CHARACTERIZATION OF OUTER MEMBRANE PROTEINS OF *Salmonella* Enteritidis IN RESPONSE TO PHENOLIC ACIDS STRESS

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This thesis is lovingly dedicated to my invaluable family
for teaching me to struggle against difficulties instead of abandoning...

ABSTRACT

CHARACTERIZATION OF OUTER MEMBRANE PROTEINS OF *Salmonella* Enteritidis IN RESPONSE TO PHENOLIC ACIDS STRESS

Salmonella enterica serovar Enteritidis (*Salmonella* Enteritidis) is one of the most reported foodborne pathogen bacterium throughout the world that causes large outbreaks and may result in deaths. Phenolic acids, synthesized in almost all plants, are important for plant metabolism and protection of plants. They can be thought as promising antimicrobial agents against *Salmonella* Enteritidis. Outer membrane proteins are unique structures to Gram-negative bacteria including *Salmonella* genus and they are the first targets to environmental changes.

This study showed the antimicrobial effect of 3-HPAA, cinnamic acid and *o*-coumaric acid on *Salmonella* Enteritidis *in vitro*. Scanning electron microscopy was used for showing the changes in bacterial shapes. Their effects on the outer membrane protein profile were investigated via proteomic approach. According to results of 96-well microtiter plate assay used for antimicrobial effect determination, among these phenolic acids, 3-HPAA showed 100% growth inhibition and other phenolics retarded the bacterial growth. 30 mM was found as minimum inhibitory concentration (MIC) of 3-HPAA at 24^{th} hour of the experiment. SEM results demonstrated that 3-HPAA causes bacteria to have collapses on cell surface while cinnamic acid and *o*-coumaric acid cause cells to elongate abnormally. SDS PAGE of the OMPs of bacteria who were exposed to phenolic acids showed changes in amounts of proteins in some protein bands compared to control OMPs. 2-D PAGE results of OMPs of control bacteria via OMPs of 3-HPAA, cinnamic acid and *o*-coumaric acid treated bacteria, changes in sizes of spots were observed.

ÖZET

FENOLİK ASİT STRESİNDE *Salmonella* Enteritidis'in DIŞ MEMBRAN PROTEİNLERİNİN KARAKTERİZASYONU

Salmonella enterica serovar Enteritidis (*Salmonella* Enteritidis) en sık rastlanan gıda patojenlerinden biridir. Salgınları sık görülür ve ölümle de sonuçlanabilir. Fenolik asitler hemen her bitki tarafından sentezlenir ve bitkilerdeki çeşitli süreçlerde etkili olmasının yanısıra, bitkilere korunma sağlar. Bu nedenle *Salmonella* Enteritidis'e karşı antimikrobiyal maddeler olarak kullanılabileceklerine dair beklentiler yüksektir. Bu çalışmada kullanılan *Salmonella* cinsine dahil olan bakteriler de Gram negatif özellik göstermektedirler. Dış zar proteinleri sadece Gram negatif bakterilerde görülür ve çevresel değişimlere karşı ilk cevap veren yapılardır.

Bu çalışmada, 3-hidroksifenilasetik asit (3-HPAA), sinamik asit (CA) ve o-kumarik asidin *Salmonella* Enteritidis üzerindeki antimikrobiyal etkisi laboratuvar ortamında kanıtlanmıştır. Taramalı elektron mikroskobu (TEM) kulanılarak bu maddelerin bakteri hücresinin şekline etkisi aydınlatılmıştır. Aynı zamanda bu maddelerin bakterinin dış zar proteinlerinde yol açtığı değişimler proteomik yöntemler kullanılarak gösterilmiştir . 96 kuyucuklu plaka yöntemiyle bulunan sonuçlara göre, test edilmiş olan üç fenolik asit arasında 3-HPAA bakteri üremesini %100 engellerken, diğerleri bakteri üremesini geciktirmiştir. 24. saatteki yüzde inhibisyon değerlerine göre 3-HPAA için minimum inhibisyon konsantrasyonu 30 mM olarak bulunmuştur. TEM sonuçları 3-HPAA uygulamasının bakterilerin hücre yüzeylerinde göçüklere neden olduğunun, sinamik asidin ve o-kumarik asidin ise hücrelerin uzamasına neden olduğunun bulunmasını sağlamıştır. Madde uygulanan bakterilerin dış zar proteinleri SDS PAGE yöntemiyle ayrılmıştır. Kontrol bakterilerinin SDS PAGE sonuçlarıyla karşılaştırılınca, fenolik madde uygulanmış bakterilerin proteinlerinde, birkaç banttaki protein miktarlarında değişiklikler olduğu görülmüştür. 2-D PAGE ile ayrımın sonucunda 3-HPAA, sinamik asit ve o-kumarik asidin uygulanmasının bazı spotların boyut değişikliğine uğramasına sebep olduğu gösterilmiştir.

