lowering pH is a cost-effective method to control *L. monocytogenes* growth in food, the concentration required may influence the sensory quality of the treated food, since high salt concentrations and strong acidic conditions (16% NaCl and pH < 4.4) are required to achieve listeriostatic effect at ≤4 ºC (Hudson, 1992). Furthermore, as mentioned previously, exposure to salts or sub-lethal acidic pH may confer cross-protection to other stresses, such as salts, acid, and possibly heat treatment as well.

Sodium nitrite is a curing agent frequently used in meat, fish and sometimes cheese as a preservative (Lado & Yousef, 2007). Nitrite derived from this curing agent had a slight listeriostatic effect (Lado & Yousef, 2007). To inhibit the growth of *L. monocytogenes*, 30 ppm (at minimum) is required to enhance the anti-listerial effect of lactate (≤3.2%) and diacetate (≤0.24%) in RTE poultry products with 0.8% ~ 3.6% of NaCl stored at 4 or 7ºC (Glass, McDonnell, Sawyer, & Claus, 2008). However, 103 ppm of sodium nitrite at pH 6.3 and 32ºC with 3.5% NaCl in meat does not control the growth of *L. monocytogenes* (Glass & Doyle, 1989), suggesting that nitrite alone is not an effective preservative to inhibit the growth of *L. monocytogenes* in food. Furthermore, the International Agency for Research on Cancer evaluated nitrate or nitrite in red and processed meat under the conditions forming endogenous N-nitroso compounds are likely to be carcinogenic to humans (Larsson, Orsini, & Wolk, 2006).

Smoke can be used to preserve meat and fish while enhancing the flavor of a food product. Commercial liquid smoke contains phenolic compounds and acetic acid that both have anti-listerial effects (Lado & Yousef, 2007). Addition of 0.2 to 0.6% of commercial liquid smoke

into liquid from wieners inhibits the growth of *L. monocytogenes* (Faith, Yousef, & Luchansky, 1992). However, *L. monocytogenes* is frequently detected in cold smoked fish according to a study from the UK (Jørgensen & Huss, 1998). Furthermore, addition of smoke into food may not be suitable for all foods due to the sensory profile it brings.

1.4.3 Phenolic compounds and antioxidants from plants

Spices, herbs, and plants extracts are often added in food as flavoring and seasoning agents, and several of these plants are rich in phenolic compounds that have antibacterial activity. Thus, plants and their extracts have gathered significant interests as an alternative food preservative (Cleveland, Montville, Nes, & Chikindas, 2001; Roller & Lusengo, 1997; Sandis, Leonard, & Viljeon, 2010). Phenolic compounds that have expressed notable listeriostatic or listeriocidal activity included carvacrol, cinnamaldehyde, eugenol, geraniol and thymol (Lado & Yousef, 2007).

Similar to phenolic compounds, some plant antioxidants have anti-listerial activity. According to Pandit and Shelef (1994), antioxidant extract from rosemary (0.3%) and encapsulated rosemary oil (5%) inhibits the growth of *L. monocytogenes* in pork liver sausage during 50 days of storage at 5ºC. Interestingly, synthetic antioxidants were also reported to have anti-listerial activity. Although synthetic antioxidants are mainly added into food to prevent lipid oxidation (rancidity), some were reported to have anti-listerial activity. Butylated hydroxyanisol, butylated hydroxytoluene (BHT), tertiary butylhydroquinone and propyl gallate are antioxidants used in the food industry and have relatively strong inhibitory activity against *L. monocytogenes* ($\leq$256 ppm, except for BHT at >512 ppm) (Lado & Yousef, 2007).

Phenolic compounds and antioxidants are promising alternatives as anti-listerial additives in food. However, some naturally produced phenolic compounds or antioxidants are expensive to extract and may require high concentrations to have anti-listerial effect. Furthermore, high concentrations of certain spice, herb and plant extracts may alter the sensory profile of food. Thus, it is important to find balance between acceptable sensory modification and antimicrobial effectiveness in the applied research.

1.4.4 Bacteriocins

Bacteriocins are polypeptides made by bacteria, such as *Lactococcus lactis* ssp. *lactis* (Lado & Yousef, 2007). Nisin and pediocin are the most investigated bacteriocins against *L. monocytogenes*, but nisin is the only bacteriocin approved as a food preservative (Amrouche, Noll, Wang, Huang, & Chikindas, 2010). Nisin can be incorporated as dried concentrated powder during processing of foods, such as dairy and canned goods (Amrouche, Noll, Wang, Huang, & Chikindas, 2010; Balciunas, et al., 2013). It is used in Europe and America in processed cheese production, and the regulation on the dosage allowed in cheese can range from no upper limit to as low as 100 International Units (IU) per gram cheese depending on the country (Arauz, Jozala, Mazzola, & Penna, 2009; Gharsallaoui, Oulahal, Joly, & Degraeve, 2016). Nisin demonstrated significant listeriostatic activity on meat, salmon and cheese stored at refrigeration temperature (Fang & Lin, 1994; Abee, Krockel, & Hill, 1995; Szabo & Cahill, 1999; Scannell, et al., 2000).

Nisin is most effective under acidic pH and low temperature, and its activity is lost as pH and temperature increases (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016). However, it is often necessary to add significantly higher amounts of nisin than those required to inhibit *Listeria* in

the laboratory growth medium to ensure the same effectiveness in food due to the physicochemical properties of food matrices (i.e. high pH and high fat) (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016). Thus, not all foods are suitable to implement nisin as an anti-listerial agent, and it may be too costly for small scale production.

1.4.5 Sanitizers

Periodic cleaning is important to reduce the risk of contamination and remove potential buildup of biofilms and microbial counts. The washed surfaces are often sanitized to reduce microbial counts and prevent growth (Lado & Yousef, 2007). Popular sanitizers used in the food industry can be categorized into chlorine-containing compounds, quaternary ammonium compounds, acid sanitizers, ozone, and iodophors (Lado & Yousef, 2007). However, the efficiency of a sanitizer can be negatively influenced sometimes by human errors, decreased temperatures and bacterial development of biofilms (Srey, Jahid, & Ha, 2013; Mafu, Roy, Goulet, Savoie, & Roy, 1990). Repeated exposure of *L. monocytogenes* to various sanitizers (sometimes at sub-lethal concentrations) in the food environment may enhance *Listeria* tolerance to sanitizers and unrelated antimicrobials (Allen, et al., 2016).

In addition, exposure of *L. monocytogenes* to sanitizers may result in the development of antibiotic resistance due to the co-localization of genes responsible for sanitizer resistance and antibiotic resistance; this is called co-selection. Some strains of *L. monocytogenes* develop increased resistance against gentamicin, an antibiotic commonly prescribed to treat listeriosis, after exposure to sub-lethal concentrations of triclosan, a common antibacterial/fungal agent found in cleaning agents such as soaps and cleaning supplies (Christensen, Gram, & Kasbjerg,

2011). Furthermore, *L. monocytogenes* that was originally sensitive to benzalkonium chloride, a type of quaternary ammonium compound, can become less sensitive to aminoglycosides after exposure to higher concentrations of benzalkonium chloride (Romanova, Wolffs, Brovko, & Griffiths, 2006). Therefore, use of sanitizers may contribute to the development of antibiotic resistance in *L. monocytogenes* and this control strategy should be reevaluated.

1.5 Multi-Barrier Food Safety Approaches to Managing *L. monocytogenes*

Hurdle technology in the food industry is described as a preservation parameter that can be used at an optimum level to achieve maximum lethality against microorganisms by combining two or more methods and causing minimum alterations in the sensory properties of the processed food (Leistner & Gorris, 1995). The majority of food production systems apply hurdle technology during production. For instance, processed cheese can use pasteurization and nisin, and fresh beef uses refrigeration with atmospheric packaging for preservation. Therefore, food producers are incentivized to reduce production costs by improving the efficiency of current methods used in hurdle technology.

Application of hurdle technology is also topical for minimally processed foods, chilled foods, healthy foods with fewer preservatives (i.e. less salts), and less packed foods in developed countries (Leistner & Gorris, 1995). However, as mentioned previously, listeriosis is highly associated with minimally processed and RTE products, such as deli meats and cheese in North America. Thus, improvement of this technology is a dire necessity for food safety.

Achieving synergistic effects by combining two or more methods is very beneficial to improve the current hurdle technology. Synergism between two or more methods would mean

reduced input for the same (or improved) efficacy against undesirable microorganisms. Thus, finding new synergism with alternative methods can improve the efficiency of current hurdle technology.

1.5.1 Phenolic compounds/antioxidants and nisin

Synergism between nisin and new phenolic compounds is an economically attractive approach to improve the current hurdle technology. Reducing the amount of nisin used during food production by combing with purified phenolic compounds/antioxidants (instead of phenolic compounds or antioxidants rich crude extracts) would minimally affect the sensory properties of food. This approach may result in new formulated products for food preservation. The new formula can be used as an additional hurdle for bacterial growth, and potentially prevent biofilm development during processing. Moreover, nisin has received the 'Generally Recognized as Safe (GRAS)' status in the US only. Therefore, implementation of a new formula combining nisin and a purified phenolic compound/antioxidant would potentially receive little resistance from regulatory agencies and the food industry.

There have been studies that investigated the growth inhibition and antibiofilm activity against *L. monocytogenes* using nisin and phenolic compounds/antioxidants (Moosavy, Mahmoudi, Davudi, & Shavisi, 2013; Thomas & Isak, 2006; Ettayebi, El Yamani, & Rossi-Hassani, 2000; Olasupo, Fitzgerald, Narbad, & Gasson, 2004; Pol & Smid, 1999; Girardin, et al., 2005; Nostro, et al., 2010; Bolocan, et al., 2016; Lado & Yousef, 2007; Chikindas, et al., 1993). These reports demonstrated the potential of nisin and phenolic compounds to improve the hurdle technology.

1.5.1.1 Synergistic growth inhibitory activity of nisin and phenolic compounds against *L. monocytogenes*

Phenolic compounds can be a viable alternative as an adjuvant with nisin. Purified phenolic compounds carvacrol, eugenol and thymol exhibited synergistic inhibitory activity when combined with nisin against *L. monocytogenes* and *L. innocua*, a surrogate for *L. monocytogenes*, when tested for different processing conditions. In one study, combination of thymol at 0.03% (v/v) and a low concentration of nisin Z (40 IU/ml for *L. monocytogenes* and 70 IU/ml for *B. subtilis*) was reported to have a strong inhibitory effect against *L. monocytogenes* ATCC 7644 and *B. subtilis* ATCC 33712 (Ettayebi, El Yamani, & Rossi-Hassani, 2000). It has also been reported that *L. innocua* and *B. subtilis* are synergistically inhibited by nisin in combination with carvacrol, eugenol, thymol and cinnamic acid (Olasupo, Fitzgerald, Narbad, & Gasson, 2004; Girardin, et al., 2005; Nostro, et al., 2010). These studies suggest that the same formula may also exhibit synergistic activity in different species of bacteria. Furthermore, Pol and Smid (1999) reported that lysozyme (an antimicrobial enzyme that is active against Gram-Positive bacteria such as *L. monocytogenes*) enhanced the antimicrobial synergism between nisin and carvacrol.

It should be noted that there are also several studies that reported the synergistic inhibitory activity of nisin in combination with other hurdle technologies against *L. monocytogenes*. For example, growth inhibition synergism was reported when nisin was combined with sodium lactate, chitosan, zinc, aluminum chloride, lactoferrin, p-anisaldehyde, EDTA, epsilon-poly-L-lysine, and ethanol (Bhatia & Bharti, 2015; Chen, et al., 2016; Schelegueda, Zalazar, Gliemmo, & Campos, 2016; McEntire, Montville, & Chikindas, 2003; Murdock, Cleveland, Matthews, & Chikindas, 2007; Najjar, Kashtanov, & Chikindas, 2007).

Thus, synergistic growth inhibition against *L. monocytogenes* using nisin and phenolic compounds with other additives may potentially generate more potent antimicrobial synergism.

Even though nisin in combination with thymol and carvacrol are potent against *L. monocytogenes*¸ their pungent nature and the purification costs may not make this combination practically applied to foods. The use of crude extract from different sources can also pose the different food quality issue, such as undesirable pigmentation of food. Thus, it would be advantageous to find alternative phenolic compounds with minimal impacts on food quality.

1.5.1.2 Synergistic anti-biofilm activity of nisin and phenolic compounds against *L. monocytogenes*

Although *L. monocytogenes* biofilm formation is a well-researched area, studies on the anti-biofilm activity of nisin is scarce with *L. monocytogenes*. According to Nostro et al. (2010), as the concentration of nisin incorporated into poly-ethylene-co-vinyl acetate films (PEV) increases (maximum 1%), the biofilm biomass of *L. monocytogenes* ATCC 7644 on PEV films decreases. Minei et al. (2008) reported that addition of nisin to brain heart infusion (BHI) broth reduced the level of *L. monocytogenes* adhesion and biofilm formation on stainless coupons. Furthermore, nisin also reduced biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus mutans* (Shin, et al., 2016).

