MOLECULAR CHARACTERIZATION OF BIOFILM PROPERTIES OF *Listeria monocytogenes* IN THE PRESENCE OF *o*-COUMARIC ACID AND 4-HYDROXYBENZOIC ACID

A Thesis Submitted to
The Graduate School of Engineering and Sciences of
Izmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Molecular Biology and Genetics

by
Umut Ekin

AUGUST 2013
İZMİR

We approve the thesis of **Umut EKİN**

Examining Committee Members:

Assist. Prof. Dr. Ferda SOYER DÖNMEZ
Department of Molecular Biology and Genetics,
Izmir Institute of Technology

Assoc. Prof. Dr. Devrim PESEN OKVUR
Department of Molecular Biology and Genetics,
Izmir Institute of Technology

Assoc. Prof. Dr. Gülşah ŞANLI MOHAMED
Department of Chemistry,
Izmir Institute of Technology

23 August 2013

Assist. Prof. Dr. Ferda SOYER DÖNMEZ
Supervisor,
Department of Molecular Biology and Genetics,
Izmir Institute of Technology

Assoc. Prof. Dr. İkbal Agah İNCE
Co-Advisor,
Department of Biosystem Engineering,
Giresun University

Assoc. Prof. Dr. Ahmet KOÇ
Head of the Department of
Molecular Biology and Genetics

Prof. Dr. Tuğrul SENGER
Dean of the Graduate School of
Engineering and Sciences

ACKNOWLEDGEMENT

First and foremost I would like to thank to my supervisor Assist. Prof. Dr. Ferda SOYER for trust in me to study in her laboratory. She always encouraged me to overcome difficulties with sharing her experience and never interfered my thoughts to be implemented. I will always remember her patience, guidance and support.

I wish to thank Assoc. Prof Dr. Devrim PESEN OKVUR and Assoc. Prof. Dr. Gülşah ŞANLI MOHAMED for accepting to be my committee member. They were more than generous with their expertise, advice and precious time.

I would like to address special thanks to my co-advisor Assoc. Prof. Dr. İkbal Agah İNCE for his support and guidence throughout this research. Without his help, the study may not be concluded at this level.

I want to thank pretty girls of our laboratory Deniz KEMAN and Özgün Öykü ÖZDEMİR for their friendship and sharing thier experiences. I wish to thank Ender Efe CERİT, crazy man , for being my fellow during 3 years. He did not ignore me when I need help and I picked up many knowledge from him during my study. I am also very grateful to Şeyda DEMİR for her patience, advices and support to resist the difficulties during the study. She did not let me fall.

I would like to thank Dane RUSÇUKLU for her patience, help and coffees in BIOMER. She did not let me alone when needed any help.

I am deeply and forever indebted to my parents for their unwavering love and encouragement throughout my entire life. They are responsible for who I am today with their moral and finantial support.

ABSTRACT

MOLECULAR CHARACTERIZATION OF BIOFILM PROPERTIES OF *Listeria monocytogenes* IN THE PRESENCE OF *o*-COUMARIC ACID AND 4-HYDROXYBENZOIC ACID

Biofilms in medical and food industries are difficult to deal with. The formation of biofilms on every type of surfaces, ability to withstand in biofilm structures against thousand folds of lethal antibiotic doses and reasoning for different kinds of chronic diseases prove dangerousness of biofilms. Food borne pathogens that survive in biofilms can contaminate foods and feeding tubes which results food poisonings, outbreaks and infections. *Listeria monocytogenes* EGDe is one of the dangerous food borne pathogens with ability to form biofilms and cause listeriosis among elderly people, immunocompromised patients or pregnant women, which results lethal consequences.

In this study, 4-hydroxybenzoic acid and *o*-coumaric acid that have potential antimicrobial and antibiofilm effect on *Listeria monocytogenes* EGDe were examined. Furthermore, protein profiles were investigated in the presence of phenolic acids. The antimicrobial assays of selected phenolic acids revealed the MICs against *Listeria monocytogenes* EGDe which are 18 mM for 4-hydroxybenzoic acid and 10 mM for *o*-coumaric acid. In the presence of MICs, while *o*-coumaric acid reduced initial attachment at 58%, 4-hydroxybenzoic acid reduced 48%. 4-hydroxybenzoic acid and *o*-coumaric acid also showed inhibitory effects on preformed biofilms as 34% and 56% respectively. The inhibitory effects of selected phenolic acids were ensured by visualization with SEM and Flourescence Microscopy. Afterwards, *Listeria monocytogenes* EGDe was treated with MIC of phenolic acids for total protein isolation. Isolated proteins were subjected to in-gel digestion and analysed in nanoLC-ESI-MS/MS system to interpret the mode of actions of antimicrobial and antibiofilming properties

ÖZET

Listeria monocytogenes'in *o*-KUMARİK ASİT VE 4-HİDROKSİBENZOİK ASİT VARLIĞINDA BİOFİLM ÖZELLİĞİNİN MOLEKÜLER KARAKTERİZASYONU

Medikal ve gıda endüstirisinde bulunan biyofilmlerle başa çıkmak oldukça zordur. Birçok yüzeyde biofilm oluşabilmesi, öldürücü antibiyotik dozunun bin katına biyofilm yapılarının içinde direnebilme kabiliyetleri ve birçok kronik hastalığa sebep olmaları biyofilmlerin tehlikesini kanıtlamaktadır. Biyofilmlerin içerisinde hayatta kalabilen gıda patojenleri gıdalara ve beslenme tüplerine bulaşarak gıda zehirlenmelerine, salgınlara ve enfeksiyonlara sebep olmaktadır. *Listeria monocytogenes* EGDe biyofilm oluşturma yeteneğine sahip tehlikeli gıda patojenlerinden biridir ve yaşlılarda, immune sistemi düşük hastalarda ve hamile kadınlarda listerioza sebep olarak ölümcül sonuçlar doğurabilir.

Bu çalışmada potansiyel antimikrobiyal ve antibiyofilm etkiye sahip 4-hidroksibenzoik asit ve *o*-kumarik asitin *Listeria monocytogenes* EGDe üzerindeki etkileri incelenmiştir. Daha sonrasında, fenolik asitlerin varlığında protein profilleri incelenmiştir. Seçilen fenolik asitlerin *Listeria monocytogenes* EGDe üzerindeki antimikrobiyal tahlilleri 4-hidroksibenzoik asitin MİK değerini 18 mM, *o*-kumarik asitinkini ise 10 mM olarak açığa çıkarmıştır. MİK değerlerinin varlığında, , *o*-kumarik asit öncül tutunmayı %58 oranında azaltırken, 4-hidroksibenzoik asit %48 oranında azaltmıştır. 4-hidroksibenzoik asit ve *o*-kumarik asitin önceden oluşmuş biyofilmler üzerinde sırasıyla %34 ve %56 azaltıcı etki gösterdiği saptanmıştır. Fenolik asitlerin azaltıcı etkileri taramalı elektron mikroskobu ve floresan mikroskobu ile görsellenerek kanıtlanmıştır. *Listeria monocytogenes* EGDe fenolik asitlerin MİK değerlerine maruz bırakılarak total proteinleri izole edilmiştir. İzole edilen proteinler jel içi sindirime maruz bırakmış, antimikrobiyal ve antibiyofilm özelliklerinin etki mekanizmalarını tahmin etmek için nanoLC-ESI-MS/MS sisteminde analiz edilmiştir.

This thesis dedicated to my family who never leave me alone against difficulties of life

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table **Page**

LIST OF ABBREVIATIONS

APS	Amonium Persulfate
BHI	Brain Hearth Infusion
BCA	Bicinchoninic Acid
B. cereus	*Bacillus cereus*
CDC	Center for Disease Control and Prevention
Cfu	Colony Forming Unit
cv	Crystal Violet
˚C	Degree Celsius
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substances
ESI	Electrospray Ionization
E.coli	*Escherichia coli*
g	Gram
g	Gravity
h	Hour
IBAQ	Intensity based absolute quantification
l	Liter
L. ivanovii	*Listeria ivanovii*
L.monocytogenes	*Listeria monocytogenes*
LC	Liquid Chromatography
MALDI	Matrix-assisted laser desorption/ionization
MIC	Minimum Inhibitory Concentration
Min	Minute
µg	Microgram
µl	Microliter
mg	Miligram
ml	Mililiter
mM	Milimolar

M	Molar
MS	Mass Spectrometry
nm	Nanometer
OD	Optical Density
pH	Power of Hydrogen
P. Aeruginosa	*Pseudomonas aeruginosa*
rpm	Revolutions Per Minute
SEM	Scanning Electron Microscopy
SDS	Sodium Dodecyl Sulfate
S. aureus	*Staphylococcus aureus*
S.mutans	*Streptococcus mutans*
TEM	Transmission Electron Microscopy
TOF	Time of Flight
U.S	United States
V	Volt
w/v	Weight per volume
4-HBA	4-Hydroxybenzoic Acid

CHAPTER 1

LITERATURE REVIEW

1.1. Foodborne Pathogenic Bacteria & Outbreaks

Consumption of contaminated food causes serious economic and health problems. According to Centers for Disease Control and Prevention (CDC), food-borne pathogens cause 76 million cases each year in U.S. Among these cases, 325.000 patients needed medical care and 5.000 of these people lost their life. The cases of food-borne pathogens mainly related with *Campylobacter*, *E. coli O157:H7, Listeria* and *Salmonella* in U.S. Although most common infections in U.S were caused by *Campylobacter* and *Salmonella*, the severity and fatality rates proves how dangerous *Listeria* is (Mead et al., 1999). *L. monocytogenes* can be found almost every food especially raw ones such as: meat, fish, poultry, vegetables and milk products like soft cheeses and ice creams. *Listeria* has two ways of infection which are non-invasive and invasive. In non-invasive way, it causes gastroenteritis with symptoms as headache, abdominal pain and fever. In invasive infection called Listeriosis, *L. monocytogenes* reaches blood or cerebrospinal fluid that cause septicemia (blood poisoning) or meningitis. Listeriosis mainly diagnosed among pregnant women, elderly people, newborns or immunocompromised people. Although, precautions decreased the incidences, listeriosis still a serious risk for human health. The estimations in U.S showed that anually 3500 people infected by this pathogen and approximately 600 of them die (Ramaswamy et al. 2007). Outbreaks in recent years show the importance of listeriosis. In September 2011, contaminated cantaloupe infected 147 people and 33 of them died. Before 2011, 2002 outbreak which occurred because of contaminated turkey deli meat was the largest among the other outbreaks with 54 infected people, 8 deaths and 3 fetal deaths (www.CDC.gov 2013).

1.2. Listeria monocytogenes

L. monocytogenes is discovered by E. G. D. Murray, R. A. Webb, and M. B. R. Swann in 1924. They isolated it from rabbits and guinea pigs which have septicemic disease. *Listeria* genus converge 6 species which are monocytogenes, ivanovii, grayi, welshimeri, innocua and seeligeri. They are gram positive with their rod shapes. Their sizes are approximately 0.5μm wide and 1.5 μm long. The members of genus *Listeria* are facultatively anaerobic and non-spore forming. They can live nearly in every environment like soil, forage, mud, silage, water, sewage, most of raw and processed food. Among these species, only *L. monocytogenes* and *L. ivanovii* are pathogenic. While the optimum growth temperature varies between 30˚C and 37˚C, *L. monocytogenes* can grow between a wide range of temperatures between 2˚C and 45˚C. This dangerous microorganism can survive under the refrigeration temperatures as low as -7˚C. *L. monocytogenes* can also tolerate a wide range of acid stress. It can live at pH range between pH: 5.5 (which corresponds to 1 M lactic acid) and pH: 9.6. Increasing the salt concentration of foods is a method to preserve. However, the salt tolerance of *L. monocytogenes* can increase up to 30% NaCl concentrations (Gandhi and Chikindas 2007, Low and Donachie 1997). Tolerance of *L. monocytogenes* to different types of environmental conditions makes it easy to contaminate foods and make human beings unavoidable to be exposed. *L. monocytogenes* have 12 known serotypes. Among these serotypes, 1/2a, 1/2b, and 4b are the most dangerous ones with being the causative agent of 90 % of Listeriosis cases (Vazquez-Boland et al. 2001).

Listerial infections mainly divided into two forms in the host which are invasive and non-invasive. These two forms of listerial infections start with consumption of contaminated foods. In non-invasive form, *Listeria* reaches the intestines. The symptoms of gastroenteritis that are fever, abdominal pain and headache start in the patient. Listeriosis is the invasive form of listerial infection. It is clinically diagnosed when blood or cerebrospinal fluid include *Listeria*. Firstly, bacteria pass the intestines and reach the liver of the host. It begins to multiply until cell-mediated immune response. Severity of the infection increases when the host is immunocompromised or immunosupressed. In other words, severity of infection increases with weakened immunity. Afterwards, this uncontrolled multiplication in non-effective immune system causes bacteremia. Pro-longed bacteremia can cause the infection of other organs and

different lethal conclusions like abortions and septicemia as summarized in Figure 1 (Ramaswamy 2006, Vazquez-Boland et al. 2001).