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LIST OF ABBREVIATIONS

APS	Ammonium Per Sulphate
CA	Cinnamic Acid
CDC	Centre for Disease Control and Prevention
cfu	Colony Forming Unit
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
g	Gram
g	Gravity
h	Hours
IEF	Isoelectric Focusing
IPG	Immobilized pH Gradient
M	Molar
MIC	Minimum Inhibitory Concentration
mM	Milimolar
µl	Microliter
ml	Mililiter
mg	Miligram
NCTC	National Collection of Type Cultures
nm	Nanometer
OD	Optical Density
OMPs	Outer Membrane Proteins
OOWW	Olive Oil Waste Water
PAGE	Polyacrylamide Gel Electrophoresis
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscope
TEMED	Tetramethyletylenediamine
TSA	Tryptic Soy Broth
TSB	Tryptic Soy Agar
V	Volt
v/v	Volume/Volume

WHO	World Health Organisation
2-D PAGE	2-Dimensional Polyacrylamide Gel Electrophoresis
3-HPAA	3-Hydroxyphenylacetic Acid

CHAPTER 1

LITERATURE REVIEW

1.1. *Salmonella* spp.

1.1.1. Properties of Salmonellae

Salmonellae, which are zoonotic and pathogenic in animals, belong to Enterobacteriaceae family. These facultative anaerobic and non-lactose fermenting bacteria do not form spores and they are usually motile. Salmonellae can not survive below pH 3.8 and above pH 9. They can survive in the temperature range of 5 ^{o}C to 45^{o}C however their optimum growth temperature is 35-37 ^{o}C. The bacteria require at least 0.94 water activity in the environment for survival and are generally sensitive to high salt concentrations (Cliver, 2002).

The first isolation of *Salmonella* was reported by Daniel E. Salmon from a pig in 1885. Since its discovery, more than 2500 serotypes of *Salmonella* were discovered, thus, taxonomy and nomenclature of the genus is a persistant problem (Bhunia, 2008). After using several nomenclatures, nowadays it is accepted that *Salmonella* genus has two species named *Salmonella enterica* with six subspecies and *Salmonella bongori* according to Centre for Disease Control and Prevention (CDC website, 2012). *Salmonella* genus has 2579 serovars in 2007 (WHO Collaborating centre for Reference and Research on *Salmonella*, 2007) and *S. enterica* subspecies have about a total of 2500 serotypes determined depending upon their antigens somatic O, flagellar H and capsular Vi. Further subdividing of serotypes is carried out accordig to the results of 1: Biotyping which shows biochemical differences between the bacteria in same serotype. 2: Phage typing that demonstrates the distinct susceptibilities of bacteria in the same serotype to a bacteriophage (Velge et al., 2005). The way of writing the names of the species and serotypes of *Salmonella* genus is putting the genus and species name in italics while using the capitalized Roman for the serotype. The organism that is used in this study is written as *Salmonella enterica* serovar (or serotype) Enteritidis which can

be shortened as *S. enterica ser.* Enteritidis. After the first introduction of the bacteria with its whole name, it can also be shortened as *Salmonella* Enteritidis in the following usages of its name in the same text. Using the serovar name without putting species epithet (i.e. *S.* Enteritidis) is accepted as incorrect usage of nomenclature for Salmonellae (Scientific nomenclature, CDC, ISSN 1080-6059).