Although not nisin, Gomez et al. (2012) reported that after treating polystyrene microtiter plates with 25 µg/ml of enterocin AS-48 (bacteriocin produced by *Enterococcus*), there was a decrease in the attachment and formation of biofilms by mixed strains of *L. monocytogenes* for at least 24 h. Furthermore, Bolocan et al. (2016) reported cell free substrates of *Bacillus licheniformis, Lactococcus lactis,* and *B. subtilis* inhibited the biofilm formation of *L.*

monocytogenes on hydrophobic (polystyrene) materials. According to the study, the cell free substrates contains lichenicidin (produced by *Bacillus licheniformis*), nisin Z (produced by *L. lactis*) and subtilomycin (produced by *B. subtilis*), suggesting that some bacteriocins can inhibit biofilm formation in *L. monocytogenes* (Bolocan, et al., 2016).

Reduction of biofilm formation activity in *L. monocytogenes* using phenolic compounds has been reported. Thymol at 0.05 mM inhibits biofilm formation by *L. monocytogenes* at 4, 25, and 37ºC (Upadhyay, Upadhyaya, Kollanoor-Johny, & Venkitanarayanan, 2013). A similar observation was reported for ferulic and gallic acids, and morin (Borges, Saavedra, & Simoes, 2012; Sivaranjani, et al., 2016). However, there have been no reports of synergistic biofilm inhibition activity of nisin in combination with phenolic compounds against *L. monocytogenes*.

Nisin has a promising potential to be implemented by the food processors as an alternative strategy to reduce the risk of *L. monocytogenes* biofilm production. This can potentially reduce the risk of biofilm build up in a food processing facility.

1.6 Project Goals and Hypothesis

Considering the anti-biofilm and inhibitory activity of phenolic compounds and nisin against *L monocytogenes*, I hypothesized that thymol and carvacrol may not be the only purified phenolic compounds that can generate synergism combined with nisin. Furthermore, this research approach may reduce the application costs of nisin in food and prevent the development of *L. monocytogenes* biofilm when this pathogen is transferred onto various surfaces. Therefore, the objectives of the project are: i) to find binary combinations of purified phenolic compounds and nisin that exhibit synergistic inhibitory activity against the growth of *L. monocytogenes*, and

ii) to test the anti-biofilm activity of nisin, purified phenolic compounds and their combinations against *L. monocytogenes*.

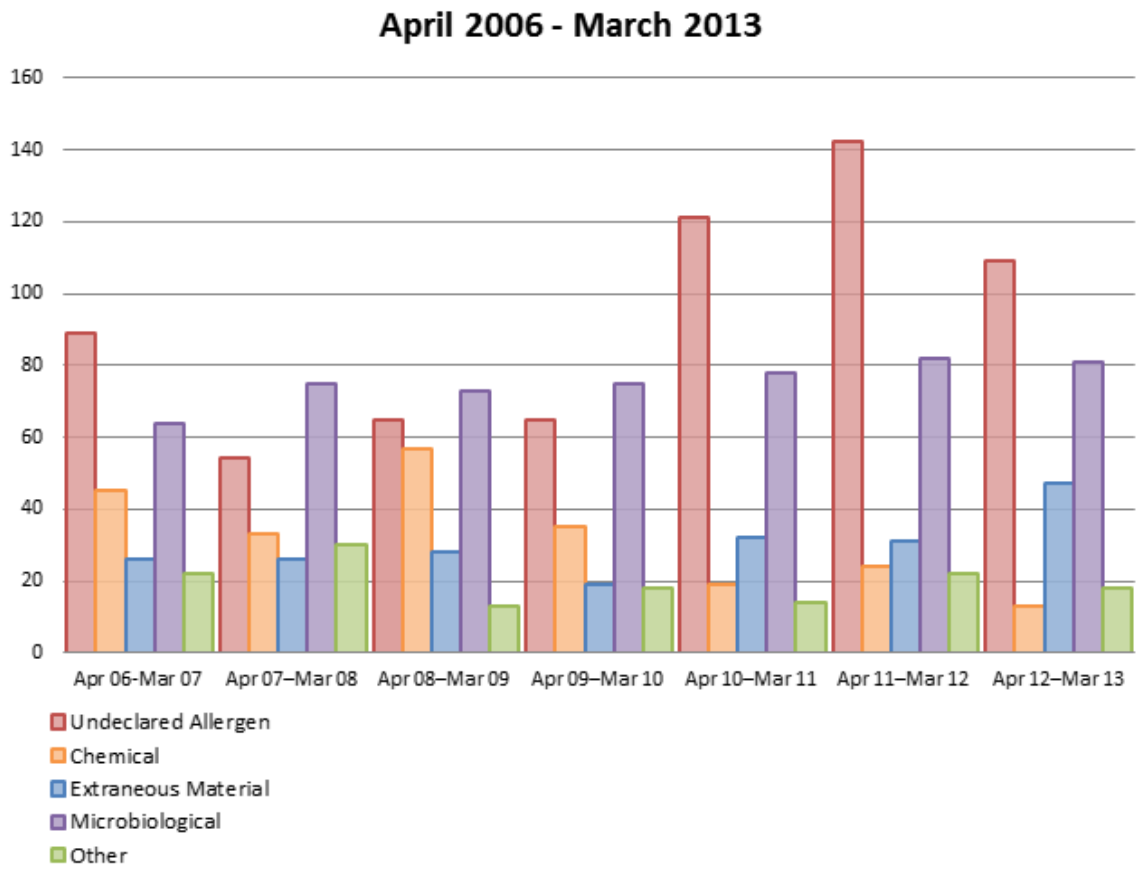


Figure 1: Food recall incidents by hazard between April 2006 to March 2013 (Canadian Food Inspection Agency, 2015).

CHAPTER 2: ANTIMICROBIAL ACTIVITY OF NISIN AND PHENOLIC ANTIOXIDANT COMBINATIONS AGAINST *L. MONOCYTOGENES*

2.1. Introduction

Listeriosis, the disease caused by *L. monocytogenes* infection, has a very high mortality rate (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014). Listeriosis outbreaks are often associated with processed meat, dairy and raw foods (Kovačević, 2007). Furthermore, the psychrotrophic nature of *Listeria monocytogenes* allows it to proliferate in food during refrigeration (Vázquez-Boland, et al., 2001). Thus, inhibition of *Listeria* growth in food at refrigeration temperatures can lower the risk of listeriosis in processed meat, dairy and raw foods.

Currently, nisin is the sole bacteriocin approved as a food additive (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016). The product is recognized by the US Food and Drug Administration (FDA) as a 'Generally Recognized As Safe (GRAS)' food additive. Nisin is added to liquid and solid foods at various temperatures (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016). Nisin has antimicrobial efficacy against *L. monocytogenes* in ready-to-eat (RTE) foods, such as ricotta-type cheese, poultry products, vacuum-packed ham and tuna, and fish roes (Lakicevic & Nastasijevic, 2017). However, the cost of nisin can be a concern for its application as a food preservative. Hence, it would be beneficial to enhance the antimicrobial activity of nisin using cost-effective antimicrobial adjuvant.

A previous study from our lab showed that phenolic antioxidants synergistically increase the antimicrobial activity of bacitracin, an antimicrobial peptide, against methicillin-resistant *Staphylococcus aureus* (MRSA) (Kim & Jeon, 2016). Therefore, we contend that phenolic antioxidants can generate synergistic antimicrobial effect with nisin against another Gram-positive bacterium, such as *Listeria*. The use of phenolic compounds as adjuvant has several

advantages. The first advantage is the potential health benefits of some phenolic compounds, such as anti-obesity, when consumed with the treated food (Kalaycioglu & Erim, 2017; Mandalari, et al., 2007). The second advantage is to enhance the preservative effect of nisin when phenolic antioxidants are added. Lastly, the antioxidant property of certain phenolics can also prevent lipid rancidity in food. The present study investigates the combined antimicrobial activity of nisin with synthetic and natural phenolic compounds. The goal of this project was to identify phenolic compounds that synergistically inhibit *L. monocytogenes* growth when combined with nisin.

2.2 Material and Methods

2.2.1. Bacterial strains and culture conditions

*L. monocytogenes*4b (ATCC 19115) was obtained from the American Type Culture Collection (ATCC). Six strains of food isolates were obtained from Dr. Lynn McMullen (Faculty of Agriculture, Life and Environmental Science, the University of Alberta). Tryptic soy broth, agar and yeast extract were obtained commercially from Becton Dickson (BD) Biosciences, VWR International and Fisher Scientific, respectively. All strains of *L .monocytogenes* were stored in 25% glycerol at -80ºC. For the experiments, strains were freshly streaked from frozen stock on to laboratory made tryptic soy broth agar (15 g/L of agar) supplemented with 0.6% yeast extract (6 g/L of yeast extract), and incubated overnight at 37^{o}C for experiments. A colony of overnight plate culture was propagated into 5 ml of tryptic soy broth with 0.6% of yeast extract (TSBYE) for overnight growth at 37^{o}C and 200 rpm. Fifty microliters of overnight broth culture was inoculated into 450 µl of fresh TSBYE and incubated under the same conditions for

6 ~ 8 h to reach the exponential phase. The broth culture was then adjusted to an OD_{600} of 1.0 ± 0.01, and 1:100 dilution was made from it for antimicrobial susceptibility testing.

2.2.2. Antimicrobial susceptibility assay

Benzoic acid, butylated hydroxytoluene (BHT), caffeic acid, catechin, chrysin, gallic acid, hesperidin, morin, naringenin, *p*-coumaric acid, quercetin, salicyclic acid, sinapic acid, syringic acid, taxifolin, *t*-cinnamic acid, and vanillic acid were purchased from Sigma-Aldrich. Stearyl gallate, octyl gallate, butyl gallate, ethyl gallate, lauryl gallate, methyl gallate, and propyl gallate were obtained commercially from the Tokyo Chemical Industry Co., Ltd (Tokyo, Japan), and nisaplin (2.5% of pure nisin) was purchased from MP Biochemicals (Santa Ana, California). Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the broth microdilution assay method ISO 20776-1 described in M07-A9 protocol from the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2012). Briefly, a ninety-six well plate was prepared in a way that phenolic compounds and nisin were serially diluted by 2-fold in a 96-well plate. Concentrations tested in this experiment ranged from 1024 µg/ml to 8 µg/ml for phenolic compounds/antioxidants, and 1000 IU/ml to 30 IU/ml for nisin. Each well was inoculated with approximately 10^6 CFU of *L. monocytogenes* strains. The prepared plate was incubated at 37^{o}C 18-20 h. The MIC was confirmed using visual inspection. MBC was determined by spotting of overnight culture from the MIC test on a TSBYE agar plate. After overnight incubation of the spotted plate, the lowest concentration with no visible colony (growth) surrounding the point of inoculation (bright dot) was considered as the MBC. The test was repeated three times.

2.2.3. Synergistic test of nisin and phenolic compounds

The pH of tryptic soy broth was adjusted to 7 before distribution into the 96 wells culturing plate. The compounds used in the experiment were mixed at sub-lethal concentrations. On a single 96-well plate, the desired concentration of phenolic compounds and nisin were prepared in each well before inoculation with 100 µl of prepared culture (CLSI, 2012). Incubation time, temperature, and method to determine growth in each well was the same as mentioned. Synergistic inhibitory activity of nisin and phenolic compounds against *L. monocytogenes* was determined using the fractional inhibitory concentration index (FIC) (Hall, Middleton, & Westmacott, 1983).

FIC was calculated by the following equation:

$$\left(\frac{MIC\ of\ A\ Combined}{MIC\ of\ A\ alone}\right) + \left(\frac{MIC\ of\ B\ combined}{MIC\ of\ B\ alone}\right)$$

where:

MIC of A combined is the MIC of a phenolic compound/antioxidant after combining with nisin. MIC of A alone is MIC of a phenolic compound/antioxidant before combining with nisin.

MIC of B combined is the MIC of nisin after combining with a phenolic compound/antioxidant. MIC of B alone is the MIC of nisin before combining with a phenolic compound/antioxidant.

An FIC range between 0.5 and 1 indicates additive activity, and an FIC below 0.5 indicates synergistic activity.

2.3. Results

2.3.1. MIC and MBC of nisin and phenolic compounds against *L. monocytogenes* 4b (ATCC 19115)

The susceptibility of *L. monocytogenes* 4b (ATCC 19115) was tested against 26 phenolic compounds and nisin (Table 1). Twenty of the 26 phenolics inhibited the growth of *L. monocytogenes* at a concentration of 512 μg/ml or above. The MIC of butyl gallate, quercetin, and propyl gallate was 256 μg/ml. Naringenin exhibited inhibitory activity against *L. monocytogenes* as low as 128 μg/ml, while lauryl gallate and octyl gallate had MICs of 64 μg/ml and 32 μg/ml, respectively. The MIC and MBC of nisin against *L. monocytogenes* were 250 IU/ml and 1000 IU/ml, respectively. The MBC was 1024 μg/ml or above for all phenolics except for propyl gallate (512 μg/ml), morin (512 μg/ml), taxifolin (512 μg/ml), lauryl gallate (256 μg/ml) and octyl gallate (64 μg/ml).