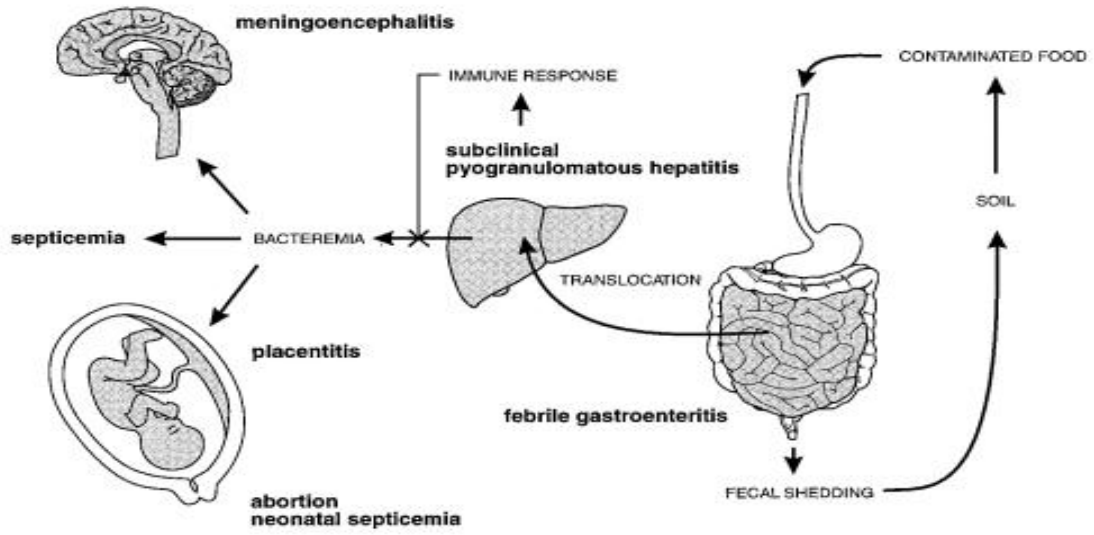

Figure 1. Pathophysiology of *Listeria* infection
(Source:Vazquez-Boland et al. 2001).

L. monocytogenes and *L. ivanovii* are pathogenic bacteria that have intracellular life cycle in phagocytic cells like macrophages and ability to enter non-phagocytic cells like endothelia cells, hepatocytes, and epithelial cells. In intracellular life cycle of *Listeria*, there are certain actors which help *Listeria* to invade the cells. The stages of invasion are summarized as in Figure 2. Firstly, invasion starts with the entry of *Listeria* to host cell by internalin protein family members A and B. Internalin A interacts with cell surface receptors (E-cadherin) of host cell. Internalin B interacts with Met so clathrin mediated endocytosis machinery is recruited and actin polymerization is triggered. Secondly, phagosomal escape of *Listeria* takes place in the host cell. LLO is key virulence factor which is member of cholesterol dependent cytotoxins that forms pores. It takes role in phagosomal maturation arrest and phagosomal lysis that allow bacteria to be released to cytoplasmic space. After replication in host cytoplasmic space, extracellular expression of ActA polymerizes actins of the host cell. Polymerized actins forms the actin tails that allow the bacteria travel in host cell and penetrate to neighbor cells (Markus Schuppler and Martin J. Loessner 2010, Javier Pizarro-Cerda 2012).

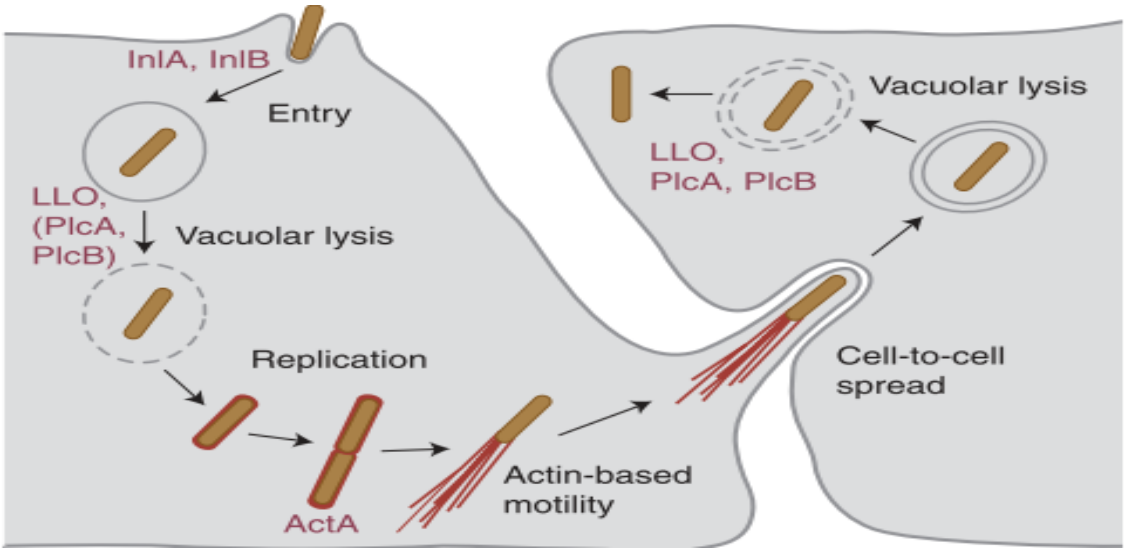

Figure 2. Intracellular cell cycle of *L. monocytogenes*.
(Source: J. Pizarro-Cerda´ et al. 2012)

1.3. Biofilms

Biofilms are complex aggregates of sessile communities attached to biotic and abiotic surfaces. In this complex aggregation, attached cells change their growth rate and gene transcription. Furthermore, they produce extracellular polymeric substances to cover cells as glue like shield. In this structure, different types of species can take part and communicate each other by quorum sensing mechanisms. This change in mode of growth has different reasons according to environmental and beneficial conditions. First of all, bacteria want to live and remain in favorable and appropriate niche. Secondly, it protects itself by forming biofilm in response to stress factors like disinfectants, antibiotics, lack of nutrients, pH changes in salty and acidic conditions. In addition to that biofilms are the reasons of most infections in host systems. From this perspective, protection from host defense systems and colonization in nutrient rich environments can be counted as the reason of biofilm formation in host systems. When change in mode of growth from planktonic to sessile form, specific genes are required in different species. In addition to that during the process of biofilm formation, different genes related with stress responses, quorum sensing, cell division, motility, adhesion, cell wall, phage and carbohydrate metabolism are up or down regulated (Donlan, R., Costerton M., William J. 2002, Kimberly K. Jefferson 2004). Besides their natural habitat like rivers, oceans, rocks and quiescent waters, biofilm formation take part problematically nearly in every

step of life. From industrial perspective, bacterial communities can be on the inner surface of pipelines that results reduction in heat transfers and corrosion of attached surfaces. In food industry, they locate themselves on meat, dairy and poultry processing areas that results food contaminations (Simoes M. et al. 2009). In addition to these, when their infectious ability combines with biofilm formation, it causes lethal or severe diseases. They form biofilms on implants, surgical devices or directly in host body. As a consequence of biofilm formation, they result chronic lung infections, middle ear infections, native valve endocarditis and ocular implant contaminations (Donlan R., Costerton M., William J. 2002). Most popular form of biofilm formation is dental plaques. These types of biofilms cause corrosions on tooth surfaces and results dental caries. (Marsh P.D. 2004).

Biofilm formation process has sequential distinct steps which begin with initial attachment of cells into target surface. The attachment of the cells determined by two main factors: 1) closeness of cells to attachment surfaces and 2) balance of attractive and repulsive forces like electrostatic, hydrophobic and van der Waals between bacteria and surface. Surface conditioning by the environment and bacterial surface structures like pili, fimbriae and flagella take role in attachment process. Attached cells produce extracellular polymeric substances that result irreversible and firm settlement to the surface. This firm and irreversible anchoring is supported by interactions between surface bound nutrients, specific receptors on pili and fimbriae, and extracellular polymeric substances which are polysaccharides, proteins, DNA and lipids. According to environmental conditions and characteristics of the species, cells show different cell growth, replication, cell-cell signaling molecules and EPS production levels. As a result of these, architecture of the biofilm constructed. This structural orientation supports transportation of signaling molecules and nutrients within the biofilm. When the biofilm structure reaches mature form, single cells disperse from the biofilm to form new ones (Michael Dunne W. 2002, Stoodley P. et al. 2002, Breyers & Ratner 2004). Although planktonic and sessile bacteria have same genetic backgrounds, this extraordinary structure of bacteria give advantages to sessile form of growth against planktonic ones. While specific doses of antibiotics kill planktonic bacteria, sessile forms in biofilm can resist up to 1000 times higher doses of antibiotics compared to planktonic form. In addition to this, biofilm structure is also advantageous for sessile community from the perspective of infectiousness. This structure of sessile community protects itself against host defense systems. Phagocytotic defense cells cannot engulf the bulk structure of

biofilms so sessile cells continue their life cycle in host system without eradication (Lewis K. 2001).

1.4. Phenolic Compounds

Plants produce different types of organic compounds which are called as primary and secondary metabolites in their life cycle. These compounds differ according to their importance and essentiality but borders of these compounds can be interchangeable in some cases. While primary metabolites take role in essential metabolic reactions like growth, development, respiration and photosynthesis, secondary metabolites take not essential but important roles such as in responses to environmental stresses, defense against invaders like pathogens and herbivores, and protection from sun based radiation (Crozier A. & Clifford M. N. 2006).

Phenolic compounds are one of the major classes of secondary metabolites in plants. Their synthesis includes pentose phosphate, shikimate and phenylpropanoid pathways (Randhir, Lin, & Shetty, 2004). Their chemical composition includes one or more aromatic ring and attached hydroxyl groups. There are thousands of phenolic compounds which sub-grouped according to their chemical structure in terms of type and number of phenol rings. More than 8000 phenolic structures take place in literature. According to the complexity of chemical structures of natural polyphenols; phenolic acids, phenylpropanoids and flavanoids can be called as simple molecules whereas lignins, melanins and tannins seen as polymerized complex compounds (Crozier A. & Clifford M.N. 2006, Bravo L. 1998). Phenolic acids are one of the subgroups of phenolic compounds. These aromatic secondary metabolites have divided into two groups that are hydroxycinnamic acids and hydroxybenzoic acids (Tripoli et al. 2005). Besides their role in plant metabolism, their significance for human health is unignorable. Their benefital effects can be exampled as anti-inflammatory, antiallergic, antimicrobial, anticarcinogenic, antiviral, antioxidant, antimutagenic and antiangiogenic (Karaosmanoglu et al. 2010, Silva F. et al. 2000, Andjelkovic et al. 2006, Chang-Seok Kong et al. 2013).

In this thesis, two candidates of phenolic acids were examined for their antimicrobial and antibiofilm properties. One candidate is the member of hydroxybenzoic acids that is 4-hydroxybenzoic acid and the other one is *o*-coumaric acid that is the member of hydroxycinnamic acids. Chemical structures of hydroxybenzoic acids and hydroxycinnamic acids are illustrated as in Figure 3.

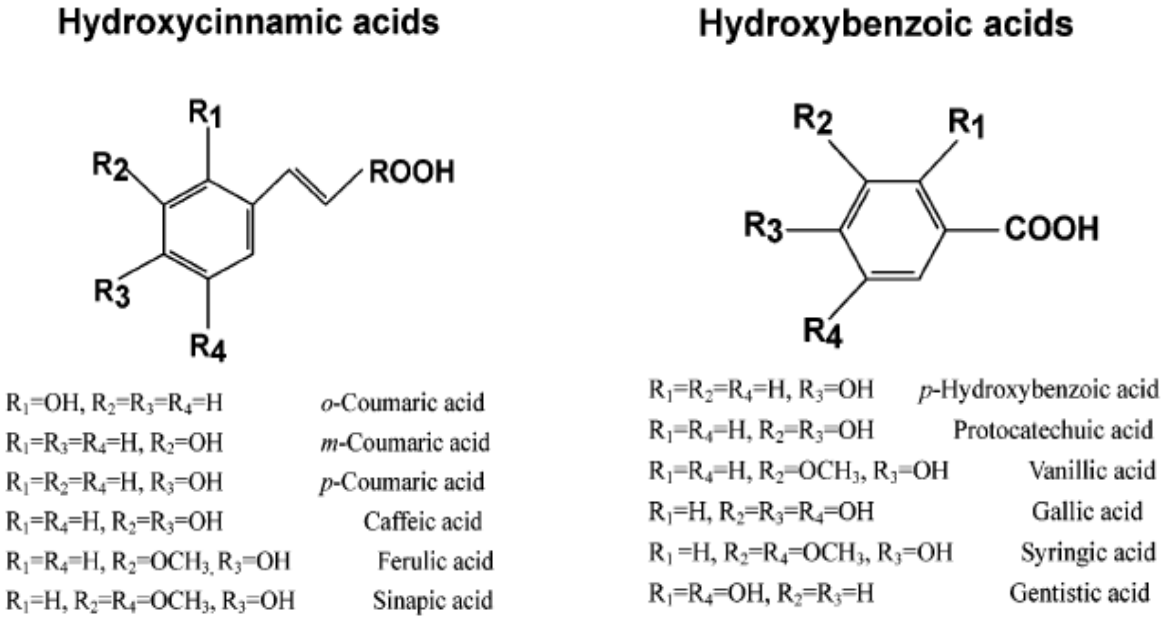

Figure 3. Chemical structures of hydroxybenzoic acids and hydroxycinnamic acids (Source: Chi-Tai Yeh and Grow-Chin Yen 2003).

1.5. Antimicrobial and Antibiofilm Properties of Plant Constituents

Antibiotic treatments are the general way of combating with bacteria. However, increase in antibiotic resistances and formation of biofilms draw the lights on plant extracts and plant compounds that have potential antibacterial and antibiofilm abilities. Plant extracts from different types of spices, fruits and folk medicinal plants were applied against bacteria (Simões M., Bennett R. N. & Rosa, E. 2009). Cranberry extracts have examined against two species of bacteria that are Staphylococcus (Gram-positive) and *E. coli* (Gram-negative). These microorganisms are capable of biofilm formation and can cause urinary tract infections. While 5 mg/mL of extract caused inhibition in Staphylococcus, the growth of *E. coli* did not show inhibition.