Table 1.1. Salmonellae Nomenclature

(Source: Su and Chiu 2007)

Taxonomic position (writing format) and nomenclature			
Genus (capitalized, italic)	Species (italic)	Subpecies (italic)	Serotypes (or serovars) (capitalized, not italic)*
Salmonella	*enterica*	*enterica* (or subspecies I)	Choleraesuis, Enteritidis, Paratyphi, Typhi, Typhimurium
		salamae (or subspecies II)	9,46:z:z39
		arizonae (or subspecies IIIa)	43:z29:-
		diarizonae (or subspecies IIIb)	6,7:l,v:1,5,7
		houtenae (or subspecies IV)	21:m,t:-
		indica (or subspecies VI)	59:z36:-
	bongori	subspecies V	13,22:z39:-
	subterranea[18]		

*: Some selected serotypes (serovars) are listed as examples.

1.1.2. Salmonellosis

Salmonella is one of the major food borne pathogenic bacterial genus in the world. Non-typhoid *Salmonella*, which includes *Salmonella* Enteritidis, is the cause of gastroenteritis called salmonellosis. After 24-48 hours of ingesting the contaminated food, the symptoms of salmonellosis are seen as fever, nausea, vomiting, abdominal cramps and diarrhea. Infectious dose of *Salmonella* to be able to cause salmonellosis is nearly 100,000 bacilli (Ricke et al., 2005). Because the pH of the stomach is about two when it is empty, high acidity does not allow bacteria to survive in the stomach if the bacteria are not taken by a contaminated food. pH of the stomach becomes nearly six while ingesting food, thus pH of the environment becomes suitable for *Salmonella* to survive and cause illness (Lawrence, 1998). Disease is usually resolved by the host, but in some cases bacteria can enter bloodstream and cause sepsis (Cliver, 2002). In high

risk population groups as very young, elderly or immunosupressed people, salmonellosis may be fatal. (Messens et al., 2005)

Major sources of the *Salmonella* that causes salmonellosis are meat, milk, poultry products and especially eggs. However tomatoes, seed sprouts, watermelons, cantaloupes, lettuce, ice cream, cheese and unpasteurised apple cider and orange juice are the other sources for salmonellosis (Guo et al., 2001; Bhunia, 2008).

Salmonella Enteritidis is the main bacterium that causes salmonellosis through eggs. Contamination can occur by two possible routes. Transovarian route, in other words vertical transmission occurs when reproductive organs of the chicken are contaminated, thus, yolk membrane or the albumen are directly contaminated before the eggshell is formed. In the other route called trans-shell route or horizontal transmission, penetration of *Salmonella* through the eggshell occurs. The faeces-contaminated eggshell contains *Salmonella* for four weeks even if it is kept at 4^{o}C. Bacterial strain, temperature, moisture, number of bacteria present, storage conditions of eggs, eggshell defects and porosity of the shell are important factors in transmission of *Salmonella* through the eggs (Messens et al., 2005).

The contamination of tomatoes can be caused from irrigating the tomatoes with contaminated water in the flowering phase. When the bacteria contaminate the flowers, they would be able to survive in the tomatoe fruit (Guo et al., 2001). For the bacteria live in the tomatoe, it is not enough to wash tomatoes to get rid of the *Salmonella*, tomatoes should be cooked before eating. Because the cooking or pasteurisation are good ways to eliminate these pathogen bacteria (Cliver, 2002).

In animals, *Salmonella* can colonize in organs other than intestine, such as lymph nodes, with no symptoms of the disease. For it is difficult to determine the contamination without symptoms of salmonellosis, a contaminated animal can be used as poultry product or meat without noticing the bacteria (Cliver, 2002). When these products are ingested by human, salmonellosis occurs. Also, the faeces of people who had salmonellosis, can include *Salmonella* for about one to three months. Thus, it is very important to pay attention to hygiene while using the public toilets.