2.3.2. Combinational inhibitory activity of nisin and butyl gallate against *L. monocytogenes*

Butyl gallate, propyl gallate, quercetin, lauryl gallate and octyl gallate were selected to examine if they had synergistic antimicrobial activity with nisin. The reason for selecting these compounds was due to their stronger inhibitory activity in comparison with other phenolics tested. Propyl gallate, quercetin, lauryl gallate and octyl gallate did not exhibit additive nor synergistic inhibitory activity with nisin against *L. monocytogenes*. Butyl gallate showed additive and synergistic inhibitory activities with nisin (Table 2); five strains of *L. monocytogenes* had their FIC at 0.5 or above, and FS-15 1/2b and Scott A had a FIC of 0.375. The results suggested that butyl gallate and nisin had synergistic inhibition activity against the environmental and food isolates. The FIC value of the ATCC 19115 strain was 0.75, indicating the combination

generated additive inhibition activity. The enhanced antimicrobial activity was clearly observed in the viability testing (Fig. 2). The results show that the combinations effectively reduced the growth and the viability of *L. monocytogenes*.

2.3.3. Effects of pH on the antimicrobial activity of nisin and butyl gallate

Since the antimicrobial activity of nisin is affected by pH, the synergistic inhibition of the combinations was investigated at different pH values. The MBC of nisin for *L. monocytogenes* ATCC 19115 at pH 7 was 1000 IU/ml, while the MBC of butyl gallate was > 512 μg/ml (Table 1). The combination of the two compounds effectively inhibited the viability of *L. monocytogenes* at significantly reduced concentrations (e.g. 128 μg/ml butyl gallate and 250 IU/ml nisin) (Fig. 2 A). The bactericidal activity of nisin was further increased at weak acidic pHs, such as pH 5~6 (Fig. 2 B and C). For example, 8 μg/ml butyl gallate and 60 IU/ml nisin effectively killed *L. monocytogenes* at pH 5 (Fig. 2 C).

2.4. Discussion

The approach of this study aims to identify possible synergistic combinations of phenolic compounds and nisin that can inhibit *L. monocytogenes*. Although tea catechins have been reported to exert strong inhibitory activity against Gram-positive pathogens, such as *S. aureus* (Cho, Schiller, & Oh, 2008), our results suggest that catechin does not have an inhibitory effect on *L. monocytogenes* (Table 1). Further study is required to determine the reason for *L. monocytogenes*' ability to tolerate catechin.

Within this study, there were four synthetic phenolic acids (butyl, lauryl, octyl and propyl gallates) and two natural phenolic compounds (naringenin and quercetin) that had MICs below 512 μg/ml (Table 1). Octyl gallate and naringenin particularly had great potential to inhibit the growth of *L. monocytogenes* due to their low MICs (Table 1). Octyl gallate was the most potent synthetic phenolic compound against *L. monocytogenes*, and naringenin exhibited the lowest MIC in the natural phenolic compounds tested in this study (Table 1). Naringenin and its derivatives have been reported to possess antimicrobial activity against *S. aureus, Enterococcus faecium, Bacillus cereus*, and reduce production of quorum sensing signaling molecules in *Pseudomonas aeruginosa* (Celiz, Daz, & Audisio, 2011; Vandeputte, et al., 2011). Although quercetin had similar MIC levels to propyl gallate (Table 1), its tendency to sediment and generate yellow pigmentation would leave it unfavorable in food applications. Based on the results, it appears that synthetic phenolics of the gallic acid derivatives are more likely to possess an inhibitory effect on *L. monocytogenes* than natural phenolics. However, it is difficult to make such a conclusion, since the number of phenolic compounds is limited in this study. It was surprising to find butyl gallate and octyl gallate to have inhibitory activity against *L. monocytogenes* at lower concentrations than natural phenolic compounds. The advantages of using synthetic phenolic as a food preservative would include the possibility of precise mass production, and the possibility of reduced production cost. Nevertheless, it is possible to use these phenolics as alternative food preservatives.

Another objective of this project was to find synergistic combinations of nisin and phenolic compounds. Aside from having the potential to lower the cost of nisin application in the food industry, a new adjuvant can increase the antimicrobial activity of nisin in food. Butyl gallate itself had inhibitory activity against *L. monocytogenes* at high concentrations (Table 1),

and butyl gallate generated synergistic inhibitory activity against *L. monocytogenes* in combination with nisin, (Table 2 and Fig. 2). Under acidic conditions (pH 5 and 6), the bactericidal effect of butyl gallate and nisin combination was enhanced significantly against strain ATCC 19115 (Fig. 3). The combined treatment only conferred synergistic bactericidal activity against *L. monocytogenes* strains Scott A in pH 7 (Fig. 2). Thus, butyl gallate can be used to enhance the bacteriostatic, but not bactericidal, effect of nisin against *L. monocytogenes* in food at neutral pH. Furthermore, butyl gallate retained its efficacy against *L. monocytogenes* ATCC 19115 under acidic pH. Further testing is required to determine the effectiveness of the combination in food matrix.

There is limited understanding on the mechanism of how phenolics synergistically enhance its antimicrobial ability with nisin. Nonetheless, it has been reported that alkyl gallate have increased the antimicrobial activities of beta-lactams and bacitracin against MRSA (Kim & Jeon, 2016; Shibata, et al., 2005), suggesting that alkyl gallates may enhance the antimicrobial activity of antimicrobial peptides and antibiotics in Gram-positive bacteria. Although it is currently unclear as to the mechanism underlying the synergism of nisin and phenolic compounds against *L. monocytogenes*, the synergism may be explained by altered bacterial physiology in *L. monocytogenes* after exposure to phenolic compounds. According to Sivarooban et al. (2008), *L. monocytogenes* exposed to nisin in combination with grape seed extract, green tea extract or the purified compounds from the major phenolic constituents of the two extracts, showed altered cell membrane structures and condensed cytoplasm. This suggests that phenolic compounds may enhance the antimicrobial activity of nisin presumably by a similar mode of action with butyl gallate.

Recent *L. monocytogenes* outbreaks indicate that current methods of decontamination, contamination control and sanitization may not be efficient enough to control the risk of *L. monocytogenes* contamination in food. The results in this study suggest that some phenolic compounds, such as butyl gallate, lauryl gallate, octyl gallate, propyl gallates, naringenin and quercetin, may be potent antimicrobial compounds to be considered as part of the processing to control *L. monocytogenes*. In addition, this study discovered that combination of butyl gallate and nisin have synergistic inhibitory activity against *L. monocytogenes*. Further studies are required to understand the mechanism underlying this observation and the antimicrobial effectiveness of the formula in food matrices.

Table 1: MICs and MBCs of phenolic compounds and nisin in *L monocytogenes* ATCC 19115.

Compound	MIC*	MBC*	Compound	MIC*	MBC*
Benzoic acid	1024	>1024	Naringenin	128	1024
BHT[a]	512	>1024	Octyl gallate	32	64
Butyl gallate	256	1024	p-AC[c]	1024	>1024
Caffeic acid	1024	1024	Propyl gallate	256	512
Catechin	512	1024	Quercetin	256	1024
Chrysin	1024	1024	Salicylic acid	1024	1024
Ethyl gallate	512	1024	Sinapic acid	512	1024
EGCG[b]	512	1024	Stearyl gallate	512	1024
Gallic acid	1024	>1024	Syringic acid	1024	>1024
Hesperidin	>1024	>1024	Tannic acid	>1024	>1024
Lauryl gallate	64	256	Taxifolin	512	512
Methyl gallate	512	1024	t-CA[d]	512	1024
Morin	512	512	Vanillic acid	>1024	>1024
Nisin (IU/ml)	250	1000			

* Concentration is in µg/ml.
[a] BHT (Butylated hydroxytoluene), [b] EGCG (Epigallocatechin gallate), [c] p-AC (p-Coumaric acid), [d] t-CA (trans-Cinnamic acid).

Table 2: MICs of nisin and butyl gallate (BG) alone, and in combinations in various strains of *L. monocytogenes*.

Strain	Nisin MIC**	BG MIC*	Combined nisin MIC**	Combined BG MIC*	FIC	Result
ATCC 15313	250	256	62(4)	64(4)	0.5	Synergy
CDC 7762 4B	500	512	125(4)	128(4)	0.5	Synergy
FS-1 1/2a	500	512	125(4)	128(4)	0.5	Synergy
FS-11 1/2b	500	512	125(4)	128(4)	0.5	Synergy
FS-15 1/2b	500	512	125(4)	64(8)	0.375	Synergy
Scott A	500	512	62(8)	128(4)	0.375	Synergy
ATCC 19115	250	512	62(4)	256(2)	0.75	Additive

Note: Fold decreases in the concentration are indicated in parentheses. The FIC value is representative of three independent experiments.
*: Units in this column is in µg/ml
**: Units in this column is in IU/ml

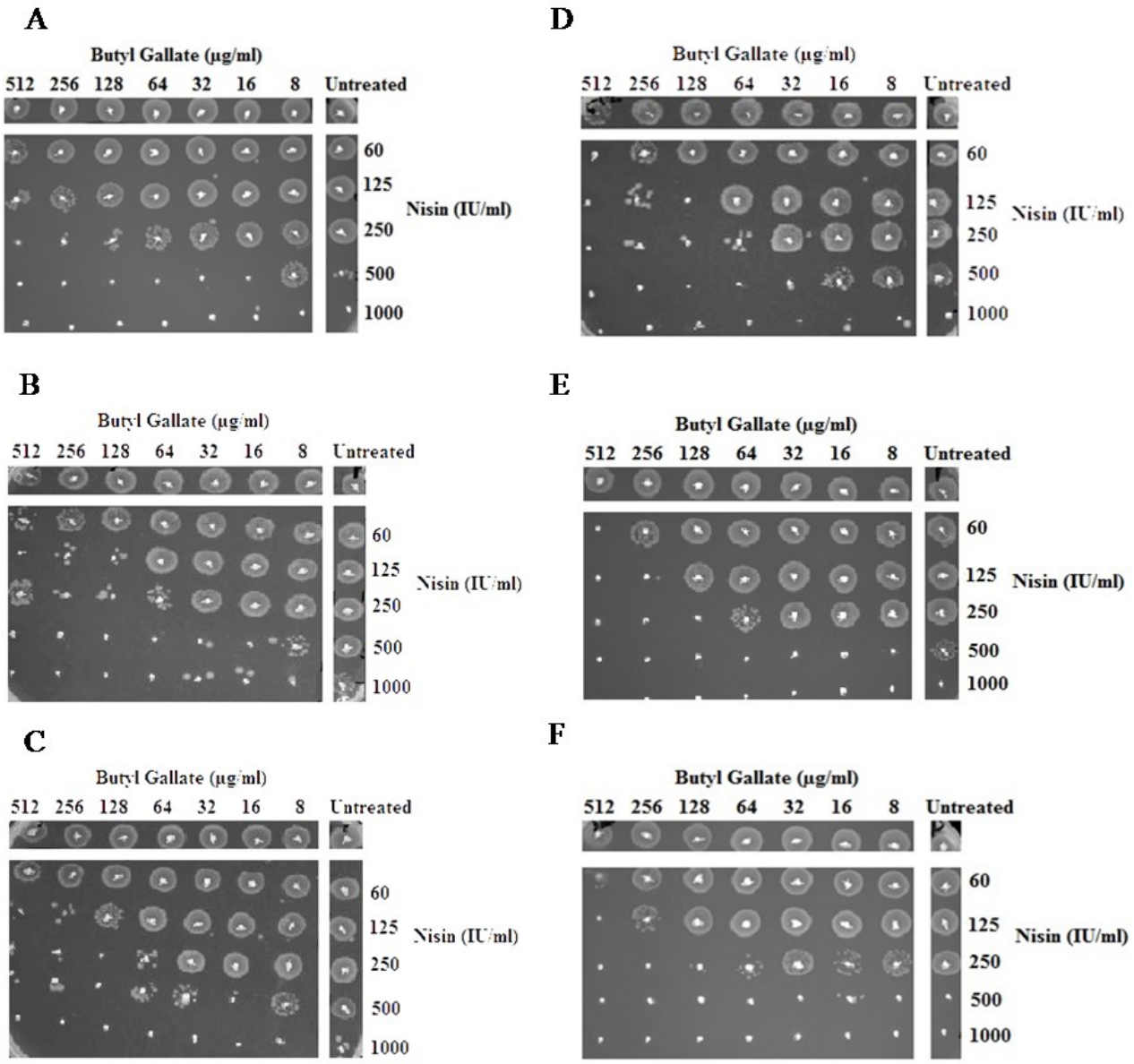
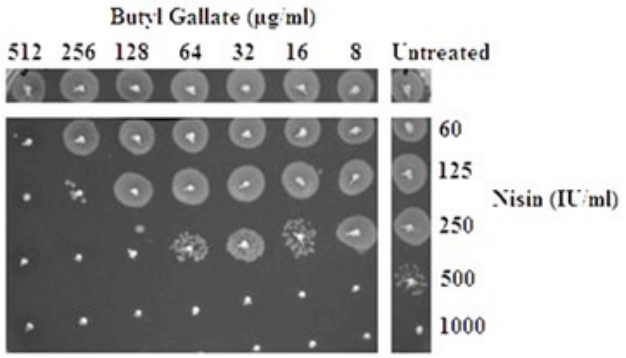
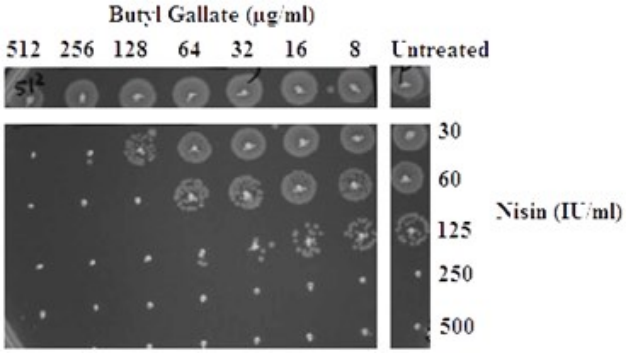