Furthermore, extracts showed antibiofilm effect in biofilm production but could not disrupt the formed biofilms (LaPlante K. et al. 2012). Although, the eradication of preformed biofilms is hard, different plant extracts can show inhibitory effect on different organism like *Listeria monocytogenes*. In (Sandasi M. et al. 2010), 15 different types of plants extracts from herbs, spices, beverages and medicinal plants investigated against *Listeria monocytogenes* based on their inhibitory effects on initial attachment and preformed biofilms. While 9 plant extracts could inhibit the initial attachment, just 3 of the plant extracts belong to *R. officinalis, M. piperita* and *Melaleuca alternifolia* showed over 50% inhibition on pre-formed biofilms. The antibiofilming extracts of plants can be enlarged by burdock leaf, oolong tea and puerh tea. While burdock leaf extracts shows antibiofilm effects on *Escherichia coli* by different ethanol elution fractions, oolong tea and puerh tea extracts inhibited the attachment of *S. mutans* (Lou Z. 2013, Wang Y. 2013). Besides their preservative and therapeutic properties, essential oils have also inhibitory effects on growth and biofilm formation of different bacteria. As an example, cassia oil, clove oil, lavender oil, peru balsam oil, red thyme oil and tea tree oil examined against *Pseudomonas aeruginosa*, *Pseudomonas putida* , *and Staphylococcus aureus*. They also compared the antibiofilm effect of antibiotics that are ofloxacin, colistin and gentamicin against the essential oils. They realized that essential oils are more effective than antibiotics that they used. Interestingly, while red thyme did not kill the planktonic cells, it showed significant antibiofilming ability (Kavanaugh N. L. & Ribbeck K. 2012). In addition to that different essential oils like thyme and oregano showed antimicrobial effects on *E. coli* and some *Salmonella* species. The reason for the inhibition considered as the presence of phenolic compounds carvacrol and thymol. Individually these phenolic compounds also showed inhibitory effects on famous pathogen bacteria: *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Edris A. E. 2007). The effects of plant extracts and essential oils take its source mainly from their components like monoterpens, flavonoids, tannins and phenolic compositions (Edris A. E. 2007, Sandasi M. et al. 2008, Karaosmanoğlu et al. 2010, Bubonja-Sonje et al. 2011 Wang Y. et al. 2013) . In the study of Annapoorani (2012), induction by phenolic and flavanoid compounds like rosmarinic acid, naringin , chlorogenic acid and mangiferin tested on the cell growth of *S. marcescens* and quorum sensing mechanisms. Different percentages of cell growth inhibitions have observed like 57% in the presence of rosmarinic acid. In another study, pathogens related with respiratory diseases are targeted by wine phenolic compounds and oenological phenolic extracts. The

antimicrobial effects of flavanoids and phenolics showed significant differences according to bacteria. In addition to that phenolic acids like gallic acid and caffeic acid showed more activity than flavanoids catechin and epicatechin (Cueva C. et al. 2012). Phenolic and natural phenolic compounds are tested on *P. aeruginosa* for both antimicrobial and antibiofilming properties. According to data, there is no notible decrease in bacterial growth but significant inhibitions were examined in biofilm formations. In terms of chemical structures, phenol, polyphenol and tannic acid showed over 69% inhibition (S. Jagani et al 2009).

Consequently, different plant extracts include different compositions and different amounts of phytochemicals. The antimicrobial applications of the phytochemicals on different bacteria change according to some variables. These variables are type of bacteria (gram positive or negative), chemical structure of phytochemicals and concentrations. These parameters significantly change the effects and different types of mode of actions occur in different antimicrobial applications. Most of the phytochemical compound targets the membrane of bacteria and affects the membrane permeability. Furthermore, inhibition of efflux pump and interference with DNA and RNA synthesis covers general mode of actions. Specifically, flavanoids inhibit the DNA gyrase activity, isoflavone changes the cell morphology like filament cells and coumarins reduce cell respiration. (Simões M., Bennett R. N. & Rosa, E. 2009, Paiva P. M. G., Gomes F. S. , Napoleão T. H. 2010). Besides these, they have quorum sensing inhibitory effect that results inhibition of biofilm and virulence factors. In *P. aeruginosa*, plant extracts of South Florida medicinal plants interfere with LasA, LasB, and pyoverdin that are virulence factors (Adonizio et al. 2008).

In this study, two antimicrobial phenolic acids: 4-hydroxybenzoic acid (4-HBA) and *o*-coumaric acid that present in Turkish extra virgin olive oils were investigated for their antimicrobial and antibiofilm properties against *Listeria monocytogenes* EGDe. The antimicrobial properties of 4-hydroxybenzoic acid were investigated against 19 different microorganism and 4-HBA showed different inhibitory effects on their growth (Jeong-Yong Cho et al 1998). *o*-Coumaric acid that also present in plant products, have antimicrobial properties (Karaosmanoglu et al. 2010). Controverserily, Dorantes L (2000) investigated that serrano chilli pepper extract include *o*-coumaric, *m*-coumaric and *p*-coumaric acids. They also applied these acids on *B. cereus, S. aureus, S. yphimurium* and *L. monocytogenes* for their potential antimicrobial properties. While *p*-

coumaric and *m*-coumaric acids showed inhibitory efects on selected bacteria, *o*-coumaric could not. It should be due to concentrations

1.6. Proteomic Insights

Consequently, the effects of plant constituents on bacteria cannot be ignored. Revealed modes of action need to be proved by proteomic researches to understand protein interactions and mechanisms. In 2010, Shu-min Yi and colleagues demonstrated the alterations of tea polyphenols on *P. Aeruginosa*. They also supported their findings with TEM analysis. Their findings showed that these polyphenols change the integrity of outer membrane and inner membranes that results leakage of cell components. They continued their study by focusing on the membrane proteins. They found 27 differentially expressed protein according to 2D gel analysis. However, they could only identify 13 of them as a result of MALDI-TOF/TOF MS analysis and database search. Among these proteins, dihdrollpoamide dehydrogenase (LPD), succinyl Co-A synthetase beta subunit, biotin caboxyl carrier protein (BCCP), elongation factor Ts (EF-Ts), 50s ribosomal protein, single-stranded DNA-binding protein (SSBP), glycine cleavage system protein T2 and polyamine transport protein were significantly changed. In the study of Longhi C. (2008), they investigated the characteristics of *Listeria monocytogenes* in the presence of serratiopeptidase (SPEP). They realized that there is significant inhibition in invade of host cells and biofilm formation. Subsequently, comparative SDS analyses were performed for treated and untreated surface proteins. Proteins were identified by MALDI-MS. Their investigations showed that proteases degraded some adhesion family members which helps attachment to environments and host cells. From the perspective of biofilm formation, we know that although the whole populations of cells have same genetic background, they show different physiological properties. As a result of these, not only the surface proteins but also intracellular proteins should be considered in terms of understanding the biofilm formation. The reason is that attached bacteria show significant different protein profile against planktonic cells. The comparison of proteomes of sessile and planktonic cells of *Listeria monocytogenes* proves the differences (Tremoulet F. et al. 2002, Hefford M.A. et al. 2005)

CHAPTER 2

INTRODUCTION

Biofilm formation is complex aggregation of bacteria on biotic or abiotic surfaces that modulated by different intracellular and extracellular signaling mechanisms. Although all cells are genetically identical, significant physiological changes occur during transition from planktonic mode of growth to sessile (Costerton R., Donlan J. 2002). This structure is considered as the reason of many chronic and persistent diseases. This structure helps sessile growing cells become less susceptible to 10-1000 fold lethal doses of antibiotics which kill planktonic counterparts (Davies D. 2003). *Listeria monocytogenes* is one of the foodborne pathogens that can form biofilm. *Listeria monocytogenes* can attach and form biofilms on all the materials used in the food industry (Møretrø T. 2004). The consumption of contaminated foods by *Listeria monocytogenes* can cause listeriosis with lethal consequences among elder people, immunocompromised patients and pregnant women (Liu D. 2006, Lewis K. 2001).

Plant extracts, essential oils and phenolic compounds attract the attention of scientist because of their potential antimicrobial and antibiofilm characters. They are also considered as new antimicrobial agents. Phenolic acids are also plant originated secondary metabolites that have the same potential. The studies about phenolic acids started with basic antimicrobial test and nowadays reached to investigation of action mechanisms (Paiva P. M. G. et al. 2010, Borges A. 2013). However, there are still many dark points waiting to be revealed in terms of their mode of actions.

The intension of this study is the investigation of antimicrobial and antibiofilm properties of 4-hydroxybenzoic acid and *o*-coumaric acid on *Listeria monocytogenes* EGDe in proteomic point of view. Recent proteomic studies mostly cover the differences between planktonic and sessile growth modes. This study will be first by revealing key proteomic changes in the presence of phenolic stress. Beside this, these changes will give clues about antimicrobial and antibiofilming properties of phenolic acids at the same time. These findings help to reveal the insight of listerial attachment and biofilm formation. After determination of MIC values for both phenolic acids respectively, antibiofilming properties were determined under same concentrations. In

the presence of phenolic acids, Listeria showed reduction in attachment and biofilm formation. The reductions in cell densities also ensured by SEM and fluorescence microscopy techniques. The cells that subjected to phenolic acid stresses were investigated by nanoLC-ESI-MS/MS and bioinformatics toolkits. Alterations in proteomes were compared for different phenolic acids

CHAPTER 3

MATERIALS AND METHODS

3.1. Bacterial Strain and Culture Conditions

L. monocytogenes EGDe was purchased from Istanbul Tıp Fakültesi Mikroorganizma Kültür Kolleksiyonları Merkezi (KUKENS). Stock cultures were prepared in Brain Heart Infusion Broth (Fluka) with 20% Glycerol (Sigma). They were aliquoted into cryo tubes and stored at -80°C (Thermo). For experimental use, stock cultures were inoculated into soft BHI agars. After sufficient growth, they streak onto BHI agar plates. They were kept at +4°C and refreshed every 2 weeks for stable viability.

3.2. Antimicrobial Activity Assays of Phenolic Acids

3.2.1. Preparation of Bacterial Culture

A single colony of *L. monocytogenes* EGDe was inoculated into 4 ml BHI medium. The tube was vortexed than incubated overnight at 37°C (Nüve) without shaking. The optical density of overnight culture was measured by spectrophotometry at 600 nm (Thermo Multiscan Spectra Reader). Overnight culture adjusted to 0.19 OD with fresh BHI medium by the equation $C_1 \times V_1 = C_2 \times V_2$. The adjusted inoculum was serially diluted with 1/10 ratio to 10^{-6}. Plate count method used for bacterial load determination by plating 10^{-5} and 10^{-6} dilutions. It was confirmed that adjusted inoculum (0.19 OD) has 10^8 cfu/ml bacterial load.

3.2.2. Preparation of Phenolic Acids

o-Coumaric acid and 4-hydroxybenzoic acid (Sigma) were dissolved in DMSO (Amresco). The percentage (5%) of the DMSO in preparations was determined according to 2 parameters which are: max solubility of phenolic content and minimum effect on bacterial growth. After molarity calculations of phenolic acids, they were dissolved in 5% DMSO of total volume (250 µl DMSO). Then, BHI (4750 µl) was added gradually upto final volume and vortexed.

3.2.3. 96-Well Microtiter Plate Assay

Antimicrobial activity of phenolic acids on *L. monocytogenes* EGDe was determined as described in Karaosmanoglu et al. (2010). After serial dilution, 10^{-4} dilution tube that had a concentration of $1x10^4$ cfu/ml was used in the experiment. Ninety-six well plate with flat bottom (BD 351172) was loaded with different molarity ranges of phenolic acids and their controls. Hundred microliters from 10^{-4} dilution tube was loaded to 96 well plate wells. Then, 100 µl of phenolic acid solution was loaded with respect to its molarity so initial molarities were halved when loaded to wells. As blank, 100 µl of phenolic acid solutions and 100 µl of BHI were loaded for each concentration of phenolic acids. As negative control, 100 µl BHI and 100 µl bacteria, 100 µl BHI with 5% DMSO and 100 µl bacteria were loaded. All the wells were arranged as triplicate in the experiment. Optical densities of the wells in the plates were determined by Thermo Multiscan Spectra Reader at 600 nm. Measurements were repeated in every 3 hours up to 24 hours. Plate was incubated at 37°C without shaking during experiment. Experiments were repeated 3 times.

3.3. Antibiofilm Assays of Phenolic Acids

3.3.1. Bacterial Culture Preparations for Antibiofilm Assays

The same procedure was followed as described in antimicrobial activity assay until plate load. After serial dilution, 10^{-2} dilution tube that has a bacterial load of $1x10^6$

cfu/ml was used to load. In order to confirm the initial adjusted bacterial load as 10^8 cfu/ml, 100 µl of 10^{-5} and 10^{-6} dilution tubes were plated respectively.

3.3.2. Phenolic Acid Preparation for Antibiofilm Assays

o-Coumaric acid and 4-hydroxybenzoic acid were dissolved in DMSO as previously described in antimicrobial activity assay. As a result of antimicrobial activity assays, minimum inhibitory concentrations (MIC) of phenolic acids: 18 mM for 4-hydroxybenzoic acid and 10 mM for *o*-coumaric acid were used in antibiofilm assays by considering the final concentrations in the wells.

3.3.3. Microtiter Plate Biofilm Assays

Two different assays have been carried out for determination of inhibition in initial attachment and inhibition of preformed biofilm. Two assays are nearly similar except the addition time of phenolic compounds and aeration of the plates. Bacterial cultures were prepared as described in section 3.2.1. In each well, 100 µl from 1x 10^6 cfu/ml culture and 100 µl BHI was loaded as triplicate. In order to see the inhibitory effect of phenolic acids on pre-formed biofilms, 96 well plate was incubated at 37°C for 4 hours without shaking and sealed with parafilm to induce attachment of the cells. After that, 100 µl of phenolic acid solutions at MIC concentrations were added onto bacterial culture so every total volume in the wells reached to 200 µl. Then, the plate was incubated at 37°C for additional 20 hours without shaking and sealing. In initial attachment assay, phenolic acids were added immediately to wells with bacteria and incubated at 37°C without sealing and shaking. For both assays, when the incubation completed, supernatants of the wells removed. Plates were submerged in distilled water and vigorously shaked to remove planktonic and loosely attached bacteria. This washing step was repeated 3 times and plates were air dried for 45 minutes. It was followed by oven dry at 60°C for 45 minutes. In order to stain the attached cells, 100 µl of 0.1% crystal violet (cv) solution was added to wells and incubated at room temperature for 15 minutes. Crystal violet solution was removed and plates were washed 3 times with distilled water. Plates were inverted on paper towels and taped on

to remove excess water and left for air dry. When plates were completely dry, 125 µl ethanol solution (96%) was added and destained for 10 minutes. Hundred microliters of ethanol-cv solution from wells were transferred to a new 96 well plate. New plate was read by Thermo Multiscan Spectra Reader at 590 nm.

3.4. Microscopy Analysis for Antibiofilm Assays

For microscopic analysis, plates were prepared as described in antibiofilm assays. After incubations, supernatants were removed by topping on the plates. Plates were washed for removing planktonic and loosely attached bacteria by shaking the plate in water. The plates were washed 3 times and plate was air dried for 45 minutes.

3.4.1. Scanning Electron Microscopy

Antibiofilm assays of 0 hour and 4 hour treatments were visualized by scanning electron microscopy technique. After air dry, the wells were cut out by the help of radio antenna. Tube shaped antenna was heated and pinned through the wells. Bottom of the wells were snatched out. Circular cut samples were golden coated and visualized by Quanta 250 FEG-SEM.

3.4.2. Flourescence Microscopy

Inhibitory effects of phenolic acids of 0 hour and 4 hour treatments were visualized by fluorescence microscopy (Olympus) technique. Air dried plates loaded with 100 µl of 1% acridine orange solution. Plates were incubated at room temperature in the dark for 10 minutes. Excess amount of solution from the wells were pipetted out and wells were rinsed with 1% PBS. Plates were visualized under 20X magnification for 1 second exposure.

3.5. Protein Isolation from Unattached Cells in Phenolic Acid Stress

3.5.1. Bacterial Culture Preparation

As previously described, bacterial culture preparation was carried out in same way upto serial dilution step. After serial dilution, 1.5 ml of $1x10^7$ cfu/ml and 13.5 ml of fresh BHI were transferred to non treated tissue culture flasks (BD 353133) in order to reach final bacterial load as $1x10^6$ that upscaled to mimic the conditions of attachment assay.

3.5.2. Phenolic Acid Preperation

o-Coumaric acid and 4-hydroxybenzoic acid were dissolved in 750 µl DMSO to maintain final 2.5% DMSO concentration in flasks. Phenolic acids were prepared 54 mM for 4-hydroxybenzoic acid and 30 mM for *o*-coumaric acid in 10 ml (750 µl DMSO + 9250 µl BHI) to have MICs in flasks (30 ml).

3.5.3. Protein Isolation from Flasks

Four different types of flasks were prepared according to Table 1 in triplicate. Flasks were incubated for 24 hours at 37°C without shaking. Each supernatant was transferred to a falcon tubes. Adherent cells were washed 2 times with 1% PBS to collect loosely attached cells and transferred to the falcon tubes. Planktonic cells were collected by centrifugation at 6000 g for 10 minutes and pellets were washed with 1% PBS. After centrifugation, pellets were dissolved in 700 µl of 4x protein sample buffer (40 mM Tris/HCl (pH 8), 4 mM EDTA, 8% SDS, 40% Glycerol and ddH_2O). Sonication (Bandelin Sonopuls) was performed for 30 Sec on 15 sec off for 8 cycles with 70% power in eppendorf tubes that submerged in absolute ethonal, NaCl and ice mixture. Sonicated cells were centrifuged at 12000 g (Dynamica) for 15 minutes at 4°C. Protein extracts were diluted in 1:10 ratio and concentrations were determined by BCA Protein Assay Kit (Santa Cruz).

Table 1. Contents of 4 different flasks for protein isolation

	Fresh BHI	Bacterial Culture	Phenolic	DMSO
Flask 1	28.5 ml	1.5 ml	-	-
Flask 2	27.75 ml	1.5 ml	-	750 µl
Flask 3	18.5 ml	1.5 ml	10 ml	-
Flask 4	18.5 ml	1.5 ml	10 ml	-

3.5.4. Sample Preparation for In-Gel Digestion and Mass spectroscopy

After determination of protein concentrations, equal amounts of proteins loaded to 12% SDS polyacrylamide gels. Four milliliters of acrylamide/bisacrylamide, 3.35 ml distilled water, 2.5 ml 1.5 M Tris-HCL, pH:8.8, 100 µl 10% (w/v) SDS, 50 µl 10% APS and 5 µl TEMED were used for 12% separating gels and 1.3ml of acrylamide/bisacrylamide, 6.1 ml distilled water, 2.5 ml 0.5 M Tris-HCL pH:6.8, 100 µl 10% (w/v) SDS, 50 µl 10% APS and 10 µl TEMED were used for stacking gels. Protein concentrations were equalled with ddH_2O in 40 µl volume that includes 8 µl 5x sample loading buffer for all groups. Mixtures were incubated at 100˚C for 10 minutes to denature the proteins. Due to insufficient capacity of wells, 20 µl of samples were loaded initially and empty wells were loaded with 8 µl sample loading buffer. Gels were run for 3 minutes at 80 V in 1X running buffer by Mini-PROTEAN Tetra System BIO-RAD. Then, 20 µl of remaining samples were added to same wells. After that, gels were run at 100 V for 100 minutes and incubated in fixer solution for 30 minutes. After that gels were stained with Coomassie Blue G-250 (SIGMA) and destained with destaining solution that includes methanol, acetic acid and distilled water. Then, gels were visualized with VersaDoc imaging system to confirm equal protein loads. Gels were washed with 50 mM ammonium bicarbonate (NH_4HCO_3) solution. Next, gels were treated with 50 mM ammonium bicarbonate solution that contains 50 mM dithiothretiol (DTT) at 60˚C for overnight without shaking. At the end of the period, gels were rinsed with 50 mM ammonium bicarbonate that include 100 mM iodoacetamide and taken into incubation with gentle shaking in the dark for 3 hours. Afterwards, every gel column

was dissected individually in 1 mm^3 sizes and transferred to low binding eppendorf tubes. Tubes were filled with 50 mM ammonium bicarbonate solution. Tubes were subjected to freeze-thaw at -80˚C for 5 minutes and taken to room temperature. Tubes were centrifuged for short time and buffer pipetted out to get rid of gel contaminants. Twenty microliters of 10 ng/µl (Roche) in 50 mM ammonium bicarbonate added onto gel pieces, and fulfilled with 50 mM ammonium bicarbonate upto adequate volume. After that, tubes were shaked gently overnight at room temperature. Following day, buffer that includes digested proteins were transferred into new tubes and pH of the buffers were adjusted with 10% trifluoroacetic acid that makes them suitable for nanoLC-ESI-MS/MS system. After samples concentrated with vacuum, samples were loaded to system for analysis.

Mass data were mined with MaxQuant and Andromeda software tools. KEGG, Swiss-Prot and NCBI databases were searched and proteins were identified. Further analyses were carried out by Perseus program. According to t-Test differences and IBAQ intensities, proteins were determined and graphs were plotted in Microsoft Office Excel.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Antimicrobial Characteristics of Phenolic Acids

Microtiter plate assays were performed in order to determine the minimal inhibitory concentrations (MIC) of *o*-coumaric acid and 4-hydroxybenzoic acid against *Listeria monocytogenes.* Furthermore, the inhibitory percentages of every concentration of phenolic acids were determined for different incubation periods as 15th, 18th, 21st and 24th hours. Both phenolic acids could inhibit the growth of *Listeria monocytogenes* EGDe.

4.1.1. Growth Inhibition of 4-Hydroxybenzoic Acid

Different concentrations of 4-hydroxybenzoic acid were applied on *Listeria monocytogenes* EGDe and growth inhibition was presented in Figure 4. While the concentrations up to 18 mM decreased the growth of *Listeria monocytogenes* EGDe, concentrations above 18 mM inhibited bacterial growth.

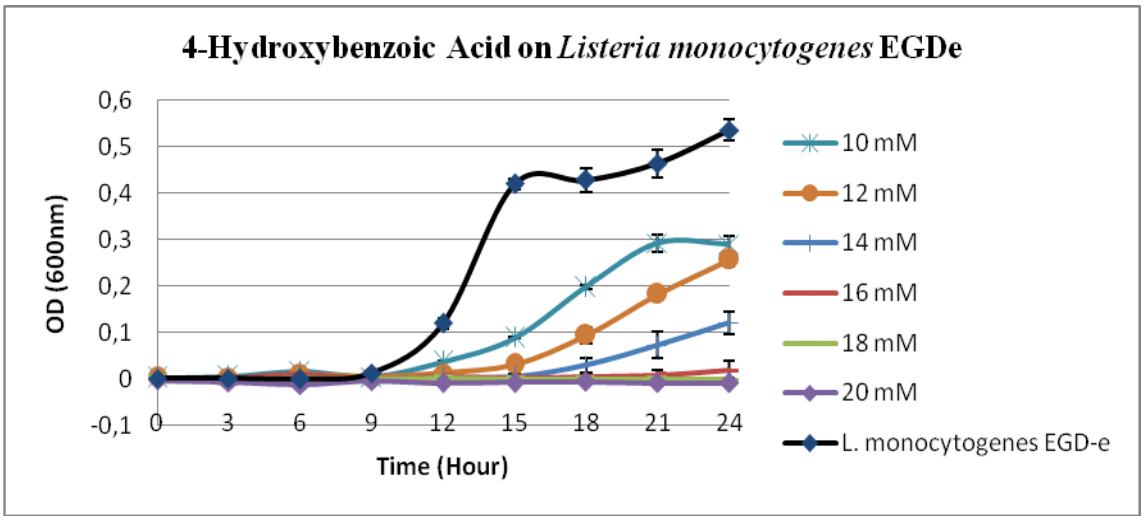

Figure 4. Growth of *Listeria monocytogenes* EGDe in the presence and absence of 4-hydroxybenzoic acid.

Percent inhibition rates of 4-hydroxybenzoic acid were shown in Table 2. Eighteen millimolars of 4-hydroxybenzoic acid were determined as MIC for the growth of *Listeria monocytogenes* EGDe.

Table 2. Percent inhibition rates of different concentrations of 4-hydroxybenzoic acid.

Concentrations of 4-Hydroxybenzoic Acid in wells	Percent Inhibition Rates at 15th Hour	Percent Inhibition Rates at 18th Hour	Percent Inhibition Rates at 21st Hour	Percent Inhibition Rates at 24th Hour
10mM	78	54	37	46
12mM	92	78	61	52
14mM	99	93	84	77
16mM	99	99	98	97
18mM	100	100	100	100
20mM	100	100	100	100

4.1.2. Growth Inhibition of *o*-Coumaric Acid

Growth of *Listeria monocytogenes* was also inhibited by *o*-coumaric. However, unlike 4-hydroxybenzoic acid, much lower concentrations of *o*-coumaric acid were affective on the growth inhibition, as shown in Figure 5. Percent inhibition rates of 4-hydroxybenzoic acid were presented in Table 3. Ten millimolars of *o*-coumaric acid were determined as MIC for *Listeria monocytogenes* EGDe.

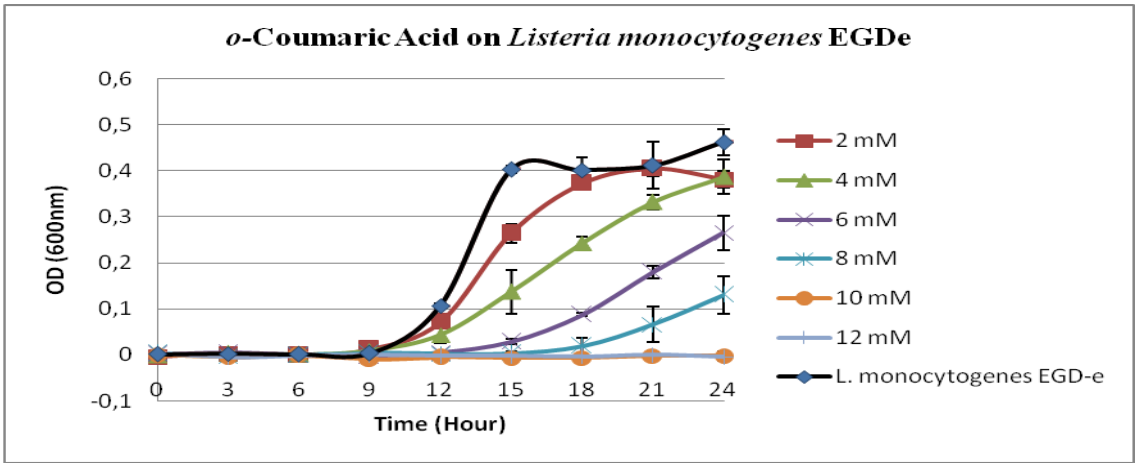

Figure 5. Growth of *Listeria monocytogenes* EGDe in the presence and absence of *o*-coumaric acid.

Table 3. Percent inhibition rates of different concentrations of *o*-coumaric acid.

Concentrations of *o*-Coumaric Acid in wells	Percent Inhibition Rates at 15th Hour	Percent Inhibition Rates at 18th Hour	Percent Inhibition Rates at 21st Hour	Percent Inhibition Rates at 24th Hour
2mM	34	7	2	18
4mM	66	40	19	16
6mM	93	78	56	43
8mM	99	95	84	72
10mM	100	100	100	100
12mM	100	100	100	100

According to antimicrobial characteristics of phenolic acids, both phenolic acids inhibited the growth of *Listeria monocytogenes* EGDe. According to Figure 3 and 4, the bacteria entered the logarithmic growth phase about 9 hours after start of the incubation. While the concentrations of phenolic acids increased, the entry time to exponential phase delayed. The concentrations below MIC proved that 4-HBA and *o*-coumaric acid have bacteriostatic effects. As the incubation time increased, inhibitory effect of phenolic acids decreased.