Figure 2: Synergistic antimicrobial killing activity of nisin and butyl gallate against various *L. monocytogenes* strains CDC 7762(A), FS 1(B), FS 11(C), FS 15(D), Scott A(E), ATCC 15313(F) at pH 7. The bright dot at the center of each halo (colony growth) is the point of inoculation.

A



B



C

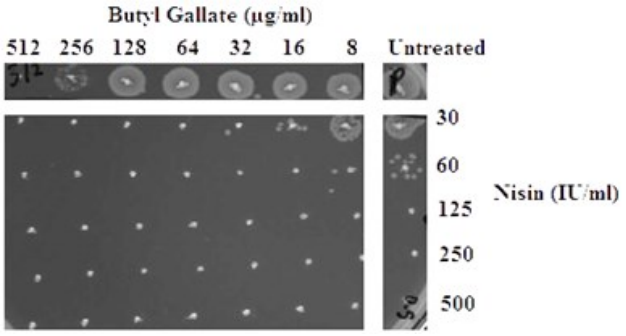


Figure 3: Differential synergistic inhibition of ATCC 19115 at pH 7 (A), pH 6 (B) and pH 5 (C). The bright dot at the center of each halo (colony growth) is the point of inoculation.

CHAPTER 3: ANTI-BIOFILM ACTIVIY OF PHENOLIC COMPOUNDS AND NISIN AND THEIR COMBINATIONS AGAINST *L. MONOCYTOGENES*

3.1. Introduction

The ability of *L. monocytogenes* to form biofilms is one of the primary reasons for the persistency of this pathogen in food processing facilities (Ferriera, Wiedmann, Teixeira, & Stasiewicz, 2014; Vikram, Jayaprakasha, Jesudhasan, Pillai, & Patil, 2010). It is well known that biofilm communities are difficult to remove once attached to a surface (Srey, Jahid, & Ha, 2013). The food processing industry relies on hygiene control to prevent the dispersion of *L. monocytogenes* biofilm (Swaminathan & Gerner-Smidt, 2007). Popular industrial practice to prevent biofilm dispersion includes periodic cleaning coupled with application of disinfectants, such as chlorine, hydrogen peroxide, iodine, ozone or/and peracetic acid to prevent the proliferation of *L. monocytogenes* biofilm. However, these processes often require high temperature, pressure and large volumes of water, and sometimes human errors can negatively influence the degree of cleanliness (Srey, Jahid, & Ha, 2013; Mafu, Roy, Goulet, Savoie, & Roy, 1990).

Bacterial cells in biofilms are physiologically different from planktonic cells and can be more resistant to antibiotics and disinfectants than planktonic cells (Hall-Stoodley, Costerton, & Stoodley, 2004). Thus, the biofilm community may encourage the growth of bacteria that are resistant to antibiotics, disinfectants, and sanitizers. The spread of *L. monocytogenes* resistant to sanitizers and disinfectants used by the food industry have been documented. One study found that 7 out of 77 environmental, food, human and animal isolates of *L. monocytogenes* had higher minimum inhibitory concentrations (MICs) to quaternary ammonium compounds than the control strains (Ghandi & Chikindas, 2007). This may explain why *L. monocytogenes* can persist

in the food processing facilities despite cleaning of processing equipment with disinfectants. *L. monocytogenes* also exhibited ability to adapt and develop resistance when exposed to sub-lethal levels of disinfectants (Mereghetti, Quentin, Marquet-Van Der Mee, & Audurier, 2000). Thus, rotation of different disinfectants or sanitizers was thought to be an effective method for regulating the development of resistance to a single disinfectant or sanitizer. However, exposure to various disinfectants or sanitizers can confer resistance to antibiotics by co-selection (Allen, et al., 2016; Christensen, Gram, & Kasbjerg, 2011). This brings concerns whether disinfectant rotation is effective in controlling the development of antimicrobial resistance in *Listeria* (Lunden, Miettinen, Autio, & Korkeala, 2000; Djordjevic, Wiedmann, & McLandsborough, 2002).

Recent outbreaks of *L. monocytogenes* indicate there is a need to develop alternative methods to control *L. monocytogenes* growth for the food industry. Since biofilms produced by *L. monocytogenes* are difficult to remove and may serve as a reservoir for further dissemination and cross-contamination, the reduction of biofilm production by *L. monocytogenes* during food processing will play a critical role in ensuring food safety associated with *Listeria* contamination. Based on the synergistic antimicrobial activity of nisin and phenolics in the previous study, the present study aimed to investigate if nisin and phenolic combinations would have anti-biofilm activity against *L. monocytogenes*.

3.2. Materials and Methods

3.2.1. Bacterial strains, culture conditions and chemicals

L. monocytogenes ATCC 19115 was purchased from the American Type Culture

Collection (ATCC), and three food isolates (FS-1 1/2a, FS-11 1/2b, and FS-15 1/2b) were kindly provided by Dr. Lynn McMullen (University of Alberta). All the strains were cultured at 37 ºC overnight on Tryptic Soy agars (TSA) supplemented with 0.6% yeast extract. Benzoic acid, butylated hydroxytoluene (BHT), caffeic acid, catechin, chrysin, gallic acid, heperidin, morin, naringenin, *p*-coumaric acid, quercetin, salicyclic acid, sinapic acid, syringic acid, taxifolin, *t*-cinnamic acid, and vanillic acid were purchased from Sigma-Aldrich. Stearyl gallate, octyl gallate, butyl gallate, ethyl gallate, lauryl gallate, methyl gallate, and propyl gallate were obtained commercially from the Tokyo Chemical Industry Co., Ltd, and nisin was purchased from MP Biochemicals. SYTO 9 green fluorescent nucleic acid stain was purchased from Thermo Fischer Scientific. Propidium iodide and calcofluor white were obtained from Sigma-Aldrich.

3.2.2. Biofilm assay

Biofilm assays were performed as described by Djordievic et al. (2002) with slight modifications. Briefly, a single colony of the overnight plate culture was propagated in 5 ml of Tryptic Soy Broth (TSB) with 0.6% of yeast extract (TSBYE) overnight at 32^{o}C with shaking at 200 rpm. The overnight culture of *L. monocytogenes* was diluted with fresh TSB media to 1:100 ratio, and predetermined concentrations of nisin and phenolics were added. Two hundred microliter of culture with antimicrobials was applied to each well in a flat bottomed 96 well plate. The plate was incubated at 32^{o}C for 48 h without agitation. After incubation, the plate was emptied of its liquid and washed with sterilized water at least three times and dried for at least 45 min. Each well was stained with 250 µl of 1% crystal violet for 15 min for biofilm quantification, then washed for three times with sterilized water and dried for another 45 min. For de-staining,

250 µl of 95% ethanol was added to each well for 15 min, and then extracted to a new 96-well plate for absorbance reading at OD_{600} (FLUOstar® Omega, BMG LabTech). This experiment was repeated three times. The quantitative data were analyzed using paired *t*-test with Prism 7 software (GraphPad Software, Inc) to compare the levels of biofilm formation between the control (i.e., untreated samples) and the samples treated with nisin and phenolic compounds, or between the samples treated with nisin or phenolic compounds alone and those treated with combinations of nisin and phenolic compounds.

3.2.3. Fluorescent microscopy

Small glass slides were sterilized with 100% ethanol for 30 seconds before using. Three hundred microliters of the 1:100 dilution of an overnight culture with fresh TSBYE supplemented with 5 µg/ml of naringenin, 10 IU/ml of nisin, and in combination were vortexed for homogeneity and added to each well of a 12-well plate harboring a glass slide. The plate was incubated for 24 h at 32ºC. After removing the supernatant through pipetting, the glass slides were washed 3 times with sterilized water. Each glass slide was submerged in 500 µl of 4% formaldehyde for 30 min before being washed 3 times with sterilized water. Glass slides were stained with 3 µl of SYTO 9 (5 mM solution in dimethyl sulfoxide), 3 µl of propidium iodide (≥94.0%) and a drop of (~50 µl) calcofluor white (composed of 1 g/L of Calcofluor White M2R and 0.5 g/L of Evans blue) for 20 min before washing with water at least 2 times. Stained glass slides were observed with a Series- Nr: 3527001327 Carl Zeiss Microscopy GmbH microscope equipped with Axio Imager.A2 (based in Oberkochen, Germany). Live imaging and 2.5 D rendition of the slides were presented in three colors. Green indicated staining by SYTO 9, red

represented staining by propidium iodide, and blue showed staining of exopolysaccharides by calcofluor white.

3.3. Results

3.3.1. Anti-biofilm activity of phenolic compounds and nisin in *L. monocytogenes*

In this study, anti-biofilm activity of phenolic compounds was examined by measuring the level of biofilm production of *L. monocytogenes* ATCC 19115 in the presence of 22 different phenolic compounds consisting of 16 phenolic acids (i.e., benzoic acid, butyl gallate, caffeic acid, *t*-cinnamic acid, *p*-coumaric acid, ethyl gallate, gallic acid, lauryl gallate, methyl gallate, octyl gallate, propyl gallate, salicyclic acid, sinapic acid, stearyl gallate, syringic acid, and vanillic acid), seven flavonoids (i.e., catechin, chrysin, hesperidin, morin, naringenin, quercetin, and taxifolin), and BHT. For the screening purposes, phenolic compounds were used in a biofilm assay at a fixed concentration (10 μg/ml) that was significantly lower than the MICs of the phenolic compounds measured in Chapter 2 (Table 1). *L. monocytogenes* did not form robust biofilms for the first 24 h (data not shown), and noticeable biofilm formation was observed after 48 h of incubation (Fig. 4). Interestingly, some phenolic compounds significantly inhibited biofilm formation in *L. monocytogenes*. These phenolic compounds included naringenin, *t*-cinnamic acid, octyl gallate, butyl gallate, lauryl gallate, propyl gallate, quercetin, and chrysin (Fig. 4). In particular, butyl gallate, propyl gallate, octyl gallate and lauryl gallate exhibited strong anti-biofilm activity against *L. monocytogenes* ATCC 19115 (Fig. 4).

Anti-biofilm activity of nisin was also determined. Nisin strongly inhibited biofilm formation in *L. monocytogenes* ATCC 19115 in a concentration-dependent manner (Fig. 5). Although the MIC and the MBC of nisin was 250 IU/ml and 1000 IU/ml in *L. monocytogenes*

ATCC 19115, respectively, as demonstrated in Chapter 2 (Table 1), the anti-biofilm activity of nisin was observed at concentrations as low as 8 IU/ml, which is 30-fold lower than the MIC of nisin (Fig. 5).

3.3.2. Concentration-dependent anti-biofilm activity of phenolic compounds

Based on the results of anti-biofilm testing with phenolic compounds (Fig. 4), five phenolic compounds were selected for further characterization. Even though tannic acid, quercetin and chrysin also inhibited biofilm production (Fig. 4), they were not included in the follow-up testing due to their tendency to form pigmented sedimentation after incubation at high concentrations. Anti-biofilm activity increased when *L. monocytogenes* was exposed to increased concentrations of phenolic compounds. Lauryl gallate and octyl gallate significantly reduced biofilm formation at concentrations as low as 2 µg/ml and 4 µg/ml, respectively (Fig. 6 C and D). Butyl gallate, propyl gallate and naringenin required higher concentrations to inhibit biofilm production compared to lauryl gallate and octyl gallate (Fig. 6).