4.2. Antibiofilm Properties of Phenolic Acids

Listeria monocytogenes EGDe has ability of forming biofilms. Crystal violet assays were performed to investigate antibiofilm effects of *o*-coumaric and 4-hydroxybenzoic acids beside their antimicrobial abilities. The assays gave the percentages relative to inhibition of biomasses in the presence of two phenolic acids. Antibiofilm effects of phenolic acids were examined on initial attachment and 4 hour-old biofilms with crystal violet assay. The inhibitory percentages of phenolic acids are calculated by formula: 100*(1-(sample/control)). The significance of inhibitions was tested with t-test, and P values were obtained below 0.05.

4.2.1. Antibiofilm Effects on Initial Attachment

In order to investigate the effects of *o*-coumaric and 4-hydroxybenzoic acids on initial attachment, addition of phenolic compounds and inoculation of bacteria were at the same time. Based on absorption of crystal violet by biofilm mass, inhibitory effects on initial attachments were compared. In the presence of the MIC values of both phenolic acids, the attachment was reduced (Figure 6). While *o*-coumaric acid (10 mM) reduced the attachment 58%, 4-hydroxybenzoic acid (18 mM) reduced it 48% with respect to DMSO control.

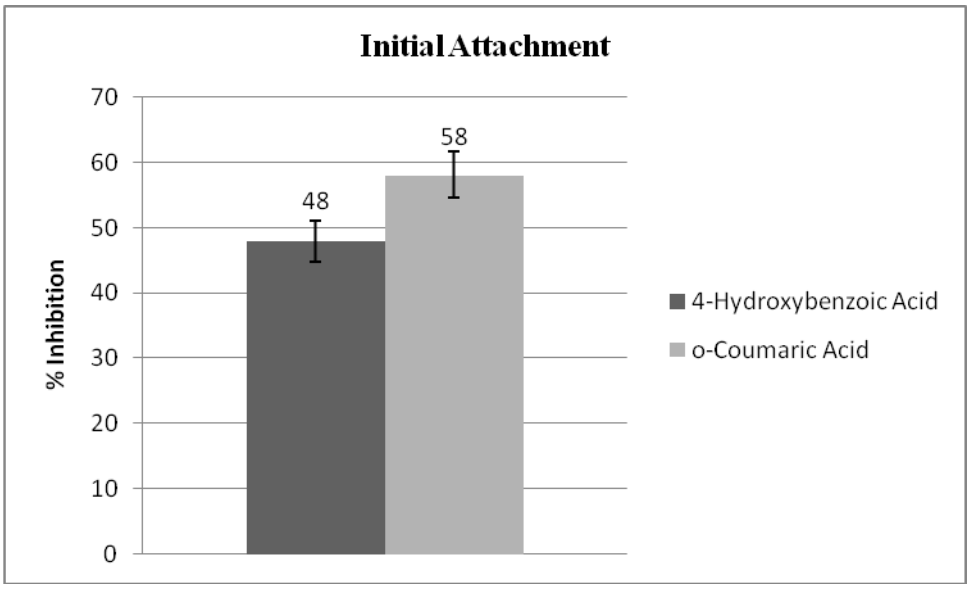

Figure 6. Percent inhibitions of *o*-coumaric acid and 4-hydroxybenzoic acid on initial attachment

According to inhibitory percentages, both phenolic acids showed significant inhibitory effects on initial attachment of *Listeria monocytogenes* EGDe to polystyrene surface. *o*-Coumaric acid showed more inhibitory effect than 4-hydroxybenzoic acid in initial attachment. In the initial attachment, there are many criteria that should be considered beside their antibiofilm activity. The presence of phenolic acids may change chemical properties of environment or the chemical forces between surface of the wells and bacteria. This may occurred by aggregation of phenolic compounds. There is also a possibility that reactions can be occurred between growth medium and phenolic acids (Armin Wen et al. 2003). In addition to that, there are surface proteins that take role in the attachment of bacteria to the surfaces like fimbriae and pili. Interaction between

phenolic acids and active sites of these proteins may be decreased the possibility of attachment of bacteria. Changes in morphology of the cells like elongation or flattening may changed the adherence capability.

4.2.2. Antibiofilm Effects on Four Hours Old Biofilms

The antibiofilm effect of *o*-coumaric acid and 4-hydroxybenzoic acid against 4h old biofilms were also investigated with the same method except the additions of phenolic acids were four hours after the bacterial inoculation. Both phenolic acids had slightly reduced antibiofilm effect on *Listeria monocytogenes* (Figure 4). While *o*-coumaric acid showed 56% inhibition on preformed biofilm, 4-hydroxybenzoic acid showed 34% inhibition.

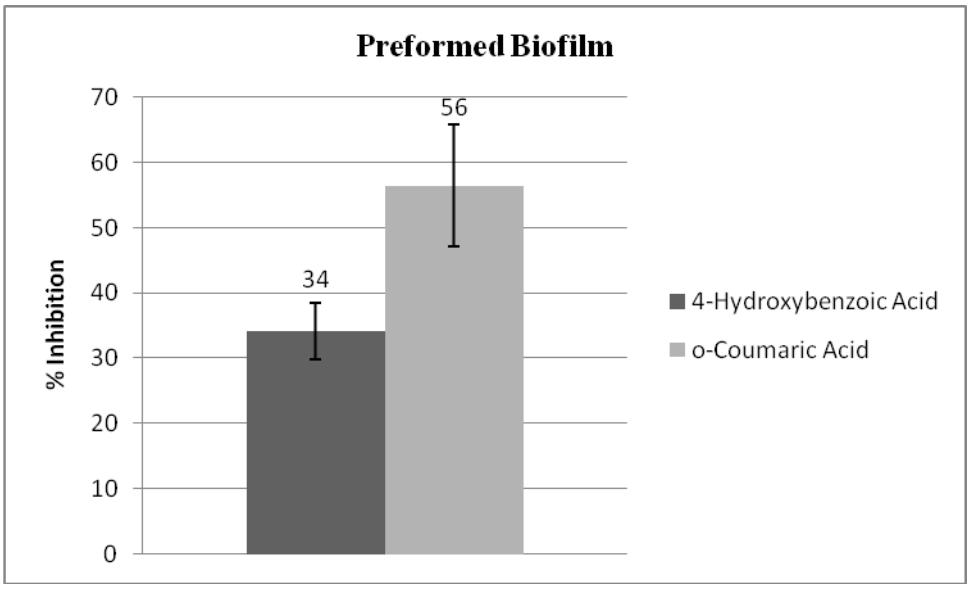

Figure 7. Percent inhibitions of *o*-coumaric acid and 4-hydroxybenzoic acid on 4h old biofilms

After initial attachment, it is very hard to eradicate the cells from surfaces. According to inhibition on 4h old biofilms, both phenolic acids still showed antibiofilming effects. Four hours old biofilm cannot be accepted as mature biofilm but it is sufficient to see the antibiofilming effect of compounds on biofilm development.

After initial attachment, the gene expressions and physiologies of cells differ. Although the inhibitory effect of 4-HBA decreased from 48% to 34%, *o*-coumaric acid did not change significantly. This should be due to the differences between modes of

action of two phenolic acids. One of the reasons can be eradication of cells from the surfaces by phenolic acids especially by *o*-coumaric acid. (Stoodley et al. 2002, Simões M., Bennett R. N., & Rosa, E. 2009, Paiva P. M. G., Gomes F. S., Napoleão T. H. 2010).

4.3. Microscopy Analysis

Antibiofilming abilities of phenolic acids were visualized with scanning electron and fluorescence microscopies. The microscopy analysis proved the inhibitory effects on initial attachment and biofilm development.

4.3.1. SEM Analysis

Scanning electron microscopy (Quanta 250FEG) analyses were performed to observe inhibitory effects of phenolic acids on initial attachment and biofilm development.

The SEM images of biofilm structure of *L. monocytogenes* cells on polystyrene surface were presented in Figure 8. In this experiment, bacterial inoculation and phenolic acid treatments performed at the same time. Micrograph of control group (A) displayed a homogenous biofilm structure with knitted chains. Control group with DMSO (B) showed different character in density of attached cells and distribution of biofilm. The micrograph of biofilm developed in 4-HBA (C) showed decreased in cell density compared to control groups. Attached microcolonies treated with 4-HBA could not establish the chain conformation. In the presence of *o*-coumaric acid (D), there were no colonies that attached to surface. Thereby we can conclude that *o*-coumaric acid prevented initial attachment and biofilm formation.

The addition of phenolic acids 4 hours after inoculation displayed different biofilm formation (Figure 9). In control group (A), knitted chains of *L. monocytogenes* cells were homogenously attached to surface. In control group grown in DMSO (B) showed dense cell attachments. DMSO may have clustered the cells non-uniformly. Density of *L. monocytogenes* cells grown in 4-HBA (C) was decreased compared to control group. Knitted chains of *L. monocytogenes* were not observed in the presence of *o*-coumaric acid. However, small amounts of microcolonies were seen on the surface.

The comparison of two micrographs of *o*-coumaric acid treated biofilms in Figure 8 and 9 represented that *o*-coumaric acid was less effective on preformed biofilms than preventing initial attachment.

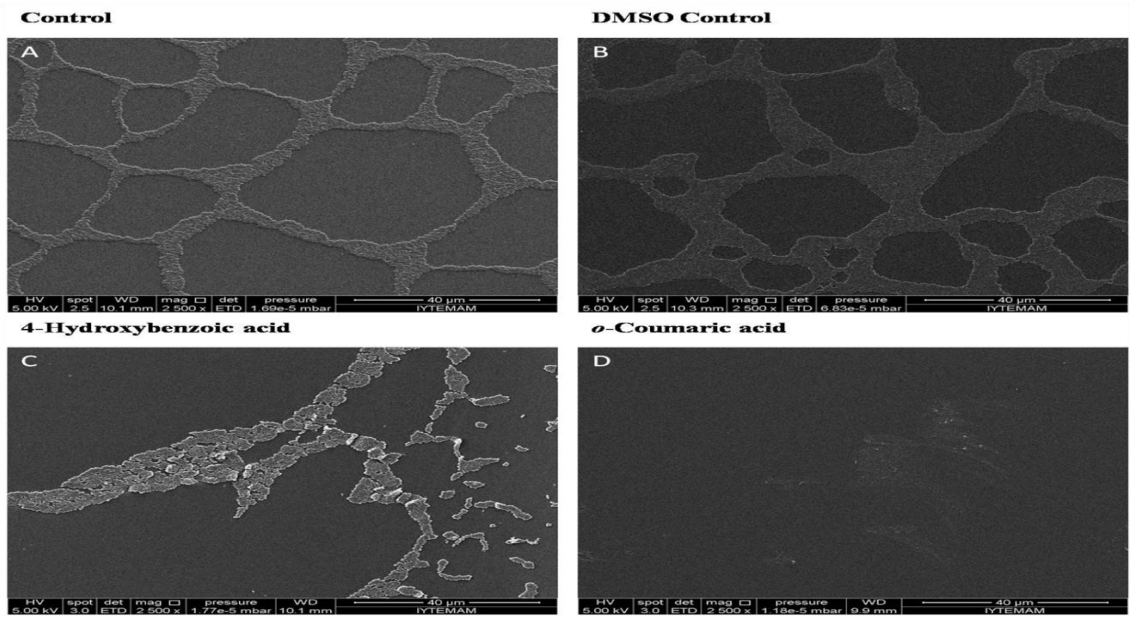

Figure 8. SEM images of 24 hours old *Listeria monocytogenes* biofilms treated with phenolic acids (added at the same time with inoculation). The images demonstrate the biofilms grown in (A) BHI broth (B) 2.5% DMSO (C) 4-hydroxybenzoic acid and (D) *o*-coumaric acid

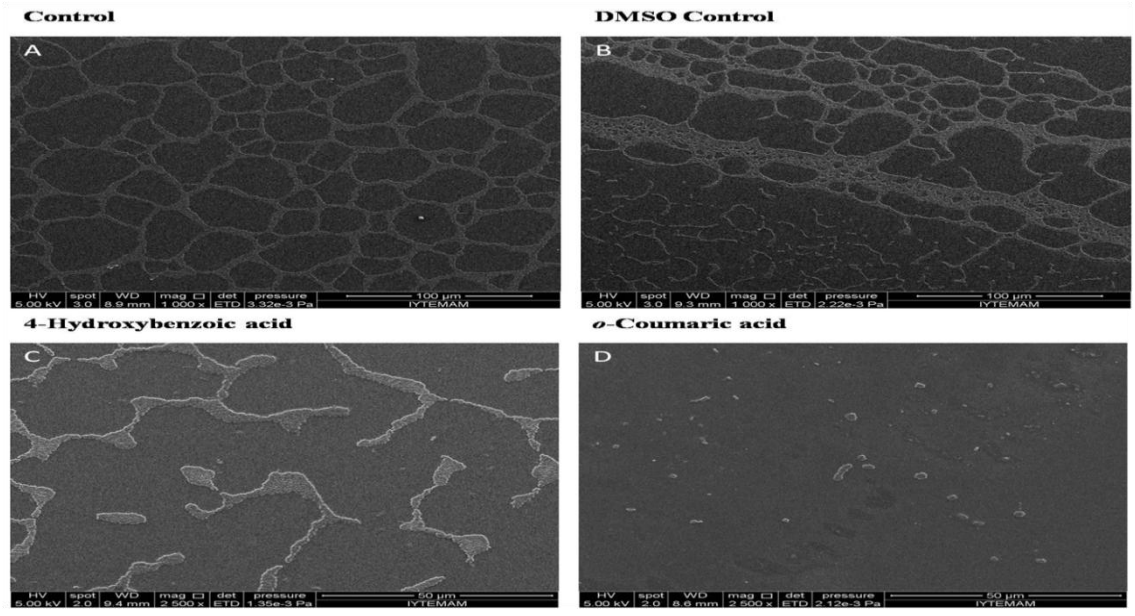

Figure 9. SEM images of 24 hours old *Listeria monocytogenes* biofilms treated with phenolic acids (added four hours after inoculation). The micrographs demonstrate the biofilms grown in (A) BHI broth (B) 2.5% DMSO (C) 4-hydroxybenzoic acid and (D) *o*-coumaric acid. Phenolic acids were added 4 hours after inoculation of bacteria.