3.3.3. Enhanced anti-biofilm activity of nisin and phenolic combinations against *L. monocytogenes* ATCC 19115

Phenolics were combined with nisin to determine if the combinations could generate synergistic anti-biofilm effects against *L. monocytogenes* ATCC 19115. Each combined treatment condition was compared with nisin or phenolic alone. The amount of biofilm in each well was quantified by staining with crystal violate and measurement of absorbance at OD_{600}. Combined treatment was deemed synergistic/enhanced, when there was less biofilm production in comparison with the treatment with nisin or phenolics alone. Butyl gallate, lauryl gallate, octyl

gallate, propyl gallate and naringenin demonstrated enhanced anti-biofilm activity when combined with nisin (Fig. 7 and 8). Octyl gallate and lauryl gallate had strong anti-biofilm activity even without nisin (Fig. 7).

3.3.4. Anti-biofilm activity of nisin-phenolic combinations in food isolates of *L. monocytogenes*

After examining the anti-biofilm activity of nisin-phenolic combinations in *L. monocytogenes* ATCC 19115, the anti-biofilm effects were investigated in different *L. monocytogenes* isolates from food. Unlike ATCC 19115, *L. monocytogenes* food isolates (FS-1 1/2a, FS-11 1/2b, and FS-15 1/2b) did not form biofilms in TSB but produced biofilms at increased nutrient concentrations (2xTSB). Compared to the anti-biofilm activity of naringenin and propyl gallate alone, combinations of nisin with naringenin or propyl gallate significantly reduced biofilm production in all tested strains at concentrations of 10 μg/ml and above (Fig. 9). Octyl gallate and lauryl gallate were very potent anti-biofilm compounds against all the food isolates, and the addition of nisin further increased the anti-biofilm activities of lauryl gallate and octyl gallate (Fig. 10). There was a strain-dependent variation in the level of biofilm reduction by nisin-phenolic combinations. FS-1 1/2a and FS-15 1/2b showed similar patterns in biofilm reduction by treatment with nisin and phenolic combinations (Fig. 9 and 10). Biofilm formation in FS-11 1/2b was significantly reduced only when nisin was combined with naringenin at 5, 10 and 20 μg/ml, propyl gallate and lauryl gallate at 10 μg/ml, and lauryl gallate at 5 μg/ml (Fig. 9).

3.3.5. Fluorescent microscopic observation of biofilms in *L. monocytogenes*

The structure of biofilms was observed with fluorescent microscopy. SYTO 9 stains nucleic acids to highlight bacteria. Propidium iodide stains nucleic acid that is not protected by

cell membrane and therefore highlights cells with compromised cell membrane. Calcoflour white stains exopolysaccharide to verify the presence of biofilm. The biofilm structure in the presence of 5 μg/ml of naringenin was similar to that of the biofilm without any treatment. Both biofilm samples contained large and concentrated groups of cells with intact and compromised cell membrane (Fig. 11 A, green and red), whereas nisin treated sample showed scattered microcolonies (Fig. 11 A, green and red). The calcofluor white staining also showed that the exopolysaccharide portion was concentrated in the control biofilm and the biofilm treated with naringenin (Fig. 11 A, blue). The calcofluor white staining in the nisin-treated samples showed reduced biofilm formation and left only microcolonies on the surface (Fig. 11 A, blue). Combined treatment of nisin and naringenin had similar distribution patterns of scattered microcolonies as the sample treated only with nisin (Fig. 11 A and B, blue).

3.4. Discussion

L. monocytogenes forms homogenous bacteria layers, ball-shaped microcolonies, and a network of knitted chains composed of elongated cells (Rieu, et al., 2008). In our study, only microcolonies were detected in nisin-treated *Listeria* biofilms (Fig. 11 A). Due to the antimicrobial activity of nisin, low concentrations of nisin were used in the biofilm assay. Although the MIC of nisin was 250 IU/ml in *L. monocytogenes* ATCC 19115, biofilm formation was inhibited by nisin at concentrations as low as 8 IU/ml (Fig. 5), indicating that biofilm inhibition by nisin is not caused by the antimicrobial activity of nisin. In addition, bacterial viability in the supernatant of the biofilm assay was also measured, and the results showed that the viability of *L. monocytogenes* was not affected by nisin at the concentrations used in the

assay (Approximately 6×10^{11} CFU/ml was measured both in treated and control samples after incubation).

Some phenolic compounds inhibited biofilm production in *L. monocytogenes* with and without nisin (Fig. 6). Specifically, octyl gallate and lauryl gallate are FDA-approved food antioxidants (U.S. Food and Drug Administration, 2014) and strongly inhibited the biofilm formation of *L. monocytogenes* in this study. Compared to non-treated control and the sample treated with 5 μg/ml naringenin, nisin at 10 IU/ml delayed the enlargement and maturation of biofilms (Fig. 11). When nisin and naringenin were combined, exopolysaccharide production was inhibited in a similar fashion as nisin alone (Fig. 11, blue). Thus, it is possible that phenolic compounds may enhance the anti-biofilm activity of nisin. Further studies are required to determine the mechanism for the synergistic activity of nisin and phenolic compounds

Naringenin is abundant in citrus fruits (Felgines, et al., 2000). The antimicrobial activities of naringenin have also been reported in different pathogens. The antimicrobial activity of naringenin extracted from citrus fruit by-products were reported to inhibit *Lactococcus lactis* at 250 μg/ml (Mandalari, et al., 2007). In addition, naringenin also affected biofilm formation and virulence in *Escherichia coli* O157:H7, *Vibrio harveyi*, and *Pseudomonas aeruginosa* PAO1 by affecting autoinducer-mediated cell-cell signaling (Vikram, Jayaprakasha, Jesudhasan, Pillai, & Patil, 2010; Vandeputte, et al., 2011). Since *L. monocytogenes* possesses quorum sensing genes, such as *agrD* and *luxS* that are associated with biofilm formation (Riedel, et al., 2009; Sela, Frank, Belausov, & Pinto, 2006), it would be an interesting future study to determine if naringenin could affect biofilm formation by influencing quorum sensing in *L. monocytogenes*.

Although *L. monocytogenes* ATCC 19115 effectively produced biofilms in TSB, the food isolates did not form biofilms effectively when grown in TSB; however, increased nutrient

concentrations facilitated biofilm development in the food isolates (data not shown). It has been reported in several studies that nutrient levels affect biofilm formation in *L. monocytogenes*. Folsom et al. reported similar differential effects of nutrients on biofilm formation in *L. monocytogenes* (Folsom, Siragusa, & Frank, 2006). Some strains produced more biofilm at high nutrient concentrations than low nutrient concentrations (e.g., a 10-fold diluted TSB), while other strains are more likely to form biofilms in diluted TSB (Folsom, Siragusa, & Frank, 2006). One study tested 30 strains of *L. monocytogenes*, and 4b serotype isolates accumulated less biofilm after growing in diluted TSB (Field, et al., 2015). In a study of Harvey et al. (2007), testing biofilm formation in 138 *L. monocytogenes* strains from various sources (animals, human and food) showed strain variations in biofilm formation and reported biofilm production for serotype 4b was higher in TSB compared to diluted TSB. In this study, nevertheless, 2X TSB allowed the food isolates to produce biofilms in the experimental conditions used.

Few studies have reported the anti-biofilm activity of nisin in *L. monocytogenes* and other Gram-positive bacteria, such as MRSA and *Staphylococcus pseudintermedius* (Bolocan, et al., 2016; Minei, Gomes, Ratti, D'Angelis, & de Martinis, 2008; Okuda, et al., 2013; Field, et al., 2015). In this study, we observed effective anti-biofilm activity of nisin against *L. monocytogenes* and the augmentation of anti-biofilm activity of nisin in combination with phenolic compounds. This is the first study to investigate anti-biofilm activity of nisin in combination with phenolic compounds in *Listeria*. Nisin is an antimicrobial peptide that is allowed for use as a food preservative, and some natural phenolic compounds used in this study are approved by the FDA as food additives. The findings in this study can provide a new approach to the control of *L. monocytogenes* biofilm. The biofilm formation of *L. monocytogenes* on the surface of foods (Carmichael, et al., 1998) and food processing equipment (Piercey,

Hingston, & Truelstrup, 2016) is a serious problem in food safety. Thus, the development of anti-biofilm formula using food-grade materials can potentially contribute to the control of *L. monocytogenes* biofilm formation in food related environment.

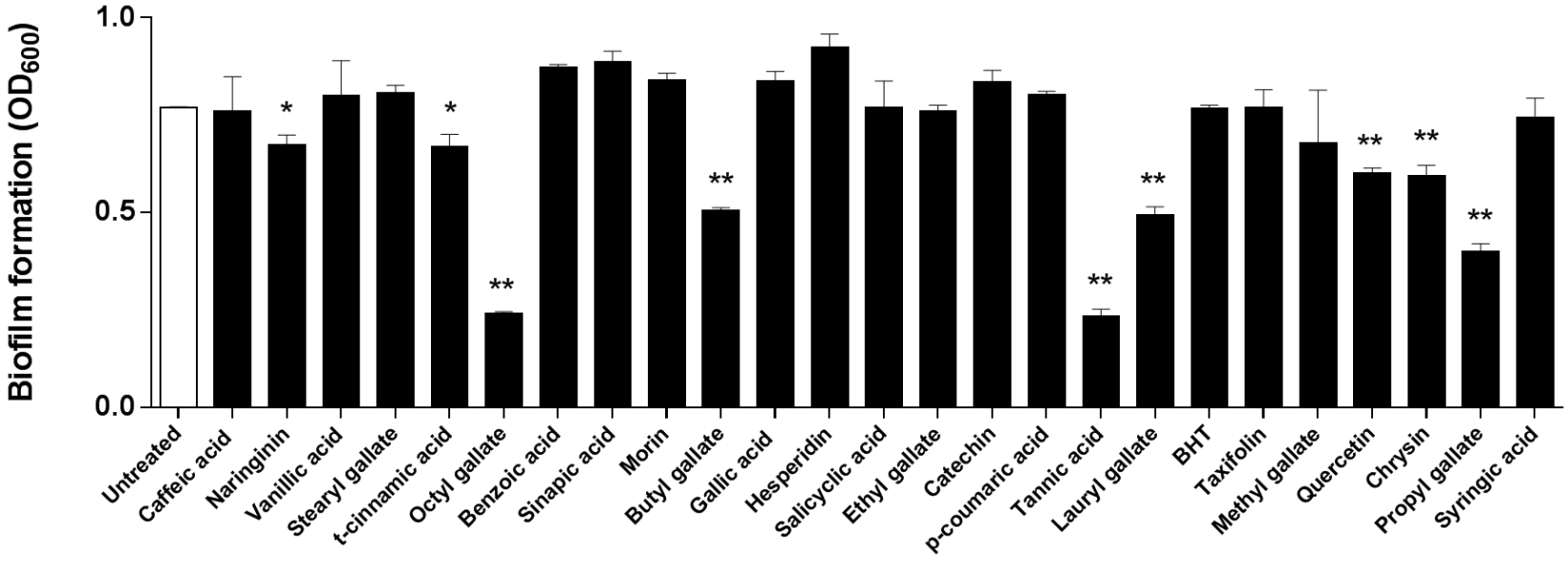


Figure 4: Inhibition of biofilm formation in *L. monocytogenes* by phenolic compounds at 10 µg/ml. The levels of biofilm were measured after 48 h incubation. The results show the mean and standard deviations of triplicate samples. Statistical significance was obtained by doing a paired *t*-test in comparison with the untreated sample and marked with * ($P \leq 0.05$) or ** ($P \leq 0.001$).

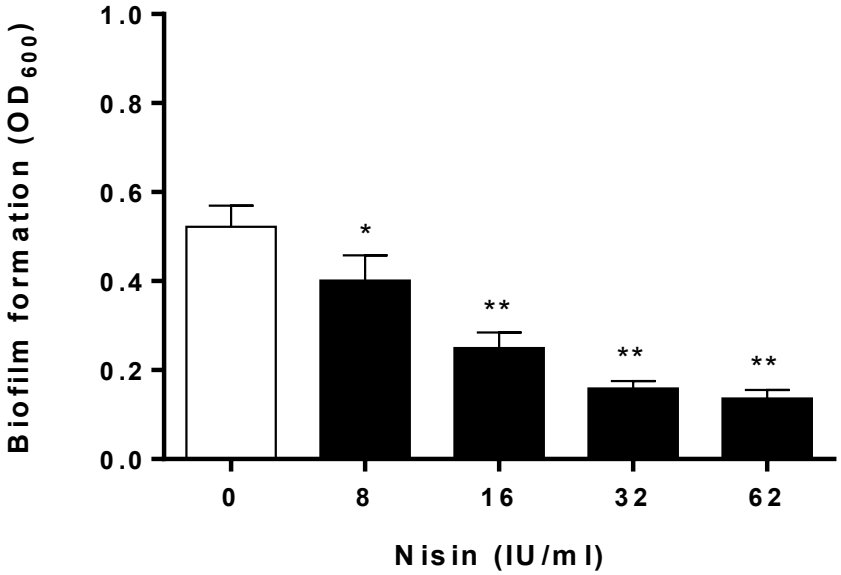


Figure 4: Anti-biofilm activity of nisin in *L. monocytogenes*. The results show biofilm production after 48 h treatment with nisin at different concentrations. Statistical significance (*: $P \leq 0.05$ and **: $P \leq 0.001$) was calculated with a paired *t*-test compared with the untreated sample.

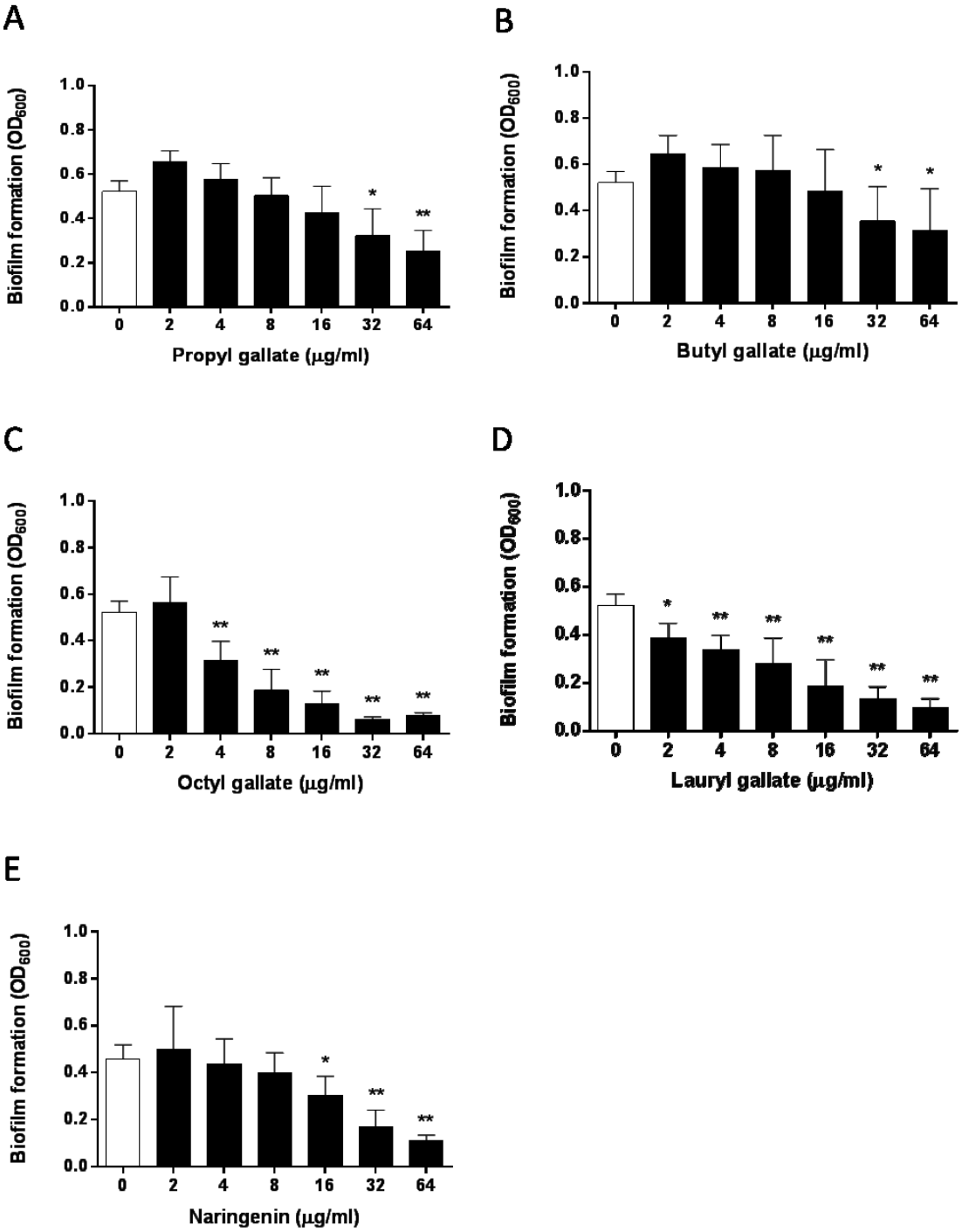


Figure 5: Dose-dependent inhibition of biofilm formation in *L. monocytogenes* ATCC 19115 by phenolic compounds, including propyl gallate (A), butyl gallate (B), octyl gallate (C), lauryl gallate (D), and naringenin (E). The results show the means and standard deviations of triplicate samples. Statistical significance was calculated with a paired *t*-test compared with the untreated sample. *: $P \leq 0.05$ and ** $P \leq 0.001$.

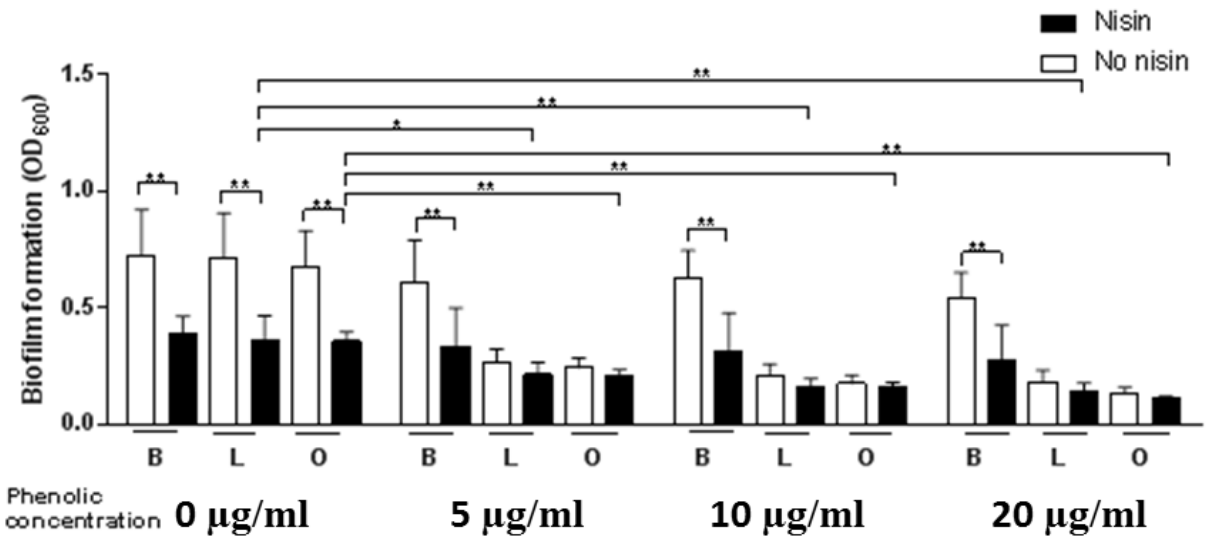


Figure 6: Enhanced antibiofilm activity of nisin against *L. mononcytogenes* ATCC 19115 in combination with phenolic compounds, including butyl gallate (B), lauryl gallate (L), and octyl gallate (O). The concentrations (0, 5, 10, 20 µg/ml) of phenolic compounds are indicated at bottom, and 10 IU/ml nisin was used in the experiment. The biofilms were incubated for 48 h. The results show the means and standard deviations of triplicate samples. * ($P \leq 0.05$) and ** ($P \leq 0.001$) indicates the difference was statistically significant basted on a paired *t*-test.

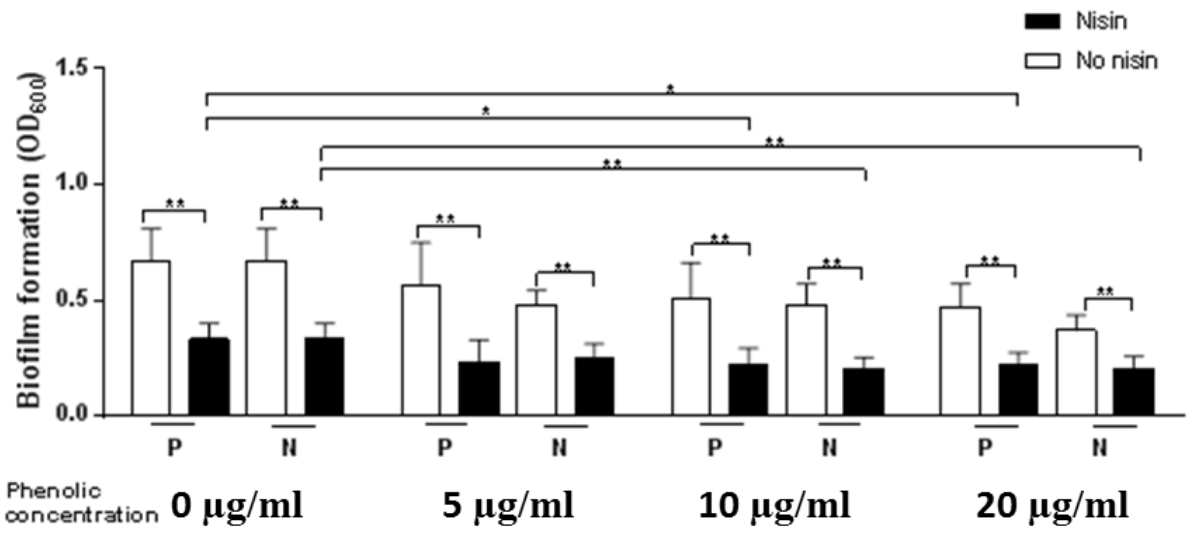


Figure 7: Enhanced antibiofilm activity of nisin against *L. mononcytogenes* ATCC 19115 in combination with phenolic compounds, including propyl gallate (P), and naringenin (N). The concentrations (0, 5, 10, 20 µg/ml) of phenolic compounds are indicated at bottom, and 10 IU/ml nisin was used in the experiment. The biofilms were incubated for 48 h. The results show the means and standard deviations of triplicate samples. * ($P \leq 0.05$) and ** ($P \leq 0.001$) indicate the difference was statistically significant basted on a paired *t*-test.

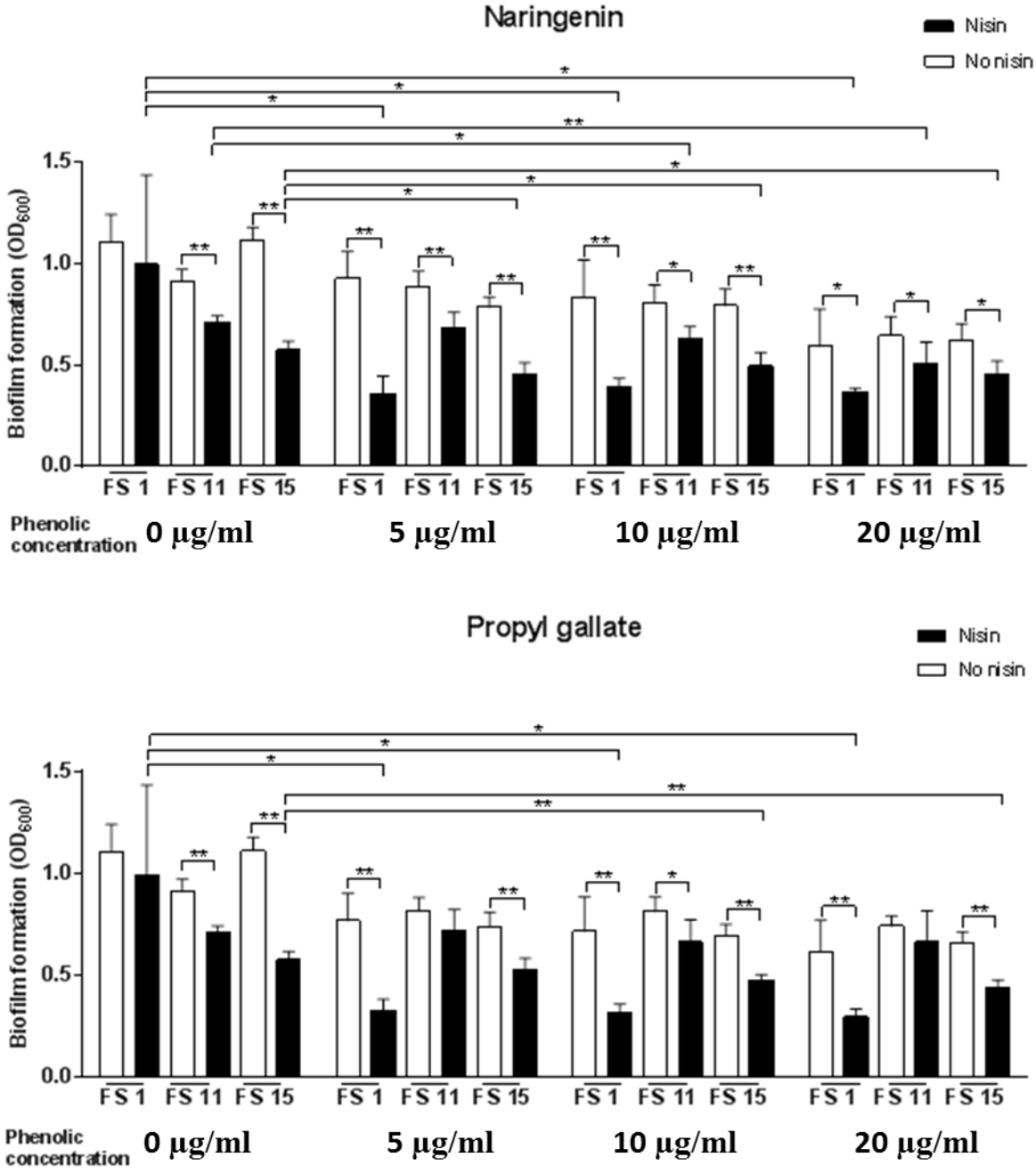


Figure 8: Enhanced antibiofilm activity of nisin and phenolic compound combinations against *L. monocytogenes* food isolates FS-1 1/2a, FS-11 1/2b, and FS-15 1/2b. The 10 IU/ml nisin was mixed with naringenin and propyl gallate at 0, 5, 10, and 20 µg/ml for the experiment. The results show the levels (means and standard deviations) of biofilm formation after 48 h incubation in 2xTSB. * ($P \leq 0.05$) and ** ($P \leq 0.001$) indicate biofilm inhibition after treatment was statistically significant basted on a paired *t*-test.