4.3.2. Fluorescence Microscopy Analysis

Fluorescence microscopy analyses were also performed to visualize the inhibitory effects of phenolic acids on biofilms. Fluorescence images were taken under 20X magnification.

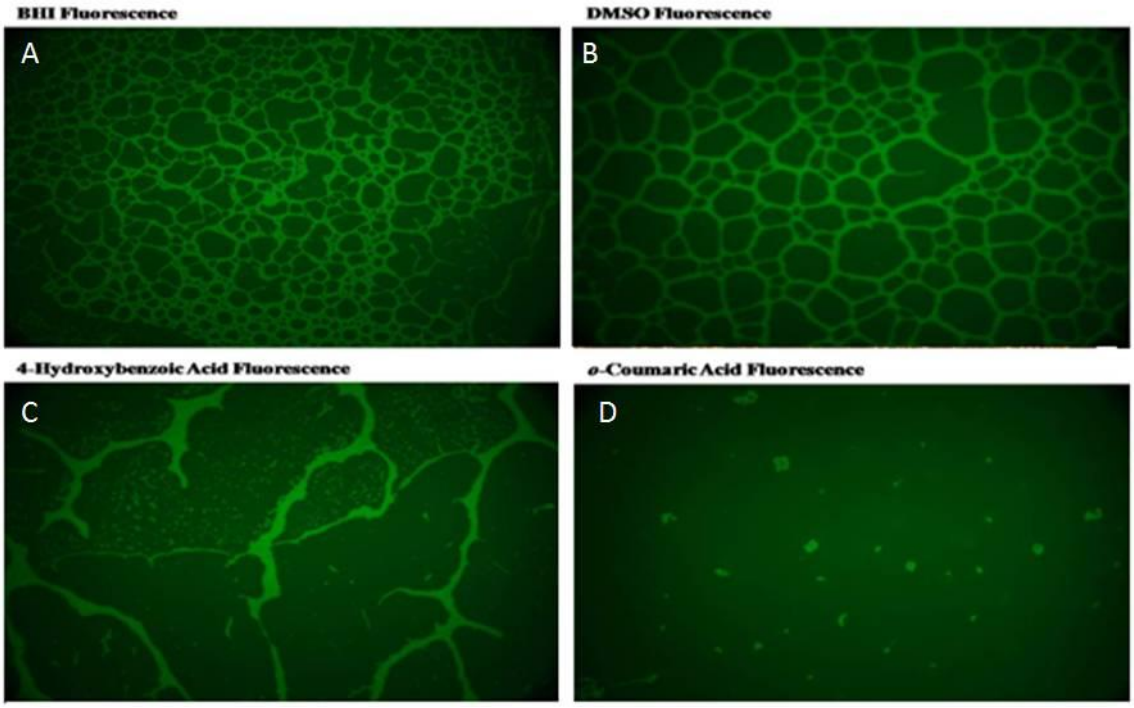

Figure 10. Fluorescence microscopy images of 24 hours old *Listeria monocytogenes* biofilms treated with phenolic acids (added at the same time with inoculation). The images demonstrate the biofilms grown in (A) BHI broth (B) 2.5% DMSO (C) 4-hydroxybenzoic acid and (D) *o*-coumaric acid.

Figure 10 demonstrates the inhibition of biofilm formation in the presence of phenolic acids that added immediately after bacterial inoculation. Fluorescence images of 24 hours old biofilms grown in BHI (A) and DMSO (B) showed similar uniform distribution on the surface of wells. However, in the presence of 4-HBA (C), amount of branched biofilms were decreased. The fluorescence images of *o*-coumaric acid (D) showed great inhibitions on biofilm formation. Only a few microcolonies were seen on the surface.

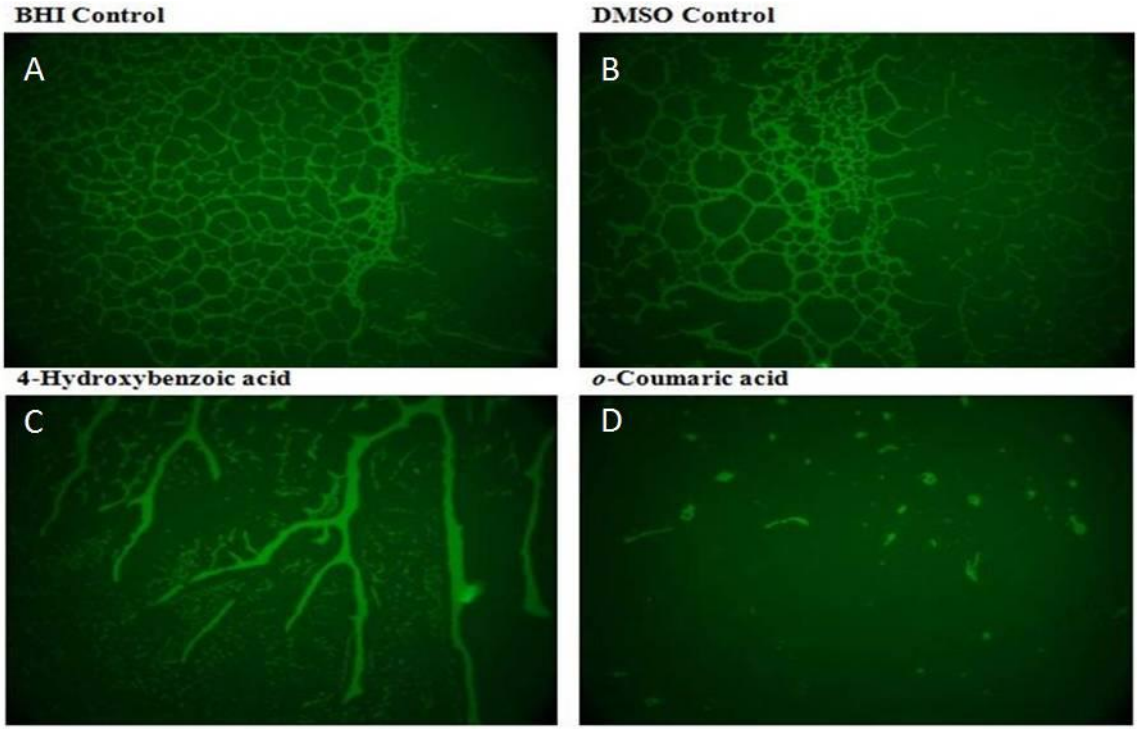

Figure 11. Fluorescence microscopy images of 24 hours old *Listeria monocytogenes* biofilms treated with phenolic acids (added four hours after inoculation). The images demonstrate the biofilms grown in (A) BHI broth (B) 2.5% DMSO (C) 4-hydroxybenzoic acid and (D) *o*-coumaric acid.

Figure 11 represents the 24 hours old biofilms formed in the presence and absence of phenolic acids. Phenolic acids were added 4 hours after inoculation of bacteria. Fluorescence images of control group (A) displayed the same homogenous pattern as knitted chains. In DMSO control (B), dense and non-uniform patterns of biofilms observed. Reduced amount of biofilm formation was seen in 4-hydroxybenzoic acid treated group (C). In the Image D, there were only small groups of attached cells. As expected, *o*-coumaric acid decreased the biofilm development.

Microscopy analyses demonstrated that both of the phenolic acids decreased the biofilm formation. Although, both phenolic acids have antimicrobial activity, *o*-coumaric acid was more effective than 4-HBA on preventing biofilm formation.

4.4. Comparative Proteomic Analysis

Phenolic acid treated and non-treated cells were collected to isolate the total proteins for comparative proteomic analysis.

The data given by Perseus program showed that 13 proteins were downregulated and 3 proteins were upregulated in the presence of 4-hydroxybenzoic acid. In the case of *o*-coumaric acid stress, 2 proteins were upregulated but no protein was detected as downregulated. Comparison of two control groups showed no significant difference in terms of protein profiles.

In the presence of phenolic acid stress, several kinds of proteins and mechanisms were altered in terms of protein abundance. The following sections contain the data for changes in the protein profiles in detail with their functions according to type of phenolic acid stress.

4.4.1. Comparison of Control Groups

DMSO was used as a solvent for phenolic acids. Although the concentration was not inhibitory in antimicrobial tests, it showed promotive effect on biofilm formation of *L. monocytogenes*. The comparison of abundance ratios with respect to IBAQ intensity ratios showed no significant change between the cells grown with DMSO and without DMSO (Figure 12). As a result of this, we could not observe any significant up or down regulated proteins between two control groups.

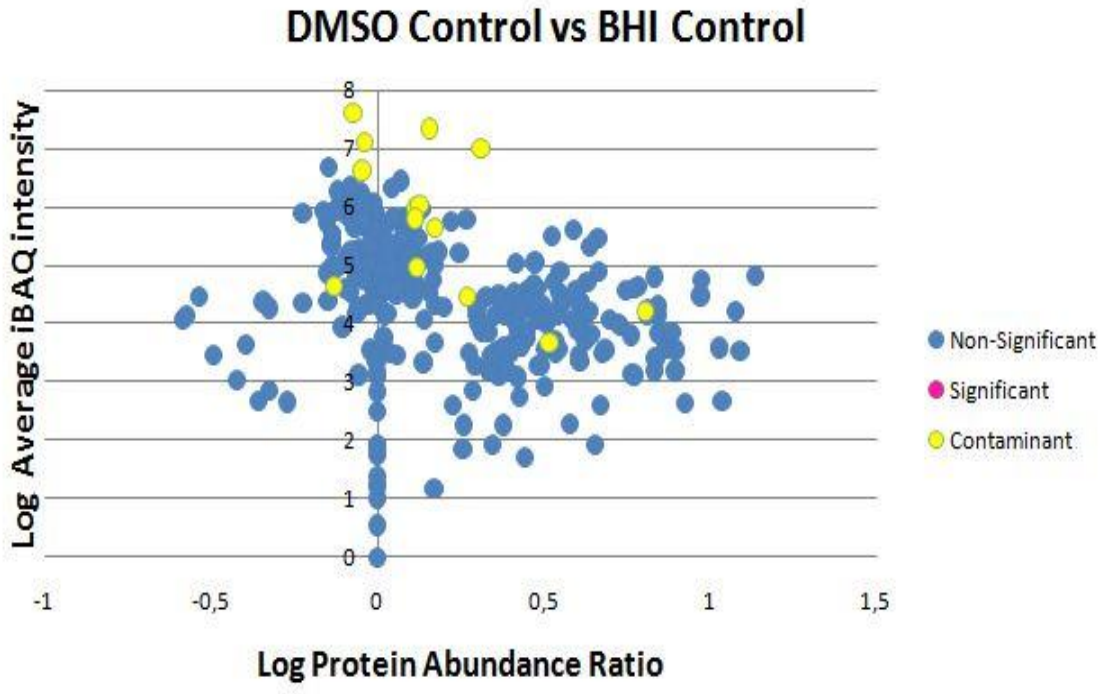

Figure 12. Comparative protein abundance graph of DMSO and BHI control group

4.4.2. Protein Profile of 4-Hydroxybenzoic Acid

The effect of 4-hydroxybenzoic acid was seen mostly as downregulation rather than up regulation of proteins. While 13 proteins were significantly downregulated, 3 proteins were upregulated in terms of the comparison of abundance ratios with respect to IBAQ intensity ratios (Figure 13).

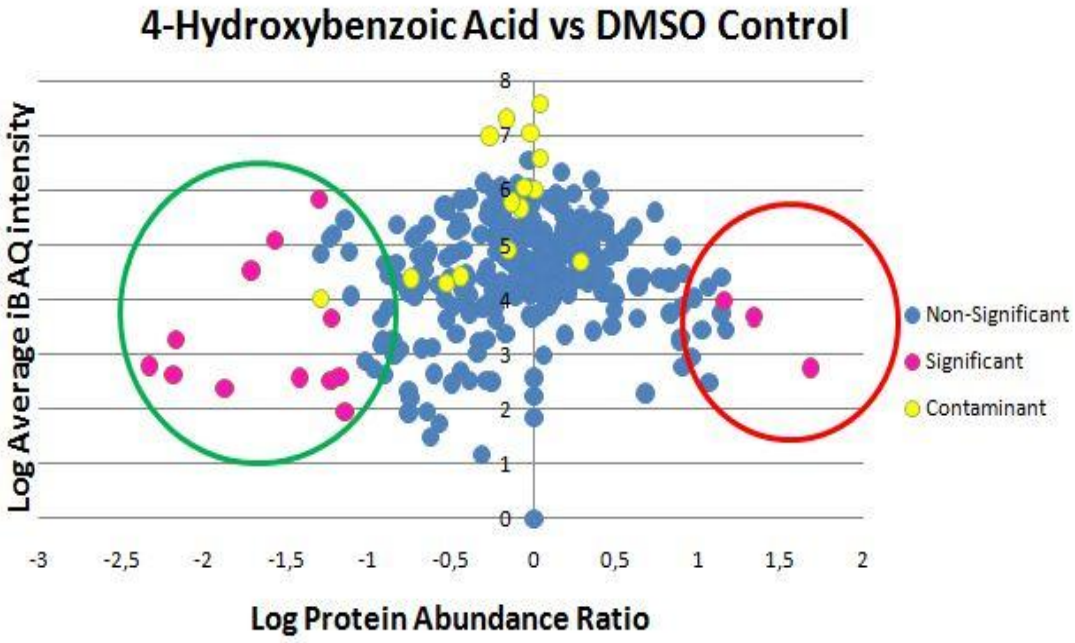

Figure 13. Comparative protein abundance graph of 4-hydroxybenzoic acid and DMSO control group. Significantly upregulated proteins circled as red, while downregulated ones circled in green

General tendency of downregulation was focused mostly on two functional categories which are namely energy metabolism and metabolite/substrate transporter systems (Table 4). Two dehydrogenases that take role in different steps of glycolysis were down-regulated. The catalytic activities of Lm4b_01645 and aldehyde-alcohol dehydrogenase enzymes produce NADH from acetaldehyde and/or alcohol. While the catalysis of acetaldehyde produces acetyl-CoA and NADH, catalysis of alcohol produces an aldehyde or a ketone with a NADH as final product. These catabolic reactions have importance in NADH reoxidation and energy conservation (Rydzak et al. 2012).