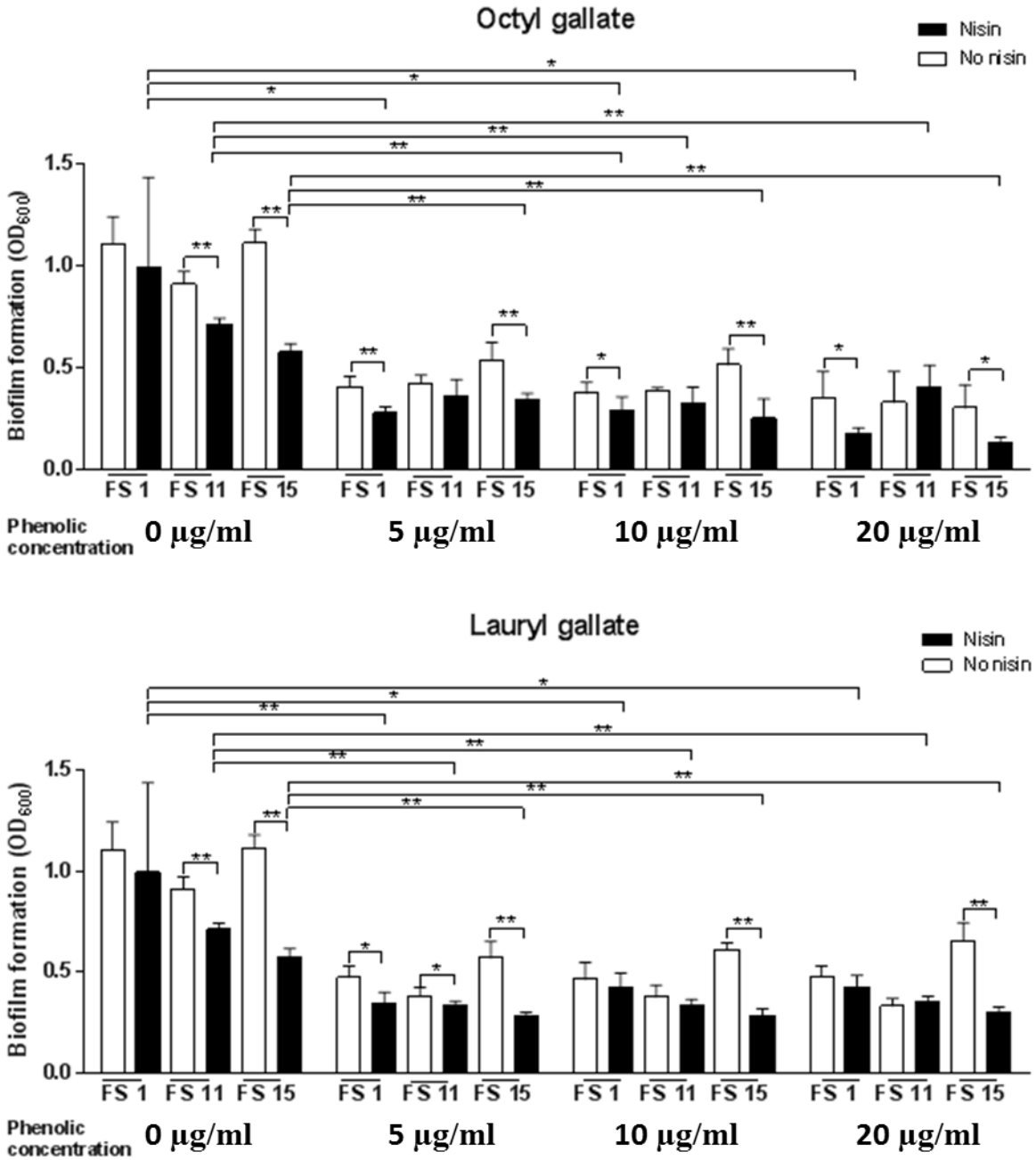


Figure 9: Enhanced antibiofilm activity of nisin and phenolic compound combinations against *L. monocytogenes* food isolates FS-1 1/2a, FS-11 1/2b, and FS-15 1/2b. The 10 IU/ml nisin was mixed with octyl gallate and lauryl gallate at 0, 5, 10, and 20 µg/ml for the experiment. The results show the levels (means and standard deviations) of biofilm formation after 48 h incubation in 2xTSB. * ($P \leq 0.05$) and ** ($P \leq 0.001$) indicate biofilm inhibition after treatment was statistically significant basted on a paired *t*-test.

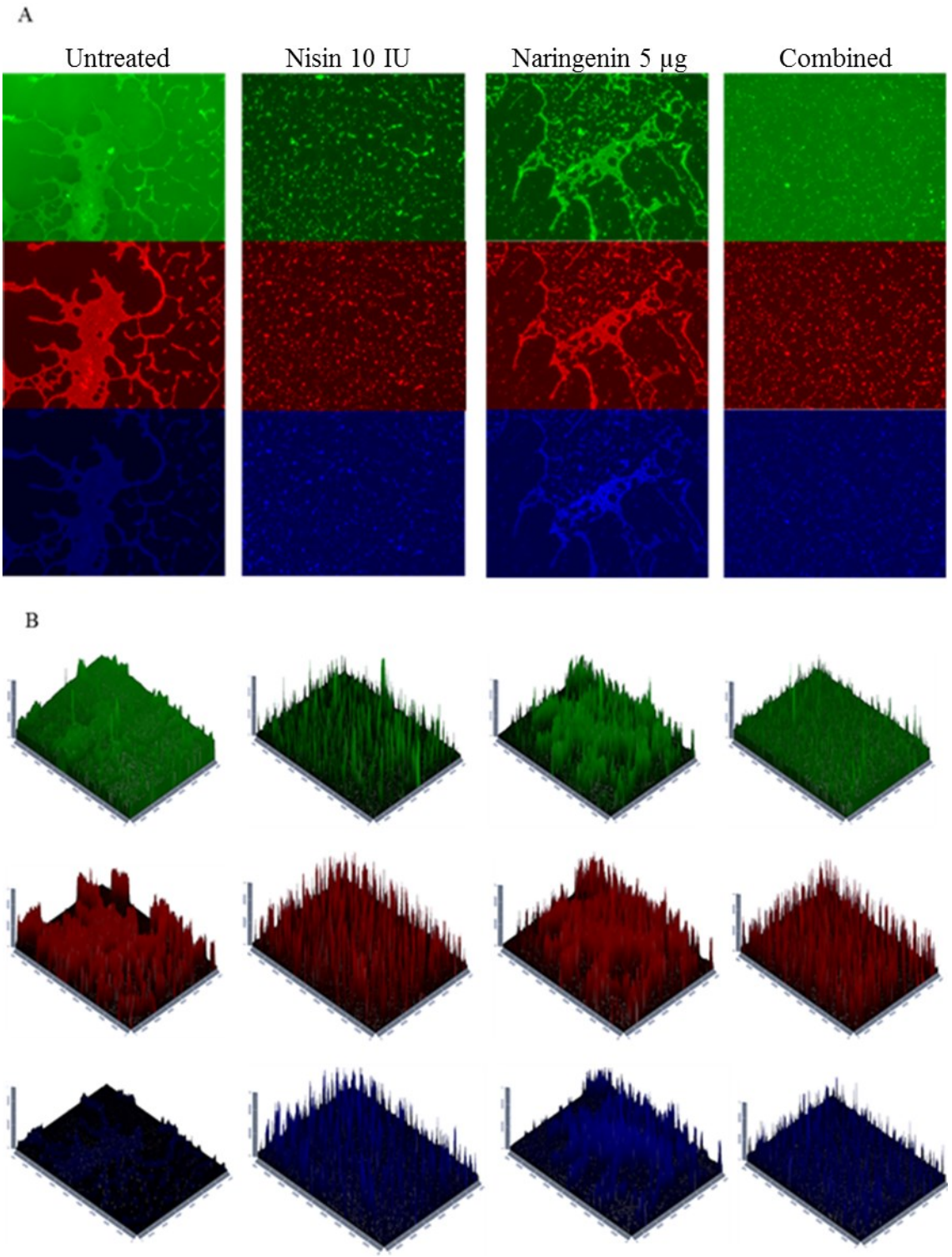


Figure 10: Fluorescent microscopy images of biofilms formed by *L. monocytogenes* ATCC 19115 (A) and biofilm images after the 2.5D rendition (B). Images of the biofilm were presented with staining of the dye SYTO 9 (green), propidium iodide (red) and calcofluor white (blue).

CHAPTER 4: GENERAL DISCUSSION

4.1 Significant Findings

The significant findings of this research were the following:

1. Butyl gallate, lauryl gallate, octyl gallate, propyl gallate, naringenin and quercetin can inhibit the growth of *L. monocytogenes* at low concentrations (256 µg/ml or less) in comparison to other phenolic compounds tested in this thesis.
2. Butyl gallate and nisin have synergistic inhibitory activity against the food isolates of *L. monocytogenes* tested in this thesis.
3. Naringenin, t-cinnamic acid, octyl gallate, butyl gallate, lauryl gallate, propyl gallate, quercetin, and chrysin at concentration as low as 10 µg/ml, or nisin at concentration as low as 8 IU/ml (30-fold lower than the MIC) can reduce the biofilm production of *L. monocytogenes* ATCC 19115 after 48 hours of incubation in TSB.
4. Butyl gallate, lauryl gallate, octyl gallate, propyl gallate, and naringenin at various concentrations (5, 10 and 20 µg/ml) demonstrated enhanced anti-biofilm activity when combined with 10 IU/ml nisin against *L. monocytogenes* ATCC 19115 in TSB.
5. Naringenin, propyl gallate, octyl gallate and lauryl gallate at various phenolic compounds in combination with nisin at 10 IU/ml demonstrated enhanced anti-biofilm activity against *L. monocytogenes* food isolates in 2XTSB.

Relevance, application, impact of these findings will be discussed in the following sections. The future research focus of these findings will be addressed at the end of the chapter.

4.2 Implication of the Significant Findings

Listeriosis is a major public health concern in North America. As mentioned before, it exhibits high mortality rates and is implicated in a significant proportion of foodborne deaths in North America. Despite all the food safety measures available, large listeriosis outbreaks have still occurred. Incidence of listeriosis outbreaks are as recent as 2016 in Canada (packaged salad products produced at the Dole processing facility in Springfield, Ohio), and very recently in 2017 in the US (a multistate outbreak of listeriosis linked to soft raw milk cheese made by Vulto creamery) (Public Health Agency of Canada, 2016; Centers for Disease Control and Prevention, 2017). These recent outbreaks suggest that current food safety methods are not sufficient in preventing listeriosis. Therefore, there is a need for developing novel methods to reduce the risk of listeriosis in minimally processed and RTE foods without altering their sensory qualities.