Table 4. Downregulated proteins of unattached *L. monocytogenes* cells in the presence of 4-hydroxybenzoic acid

Gene Identity	Protein Description	Functional Category
Lm4b_01645	Putative alcohol-acetaldehyde dehydrogenase	Glycolysis/Energy production
adhE	Aldehyde-alcohol dehydrogenase	Glycolysis/Energy production
pfl-2	Formate acetyltransferase	Glycolysis/Energy production
gpmA	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	Glycolysis/Gluconeogenesis
Lm4b_00101	Putative PTS system mannose-specific, IID component	Mannose uptake/Protein glycosylation
Lm4b_00863	Putative Glutamine ABC transporter	Aminoacid Uptake/Protein Synthesis
LMOf6854_1910	Manganese ABC transporter, ATP-binding protein	Mn^{+2} Transport/Enzyme Cofactor/Activity
LMOf6854_1908	ABC transporter, manganese-binding protein	Mn^{+2} Transport/Enzyme Cofactor/Activity
cadA	Cadmium-translocating P-type ATPase	Cd^{+2} Transport/Efflux
rplT	50S ribosomal protein L20	Ribosomal Integrity/Protein Synthesis
rpsL	30S ribosomal protein S12	Ribosomal Integrity/Protein Synthesis
clpC	Endopeptidase Clp ATP-binding chain C	Protein degradation
Lm4b_00315	Putative two-component response regulator	Signal Transduction/Transcriptional Regulation

Furthermore, another down-regulated protein was formate acetyltransferase which is responsible from the conversion of pyruvate to formate in the presence of acetyl-CoA. Underproduction of this enzyme was also observed in *L. monocytogenes* as a response to the gastric acid stress (Melo J. et al. 2013). Abundance of this enzyme might have been decreased at low pH. In addition, 2, 3-bisphosphoglycerate-dependent phosphoglycerate mutase which plays role in carbohydrate metabolism was also downregulated. Phosphoglycerate mutase is a general stress protein that regulated by RNA polymerase sigma factor SigB. Beside 4-hydroxybenzoic acid stress, this enzyme also downregulated as a response to heat-shock stress (Van der Veen S. et al. 2007).

Five proteins that play role in active transport systems located on membrane were downregulated. The downregulation in one of those proteins, Lm4b_00101, a mannose specific PTS system protein might have leveled the uptake and/or the phosphorylation of mannose. Decreased levels of mannose could lead to attenuated levels of protein glycosylation. Another downregulated protein was associated with uptake mechanism of glutamine. The lack of glutamine might have lead to protein synthesis inhibition. Additional two ABC transporter proteins associated with active transport of manganese were downregulated. Manganese is the cofactor of enzymes that degrades reactive oxygen species (Culotta and Daly 2013), for that reason an oxidative stress might have been induced in cell due to the decreased levels of manganese. Cadmium-translocating P-type ATPase that responsible from the efflux of cadmium ion was downregulated. Absence of this protein may lead to inhibition of efflux of cadmium which is toxic for the cell when accumulated (Wu C. et al. 2006). Beside the transporters, two ribosomal proteins were downregulated under 4-hydroxybenzoic stress. While *rpsL* transcribes the S12 unit of 30S ribosomal complex, *rplT* transcribes L20 unit of 50S.Downregulation of aforementioned proteins might have negatively affected translational accuracy and/or protein synthesis. The direct interaction between 4-hydroxybenzoic acid and these ribosomal proteins may have affected their own regulation in negative manner (Sander P. et al. 2001, Choonee N. et al. 2006). One of the protein belongs to heat-shock HSP-100/Clp family, Clp ATP-binding chain C protein, was also downregulated. It is known that this protein plays role in the virulence of *Listeria monocytogenes* (Rouquette C. et al. 1998).

Three proteins were upregulated in response to 4-hydroxybenzoic acid stress as listed in Table 5.

Table 5. Upregulated proteins of unattached *L. monocytogenes* cells in the presence of 4-hydroxybenzoic acid

Gene Identity	Protein Description	Functional Category
Lm4b_01002	Putative glucanase and peptidase	Protein folding /Protein turnover
Lm4b_00315	Putative two-component response regulator	Signal Transduction/Transcriptional Regulation
alsS	Acetolactate synthase	Essential Amino Acid Synthesis/Protein Synthesis

Two component systems sense and respond according to environmental changes. Expression of two different two-component response regulators was altered. While one of the regulators downregulated, another one was upregulated. This alteration might have been due to the stress induced by 4-hydroxybenzoic acid (Kallipolitis B. H. et al. 2001).

One of these upregulated proteins, putative glucanase and peptidase protein was also upregulated under gastric acid stress (Melo J. et al.2013). It seems like under acidic conditions, this protein was induced to affect protein folding in a positive manner. Acetolactate synthase which has a role in branched-chain amino acid synthesis is upregulated. Upregulation of this protein might have indicated protein synthesis requirements of bacteria in the presence of phenolic acid stress.

4.4.3. Protein Profile of *o*-Coumaric Acid

Figure 14 presents the distribution of proteins in comparison between *o*-coumaric acid treated test group and DMSO control group according to protein abundance. Under *o*-coumaric acid stress, no significantly downregulated proteins were detected. However, two proteins were found to be upregulated.

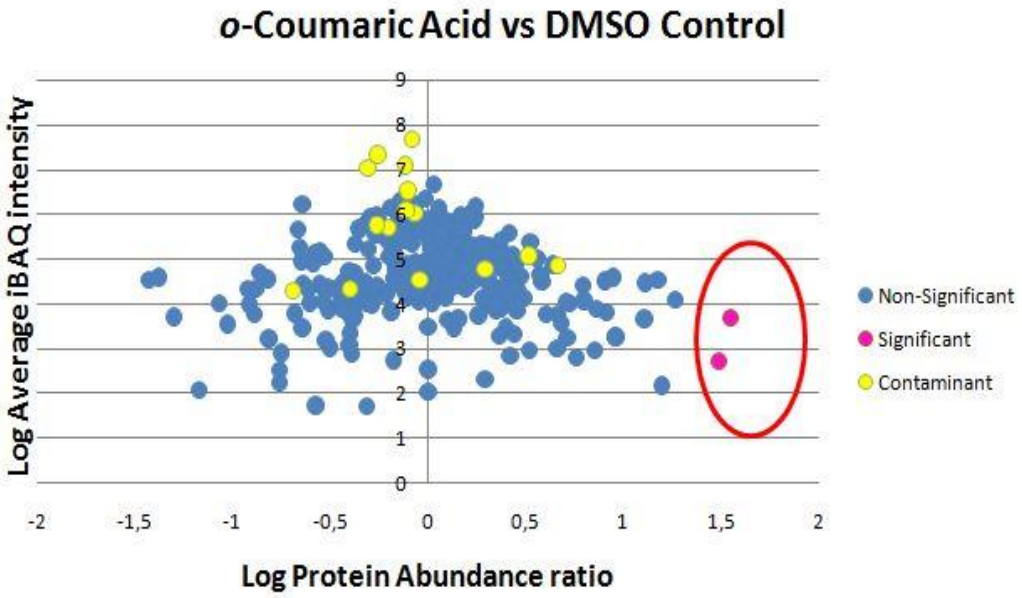

Figure 14. Comparative protein abundance graph of *o*-coumaric acid and DMSO control group. Significantly upregulated proteins circled as red, while downregulated ones circled in green.

Upregulated proteins of *o*-coumaric acid treated cells were listed with their functional category in Table 6. Acetolactate synthase, one of aforementioned upregulated protein in the presence of 4-hydroxybenzoic acid was also upregulated in the presence of o-coumaric acid. Carbonic anhydrase which controls CO_2/HCO_3 conversion under aerobic conditions in facultative anaerobic bacteria was also upregulated. According to Bowman et al. (2012), this protein might contribute to acid resistance of *Listeria monocytogenes* due to its function above.

Table 6. Upregulated proteins of unattached *L. monocytogenes* cells in the presence of *o*-coumaric acid

Gene Identity	Protein Description	Functional Category
alsS	Acetolactate synthase	Essential Amino Acid Synthesis
cah	Carbonic Anhydrase	pH Balance

CHAPTER 5

CONCLUSION

In this study, we investigated the antimicrobial and antibiofilm effects of 4-hydroxybenzoic and *o*-coumaric acids on *L. monocytogenes* EGDe with a proteomic approach. We determined MIC of *o*-coumaric and 4-hydroxybenzoic acids as 18 mM and 10 mM, respectively. In addition to these, we highligthed the inhibitions in initial attachment and biofilm development processes in the presence of aforementioned phenolic acids. Both inhibitions were proved by SEM and fluorescence microscopy techniques.

Our investigations revealed that both phenolic acids significantly inhibited initial attachment and biofilm development under individual treatments. The inhibition percentages and microscopy analyses showed that *o*-coumaric acid was more effective than 4-hydroxybenzoic acid as an antibiofilming agent. In addition, while high concentrations of DMSO as a solvent were toxic for bacteria, a non-toxic concentration (2.5%) of DMSO induced biofilm formation.

Comparative proteomic data showed significantly different variations in protein profiles under phenolic acid stress. Interestingly, although *o*-coumaric acid effectively inhibited the biofilm formation, only two proteins were found to be significantly upregulated and no significantly downregulated protein was detected. Unlike *o*-coumaric acid, 13 significantly downregulated and 3 significantly upregulated proteins were observed in the presence of 4-hydroxybenzoic acid. Most of the downregulated proteins under 4-hydroxybenzoic acid stress were related with energy metabolism and transporters on the membrane. The results indicated altered membrane integrity. Unlike 4-hydroxybenzoic acid, it was hard to speculate on targets of *o*-coumaric acid with only 2 variations in the protein profile. However, extracellular proteins that play vital roles in biofilm formation or lipids in the bacteria may be the reasons behind the antibiofilming properties of *o*-coumaric acid.

REFERENCES

Adonizio, A., Kong, K.-F., & Mathee, K. (2008). Inhibition of quorum sensing-controlled virulence factor production in Pseudomonas aeruginosa by South Florida plant extracts. *Antimicrobial agents and chemotherapy*, *52*(1), 198–203. doi:10.1128/AAC.00612-07

Andjelković, M., Camp, J. Van, Meulenaer, B. De, Depaemelaere, G., Socaciu, C., Verloo, M., & Verhe, R. (2006). Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry*, *98*(1), 23–31. doi:10.1016/j.foodchem.2005.05.044

Annapoorani, A., Parameswari, R., Pandian, S. K., & Ravi, A. V. (2012). Methods to determine antipathogenic potential of phenolic and flavonoid compounds against urinary pathogen Serratia marcescens. *Journal of microbiological methods*, *91*(1), 208–11. doi:10.1016/j.mimet.2012.06.007

Borges, A., Ferreira, C., Saavedra, M. J., & Simões, M. (2013). Antibacterial Activity and Mode of Action of Ferulic and Gallic Acids Against Pathogenic Bacteria. *Microbial drug resistance (Larchmont, N.Y.)*, *00*(0). doi:10.1089/mdr.2012.0244

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews*, *56*(11), 317–33.

Bryers, J. D., Ratner, B. D., & Re-, W. (2004). Bioinspired Implant Materials Befuddle Bacteria, *70*(5), 232–237.

Bowman, J. P., Hages, E., Nilsson, R. E., Kocharunchitt, C., & Ross, T. (2012). Investigation of the Listeria monocytogenes Scott A acid tolerance response and associated physiological and phenotypic features via whole proteome analysis. *Journal of proteome research*, *11*(4), 2409–26. doi:10.1021/pr201137c

Bubonja-Sonje, M., Giacometti, J., & Abram, M. (2011). Antioxidant and antilisterial activity of olive oil, cocoa and rosemary extract polyphenols. *Food Chemistry*, *127*(4), 1821–1827. doi:10.1016/j.foodchem.2011.02.071

Cho, J.-Y., Moon, J.-H., Seong, K.-Y., & Park, K.-H. (1998). Antimicrobial Activity of 4-Hydroxybenzoic Acid and *trans* 4-Hydroxycinnamic Acid Isolated and Identified from Rice Hull. *Bioscience, Biotechnology, and Biochemistry*, *62*(11), 2273–2276.

Choonee, N., Even, S., Zig, L., & Putzer, H. (2007). Ribosomal protein L20 controls expression of the Bacillus subtilis infC operon via a transcription attenuation mechanism. *Nucleic acids research*, *35*(5), 1578–88. doi:10.1093/nar/gkm011

Crozier, A., & Clifford, M. N. (n.d.). *Plant Secondary Metabolites Occurrence , Structure and Role in the Human Diet Edited by*.