A better control of *Listeria* contamination will alleviate the economic burdens associated with listeriosis. According to Thomas et al. (2015), the economic cost of a *L. monocytogenes* outbreak in 2008 (57 cases of listeriosis and 24 deaths linked to delicatessen meat from an Ontario RTE meat processing plant) was estimated to be $242 million Canadian dollars (including medical costs, nonmedical costs, productivity costs and those incurred by the implicated plant and federal agencies responding to the outbreak). Considering the potential economic cost of a single large outbreak, it is cost effective to invest in preventative measures. According to an estimate derived from published economic analysis of different methods (i.e. willingness to pay, cost of illness, cost function and event study), the estimated annual benefit and costs of food safety measures to control *L. monocytogenes* in the US range from $2.3 billion to $22 billion and $0.01 billion to $2.4 billion US dollars, respectively (Ivanek , Gröhn, Tauer, & Wiedman, 2005). Therefore, federal institutions encourage the food industry to invest heavily in

preventative measures to reduce listeriosis. However, additional safety measures can result in increased retail price, and that is a barrier to the implementation of new safety measures in the food industry. Hence the significant findings of this study can enhance the effectiveness of current methods instead of replacing them. This could ultimately reduce the cost of *L. monocytogenes* outbreaks by lowering the scale of outbreaks, or preventing outbreaks from occurring.

The hypothesis of this project was that there would be phenolic compounds/antioxidants other than thymol and carvacrol that also generate synergistic anti-listerial activity. Finding new synergistic combinations between nisin and phenolic compounds/antioxidants can significantly reduce the amount of time and costs for discovering new anti-listerial compounds and testing their toxicity. The findings from this thesis support this hypothesis, and phenolic compounds/antioxidants, to which *L. monocytogenes* is sensitive, generated additive and synergistic antimicrobial and anti-biofilm activities in combination with nisin.

4.2.1 Phenolic compounds and nisin as food additives

Data presented in this thesis highlighted the potential of phenolic compounds as adjuvant to enhance the anti-listeria activity of nisin. The screening data demonstrated that gallic acid derivatives have strong inhibitory activity against *L. monocytogenes* ATCC 19115, and octyl gallate exhibited significantly lower levels of MIC (32 µg/ml) and MBC (64 µg/ml) than the other phenolic compounds screened (Table 1). The MIC range of octyl gallate in this study is similar to a previous study reported by Mejia et al. (2013) (octyl gallate MIC for *L. monocytogenes* LNSP 031212 was 40 µg/ml). In the same study, higher percentage of inhibition was achieved against *L. monocytogenes* LNSP 031212 than *E. coli* NRRLB 14128 when octyl

gallate concentration was greater or equal to 16 μg/ml Mejia et al. (2013). According to a previous study from our laboratory, the MIC and MBC of octyl gallate in MRSA USA300 were 32 μg/ml and 64 μg/ml, respectively (Kim & Jeon, 2016). Octyl gallate appears to be more effective against Gram-positive bacteria than Gram-negative bacteria, presumably the lack of an outer cell membrane could be a major factor limiting bacterial sensitivity to octyl gallate.

The combination of nisin and butyl gallate generated synergistic inhibitory activity against *L. monocytogenes*. It is interesting that Kim and Jeon (2016) also observed synergistic bactericidal effect against MRSA USA300 when bacitracin (at concentration as low as 1 μg/ml) was combined with octyl gallate, butyl gallate, methyl gallate, ethyl gallate, propyl gallate, stearyl gallate or dodecyl gallate. According to the report, octyl gallate significantly increased the antimicrobial activity of bacitracin against MRSA USA300. It is unclear why only butyl gallate generated synergistic inhibitory activity against *L. monocytogenes* ATCC 19115. This may be explained by the differences in bacteria physiology between *L. monocytogenes* and *S. aureus*. Furthermore, it is also possible that alkyl gallates generate antimicrobial synergism against Gram-positive bacteria through different mechanisms when combined with either nisin or bacitracin. The mechanism of observed growth inhibition synergism can also be explained by the membrane destabilization and enzyme inhibition activity of butyl gallate. According to a study, phenolic products such as alkyl gallates, phenolic acids and alkyl esters can destabilize bacterial cell membranes, increase the membrane permeability, and affect the function of some enzymes (Borges, Ferreira, Saavedra, & Simoes, 2013). The mode of action of nisin involves: i) binding to the lipid II in the cell membrane to inhibit cell wall biosynthesis, ii) lysis of cell wall, and iii) formation of nisin-lipid II complexes that assemble into stable transmembrane pores and lead to increased permeability. This may consequently cause cell death due to the dissipation of

membrane potential and the release of small cytoplasmic molecules (Punyauppa-path, 2015). Butyl gallate may enhance the activity of nisin by destabilizing the membrane stability and allow for easier integration of nisin into cell membrane. It is also possible that the transmembrane pores formed by nisin may provide butyl gallate with easier access to its target site within the cell. Further studies involving permeability and cytoplasm enzymatic activity are required to determine the mechanisms of the synergistic antimicrobial activity of butyl gallate and nisin against *L. monocytogenes*.

The first limitation of this project is the number of phenolic compounds tested. Based on the result of this project, it appears that the *L. monocytogenes* growth inhibitory activity of synthetic phenolic compounds/antioxidants was more effective than natural phenolic compounds. However, it is difficult to make such assumption without testing more phenolic compounds and more *L. monocytogenes* strains. The second limitation is the lack of testing in various food matrices. As mentioned earlier, increased amount of nisin is needed to test antimicrobial activity in food than the laboratory growth medium. The types of food to investigate should include dairy products, preserved meat, and frozen vegetables. Nevertheless, this is the first report on the synergistic growth inhibitory activity of butyl gallate and nisin against *L. monocytogenes*.

4.2.2. Enhanced anti-biofilm activity of nisin and phenolic compounds

The results of the biofilm study showed that nisin and phenolic compounds by themselves reduced biofilm production in *L. monocytogenes*. When combined, phenolic compounds can enhance the anti-biofilm activity of nisin. The screening data revealed 10 μg/ml of tannic acid, naringenin, t-cinnamic acid, octyl gallate, butyl gallate, lauryl gallate, propyl gallate, quercetin, and chrysin reduced biofilm production in *L. monocytogenes* after 48 h of

incubation. Interestingly, it was lauryl gallate and octyl gallate that significantly reduced biofilm production by *L. monocytogenes* ATCC 19115 at concentrations as low as 2 µg/ml and 4 µg/ml, respectively. According to a study of Sivaranjani et al. (2016), morin inhibited *L. monocytogenes* biofilm production at concentrations as low as 6.25 µg/ml in Modified Welshimer Broth (a minimally define growth media); however, morin did not affect biofilm formation in *L. monocytogenes* in this study. The discrepancy may be ascribed to the different experimental setting, such as different growth media and incubation time for biofilm development.

Interestingly, nisin inhibited *L. monocytogenes* from producing biofilms at low concentrations, such as 8 IU/ml. There have been no reports showing the anti-biofilm activity of nisin in *L. monocytogenes* in a concentration-dependent manner, whereas it has been reported that nisin reduces the biofilm formation of MRSA and *Streptococcus mutans* (Shin, et al., 2016). Therefore, it is possible that nisin may interfere with biofilm formation commonly in Gram-positive bacteria. However, validation of the hypothesis awaits further studies. Lauryl gallate, octyl gallate, propyl gallate and naringenin synergistically enhanced the anti-biofilm activity of nisin in *L. monocytogenes*. To the best of my knowledge, there is no literature available that report similar observations.

The fluorescent microscopy imaging also confirmed the anti-biofilm activity of nisin in *L. monocytogenes* even at low concentrations. In my study, *L. monocytogenes* ATCC 19115 culture that was treated by 10 IU/ml of nisin developed small microcolonies. Similar observation of delayed biofilm development was reported by another research group when *L. monocytogenes*162 (isolated from a smoked salmon factory) and *L. monocytogenes*162R (nisin-resistant variant of the same strain) were treated with nisin and B3A-B3B enterocin (bacteriocin produced by *Enterococcus faecalis* B3A-B3B) at MIC (Al-Seraih, et al., 2017). Therefore, it is

possible nisin, B3A-B3B enterocin and similar bacteriocins are capable of reducing *L. monocytogenes*' biofilm development through similar mechanism. Interestingly, a similar result was also reported by Sivaranjani et al. (2016) when the *L. monocytogenes* culture was treated with 25 µg/ml of morin. According to the same study, morin also reduced the motility of *L. monocytogenes* in a concentration dependent manner (the swarming and swimming capability of *L. monocytogenes* decreased as morin concentration increased). This is interesting because Lemon et al. (2007) observed that non-motile mutants of *L. monocytogenes* were defective in biofilm formation. Hence, it is possible that morin and other phenolic compounds inhibit *L. monocytogenes* biofilm develop through different mechanism than bacteriocin.

A comprehensive study on the determinants required for biofilm formation in *L. monocytogenes* reported that deletion of the *dltABCD* operon rendered *L. monocytogenes* defective in biofilm formation (Alonso, 2014). The *dltABCD* operon is involved in the incorporation of D-alanine into lipoteichoic acids, a major constituent of gram-positive bacteria cell wall. Furthermore, reducing the amount of extracellular amino acids, such as D-alanine, can influence the ability of bacterial attachment to hydrophobic surfaces (i.e. polyvinyl chloride) due to the alteration in surface charge, and the thickness and rigidity of the cell wall (Alonso, 2014). Thus, it is possible that the synergistic anti-biofilm activity of combinations of nisin and phenolic compounds reduces biofilm formation by interfering with the cell wall synthesis. Furthermore, it is also possible that phenolic compounds may destabilize the cell membrane and surface structures anchored to the membrane as well. Since cell-to-cell interactions are important in the establishment of biofilms, the altered membrane integrity may affect biofilm formation. Further investigation is required to validate these hypotheses.

The second limitation of this project is the lack of different surfaces which biofilm can form upon. Food processing environments contain different surfaces such as stainless steel, plastic, ceramic, and glass. Lastly, because formaldehyde was added to fix the biofilm before staining with SYTO 9, propidium iodide and calcofluor white, the result does not show how effective nisin and phenolic compounds can kill bacteria inside of biofilm. Therefore, future experimental design should stain the biofilm before formaldehyde is used to fix the microbial community.

4.3 Future Research Consideration

Unlike its derivatives, gallic acid itself had no growth inhibitory activity against *L. monocytogenes* in this thesis. Therefore, future research should focus on the antimicrobial property of various gallic acid derivatives. Future study should determine the effects of alkyl chains on the anti-listerial activity. Merkl et al. (2010) showed that *Escherichia* is less sensitive to methyl ester and ethyl ester than Gram-positive bacteria, such as *Listeria* (Merkl, HrádkoVá, Filip, & Smidrkal, 2010). Therefore, future studies can investigate the impact of alkyl chain modification in gallic acid derivatives on the synergistic antimicrobial activity of nisin against *L. monocytogenes*.

The antimicrobial activity of nisin and butyl gallate combinations should be investigated using food matrices. The type of foods to be investigated should include those that are frequently associated with listeriosis in humans (i.e. processed cheese, and deli meats). Furthermore, it would be interesting to examine the growth of *L. monocytogenes* on a piece of sliced deli meat or sliced soft cheese treated with nisin and butyl gallate. If pretreatment of food with nisin and butyl gallate during production may prevent *L. monocytogenes* growth, the risk of listeriosis will be

significantly reduced. Lastly, future studies should involve different food preservation methods (i.e. refrigeration temperature, acidic/alkaline condition, and salt) to test the anti-listerial effect of nisin and butyl gallate combinations. The addition of another antimicrobial condition may further enhance the bacteriostatic/bactericidal effect of nisin and butyl gallate (as seen in Fig 3).

The results about the biofilm study present the potential of nisin and phenolic compounds to prevent the biofilm development of *L. monocytogenes*. The molecular mechanism for the anti-biofilm activity in *L. monocytogenes* has not been understood. Furthermore, it is also unclear how phenolic compounds/antioxidants enhance the anti-biofilm activity of nisin. However, according to a comparative proteomic analysis of *L. monocytogenes* ATCC 7644, exposure to a sub-lethal concentration of nisin increases the expression of proteins associated with oxidative stress response, enzymes related to the production of membrane lipids (this indicates a failure in conventional mechanisms of cell division), and flagellar and motility proteins (this indicates increased bacterial motility) (Miyamoto, et al., 2015). Therefore, it is unlikely that nisin reduces biofilm development through motility changes in *L. monocytogenes*. Future study may investigate other factors associated with biofilm formation in *L. monocytogenes* (e.g., adherence and exopolysaccharide production). It is important to determine whether nisin and phenolic compounds can prevent the adhesion of *L. monocytogenes* to a surface or prevent biofilm development after adhesion. The results from such experiments could determine whether it is more effective to apply nisin/phenolic compound combinations after sanitation (removal of biofilm) or disinfection (killing of bacteria). Lastly, it would be interesting to see if nisin/phenolic compound combinations can remove mature biofilm community more effectively than conventional sanitizers (e.g., quaternary ammonium compounds and acetic acid).

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