Cueva, C., Mingo, S., Muñoz-González, I., Bustos, I., Requena, T., Del Campo, R., Martín-Álvarez, P. J., et al. (2012). Antibacterial activity of wine phenolic compounds and oenological extracts against potential respiratory pathogens. *Letters in applied microbiology*, *54*(6), 557–63. doi:10.1111/j.1472-765X.2012.03248.x

Culotta, V.C. and M.J. Daly. 2013. Manganese Complexes: Diverse Metabolic Routes to Oxidative Stress Resistance in Prokaryotes and Yeast, *Antioxidants and Redox Signaling*

Donlan, R. M., & Costerton, J. W. (2002). Biofilms : Survival Mechanisms of Clinically Relevant Microorganisms. *Society*, *15*(2), 167–193. doi:10.1128/CMR.15.2.167

Dorantes, L., Colmenero, R., Hernandez, H., Mota, L., Jaramillo, M. E., Fernandez, E., & Solano, C. (2000). Inhibition of growth of some foodborne pathogenic bacteria by Capsicum annum extracts. *International Journal of Food Microbiology*, *57*(1–2), 125–128. doi:10.1016/S0168-1605(00)00216-6

Dunne, W. M. (2002). Bacterial Adhesion : Seen Any Good Biofilms Lately ? *Society*, *15*(2), 155–166. doi:10.1128/CMR.15.2.155

Edris, A. E. (2007). Pharmaceutical and Therapeutic Potentials of Essential Oils and Their Individual Volatile Constituents : A Review, *323*(October 2006), 308–323. doi:10.1002/ptr

Gandhi, M., & Chikindas, M. L. (2007). Listeria: A foodborne pathogen that knows how to survive. *International journal of food microbiology*, *113*(1), 1–15. doi:10.1016/j.ijfoodmicro.2006.07.008

Hedberg, C. (1999). Food-related illness and death in the United States. *Emerging infectious diseases*, *5*(6), 840–2. doi:10.3201/eid0506.990624

Hefford, M. A., Aoust, S. D., Cyr, T. D., Austin, J. W., Sanders, G., Kheradpir, E., & Kalmokoff, M. L. (2005). Proteomic and microscopic analysis of biofilms formed by Listeria monocytogenes 568. *Proteins*, *208*, 197–208. doi:10.1139/W04-129

Jagani, S., Chelikani, R., & Kim, D.-S. (2009). Effects of phenol and natural phenolic compounds on biofilm formation by Pseudomonas aeruginosa. *Biofouling*, *25*(4), 321–4. doi:10.1080/08927010802660854

Jefferson, K. K. (2004). What drives bacteria to produce a biofilm? *FEMS microbiology letters*, *236*(2), 163–73. doi:10.1016/j.femsle.2004.06.005

Kallipolitis, B. H., & Ingmer, H. (2001). Listeria monocytogenes response regulators important for stress tolerance and pathogenesis. *FEMS microbiology letters*, *204*(1), 111–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11682188

Karaosmanoglu, H., Soyer, F., Ozen, B., & Tokatli, F. (2010). Antimicrobial and antioxidant activities of Turkish extra virgin olive oils. *Journal of agricultural and food chemistry*, *58*(14), 8238–45. doi:10.1021/jf1012105

Kavanaugh, N. L., & Ribbeck, K. (2012). Selected antimicrobial essential oils eradicate Pseudomonas spp. and Staphylococcus aureus biofilms. *Applied and environmental microbiology*, *78*(11), 4057–61. doi:10.1128/AEM.07499-11

Kong, C.-S., Jeong, C.-H., Choi, J.-S., Kim, K.-J., & Jeong, J.-W. (2013). Antiangiogenic effects of p-coumaric acid in human endothelial cells. *Phytotherapy research : PTR*, *27*(3), 317–23. doi:10.1002/ptr.4718

LaPlante, K. L., Sarkisian, S. a, Woodmansee, S., Rowley, D. C., & Seeram, N. P. (2012). Effects of cranberry extracts on growth and biofilm production of Escherichia coli and Staphylococcus species. *Phytotherapy research : PTR*, *26*(9), 1371–4. doi:10.1002/ptr.4592

Lewis, K. I. M. (2001). MINIREVIEW Riddle of Biofilm Resistance, *45*(4), 999–1007. doi:10.1128/AAC.45.4.999

Liu, D. (2006). Identification, subtyping and virulence determination of Listeria monocytogenes, an important foodborne pathogen. *Journal of medical microbiology*, *55*(Pt 6), 645–59. doi:10.1099/jmm.0.46495-0

Longhi, C., Scoarughi, G. L., Poggiali, F., Cellini, A., Carpentieri, A., Seganti, L., Pucci, P., et al. (2008). Protease treatment affects both invasion ability and biofilm formation in Listeria monocytogenes. *Microbial pathogenesis*, *45*(1), 45–52. doi:10.1016/j.micpath.2008.01.007

Lou, Z., Song, X., Hong, Y., Wang, H., & Lin, Y. (2013). Separation and enrichment of burdock leaf components and their inhibition activity on biofilm formation of E. coli. *Food Control*, *32*(1), 270–274. doi:10.1016/j.foodcont.2012.11.020

Low, J. C., Donachie, W. (1997). *A Review of Listeria monocytogenes and Listeriosis* , 9–29.

Møretrø, T., Langsrud, S. (2004). Listeria monocytogenes: biofilm formation and persistence in food-processing environments. *Biofilms*, *1*(02), 107–121. doi:10.1017/S1479050504001322

Marsh, P. D. (2004). Dental plaque as a microbial biofilm. *Caries research*, *38*(3), 204–11. doi:10.1159/000077756

Melo, J., Schrama, D., Andrew, P. W., & Faleiro, M. L. (2013). Proteomic analysis shows that individual Listeria monocytogenes strains use different strategies in response to gastric stress. *Foodborne pathogens and disease*, *10*(2), 107–19. doi:10.1089/fpd.2012.1297

Paiva, P. M. G., Gomes, F. S., Napoleão, T. H., Sá, R. A., Correia, M. T. S., & Coelho, L. C. B. B. (2010). Antimicrobial activity of secondary metabolites and lectins from plants, 396–406.

Pizarro-Cerdá, J., Kühbacher, A., & Cossart, P. (2012). Entry of Listeria monocytogenes in mammalian epithelial cells: an updated view. *Cold Spring Harbor perspectives in medicine*, *2*(11). doi:10.1101/cshperspect.a010009

Ramaswamy, V., Cresence, V. M., Rejitha, J. S., Lekshmi, M. U., Dharsana, K. S., Prasad, S. P., & Vijila, H. M. (2007). Listeria--review of epidemiology and pathogenesis. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi*, *40*(1), 4–13.

Randhir, R., Lin, Y.-T., & Shetty, K. (2004). Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia Pacific journal of clinical nutrition*, *13*(3), 295–307.

Rouquette, C., de Chastellier, C., Nair, S., & Berche, P. (1998). The ClpC ATPase of Listeria monocytogenes is a general stress protein required for virulence and promoting early bacterial escape from the phagosome of macrophages. *Molecular microbiology*, *27*(6), 1235–45.

Rydzak, T., McQueen, P. D., Krokhin, O. V, Spicer, V., Ezzati, P., Dwivedi, R. C., … Sparling, R. (2012). Proteomic analysis of Clostridium thermocellum core metabolism: relative protein expression profiles and growth phase-dependent changes in protein expression. *BMC microbiology*, *12*(1), 214. doi:10.1186/1471-2180-12-214

Sandasi, M., Leonard, C. M., & Viljoen, a M. (2010). The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against Listeria monocytogenes. *Letters in applied microbiology*, *50*(1), 30–5. doi:10.1111/j.1472-765X.2009.02747.x

Sandasi, M., Leonard, C. M., & Viljoen, a. M. (2008). The effect of five common essential oil components on Listeria monocytogenes biofilms. *Food Control*, *19*(11), 1070–1075. doi:10.1016/j.foodcont.2007.11.006

Sander, P., Ellrott, K., & Bo, E. C. (2001). Mechanisms of Streptomycin Resistance : Selection of Mutations in the 16S rRNA Gene Conferring Resistance, *45*(10), 2877–2884. doi:10.1128/AAC.45.10.2877

Schuppler, M., & Loessner, M. J. (2010). The Opportunistic Pathogen Listeria monocytogenes: Pathogenicity and Interaction with the Mucosal Immune System. *International journal of inflammation*, *2010*, 704321. doi:10.4061/2010/704321

Silva, F. a, Borges, F., Guimarães, C., Lima, J. L., Matos, C., & Reis, S. (2000). Phenolic acids and derivatives: studies on the relationship among structure, radical scavenging activity, and physicochemical parameters. *Journal of agricultural and food chemistry*, *48*(6), 2122–6.

Simões, M., Bennett, R. N., & Rosa, E. a S. (2009). Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Natural product reports*, *26*(6), 746–57. doi:10.1039/b821648g

Simões, M., Simões, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology*, *43*(4), 573–583. doi:10.1016/j.lwt.2009.12.008

Stoodley, P., Sauer, K., Davies, D. G., & Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Annual review of microbiology*, *56*, 187–209. doi:10.1146/annurev.micro.56.012302.160705

Taylor, P., Wang, Y., Lee, S. M., & Dykes, G. A. (2013). Biofouling : The Journal of Bioadhesion and Biofilm Potential mechanisms for the effects of tea extracts on the attachment , biofilm formation and cell size of Streptococcus mutans, (May), 37–41.

Trémoulet, F., Duché, O., Namane, a, Martinie, B., & Labadie, J. C. (2002). Comparison of protein patterns of Listeria monocytogenes grown in biofilm or in planktonic mode by proteomic analysis. *FEMS microbiology letters*, *210*(1), 25–31.

Tripoli, E., Giammanco, M., Tabacchi, G., Di Majo, D., Giammanco, S., & La Guardia, M. (2005). The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. *Nutrition research reviews*, *18*(1), 98–112. doi:10.1079/NRR200495

Van der Veen, S., Hain, T., Wouters, J. a, Hossain, H., de Vos, W. M., Abee, T.,Wells-Bennik, M. H. J. (2007). The heat-shock response of Listeria monocytogenes comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. *Microbiology (Reading, England)*, *153*(Pt 10), 3593–607. doi:10.1099/mic.0.2007/006361-0

Vázquez-boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domi, G., González-zorn, B., & Wehland, J. (2001). Listeria Pathogenesis and Molecular Virulence Determinants Listeria Pathogenesis and Molecular Virulence Determinants. doi:10.1128/CMR.14.3.584

Wu, C.-C., Gardarin, A., Martel, A., Mintz, E., Guillain, F., & Catty, P. (2006). The cadmium transport sites of CadA, the Cd2+-ATPase from Listeria monocytogenes. *The Journal of biological chemistry*, *281*(40), 29533–41. doi:10.1074/jbc.M604658200

Yeh, C.-T., & Yen, G.-C. (2003). Effects of phenolic acids on human phenolsulfotransferases in relation to their antioxidant activity. *Journal of agricultural and food chemistry*, *51*(5), 1474–9. doi:10.1021/jf0208132

Yi, S., Zhu, J., Fu, L., & Li, J. (2010). Tea polyphenols inhibit Pseudomonas aeruginosa through damage to the cell membrane. *International journal of food microbiology*, *144*(1), 111–7. doi:10.1016/j.ijfoodmicro.2010.09.005

APPENDIX A

STANDARD CURVE OF BCA ASSAY

Protein concentrations of the samples determined by BCA Assay. BSA standards were prepared by dissolving 1mg/ml of stock BSA in 1% PBS. Stock BSA is diluted to the concentrations of 0µg/ml, 50µg/ml, 125µg/ml, 250µg/ml and 500µg/ml. Then, BCA working solution was prepared by mixing 50x Reagent A and 1x Reagent B. 25µl of corresponding BSA standard and 200 µl Reagent AB mixture was loaded to 96 well plate. Plate was incubated in 37˚C for 30 minutes and cooled for 5 minutes at room temperature. The OD of the wells were measured at 562nm. According to spectrophotometric data, standard curve of BSA was plotted in Microsoft Office Excel.

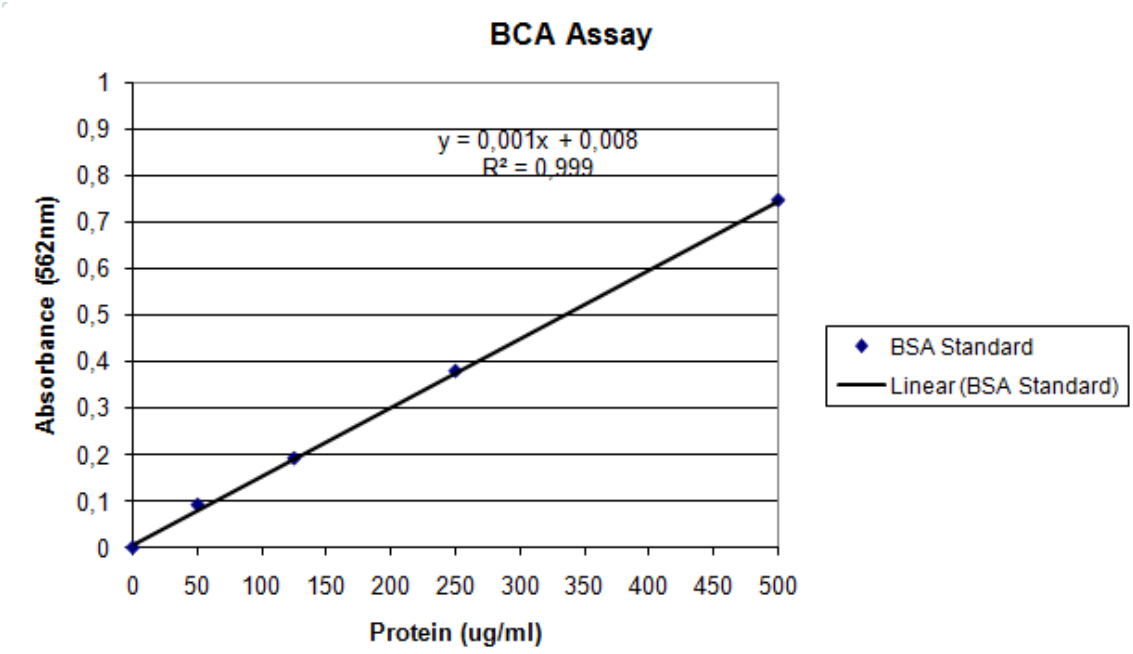

Figure 15. Standard curve of BSA series for BCA assay