One of the most arised illness worldwide is salmonellosis with the global approximation of 14-120 people per 100,000 people (Bollaerts et al., 2008). Worldwide there were 1.3 billion cases of gastroenteritis reported in 2008 according to CDC. In the report of *Salmonella* annual summary, 2010, it was shown that the incidence rate of

salmonellosis in 2009 was 17.8/100,000 while 20.4/100,000 in 2010 in the USA. That indicates a 14.5% raise of salmonellosis in year 2010 compared to year 2009. In the United States, two million cases occur annually and mortality rate is 500-1000 people. In CDC report, it was shown that the incidence of *Salmonella* Enteritidis had an increasing rate (Bhunia, 2008). It was reported that in Turkiye, 47% of human *Salmonella* isolates were *Salmonella* Enteritidis between 2000-2002 (Kalender and Şen 2008). Nevertheless the accurate percentage of salmonellosis is not known because of the reports do not demonstrate the whole cases due to the underreporting the food poisining cases in Turkiye. In USA, many outbreaks of salmonellosis caused by *Salmonella* Enteritidis have occured. In January 2012, 52 people became ill after eating lettuce, ground beef, cheese and tomatoes at mexican style restaurants. From five states of USA, a total of 25 patients were reported who had salmonellosis from Turkish pine nuts or products of it in November 2011. A total of 46 people have become ill because of the alfalfa sprouts and spicy sprouts in July 2011. All the products mentioned, which are suspected to be the cause of these food poisining cases, were investigated in laboratories in USA and *Salmonella* Enteritidis was detected in these foods. Between May and December 2010, a multistate outbreak of *Salmonella* Enteritidis with nearly 1,939 illnesses caused by contaminated shell eggs was reported (CDC, 2012).

1.2. Cell Wall Properties of Gram-Negative Bacteria

Cell envelope of Gram-negative bacteria has outer membrane structure at the outermost layer of the cell. Under the outer membrane, peptidoglycan layer which is attached to outer membrane with lipoprotein is placed. Peptidoglycan which is a sugar polymer acts as a support layer for the cell envelope (Sikkema et al., 1995). Periplasmic space that acts as a storage space for enzymes, binding proteins, oligosaccharides and toxins takes part between peptidoglycan layer and the cytoplasmic membrane which is the inner part of the cell envelope. Figure 1.1 shows the general sturucture of Gram-negative bacteria cell envelope.

Outer membrane, which consists of lipopolysaccharides, phospolipids and proteins, surrounds the cell envelope. Permeability of the cell wall depends on the phospholipid composition of the membrane and membrane proteins which interact with lipids. Adjustment of these structures according to environmental conditions is crucial

for the bacterial survival (Heipieper et al., 1991). Penetration of the molecules with molecular weight of 600-1000 daltons through the outer membrane can not occur (Sikkema et al., 1995).

Outer membrane has 2 layers, lipopolysaccharide which is the outer leaflet and phospholipid which is the inner one. Lipopolysaccharide consists of O side chain, core oligosaccharide and lipid A regions. Porins and receptor proteins are placed through the outer membrane (Bhunia, 2008). Outer membranes act as selective permeable barriers for environmental molecules. The suitable composition of the outer membrane with highly negatively charged lipopolysaccharide which helps to exclude hydrophilic molecules, and porin proteins which are hydrophilic channels permit selective permeability of the outer membrane for small hydrophilic molecules. Hydrophobic compounds encounter with a very low permeability of the outer membrane because of hydrophobic or in other words lipophilic lipopolysaccharide (Sikkema et al., 1995).

Porins are not permeable for hydrophilic molecules greater than 550-650 daltons. Although porins vary from strain to strain, it can be said that around 35,000-40,000 daltons is the general weight of the major outer membrane proteins (Hancock and Nikaido 1978). Also outer membrane proteins (OMPs) of Gram-negative bacteria act as virulance factors and used as epidemiological markers for pathogens. Immune defence factors of the host are suppressed by these proteins (Kumar et al., 2007).

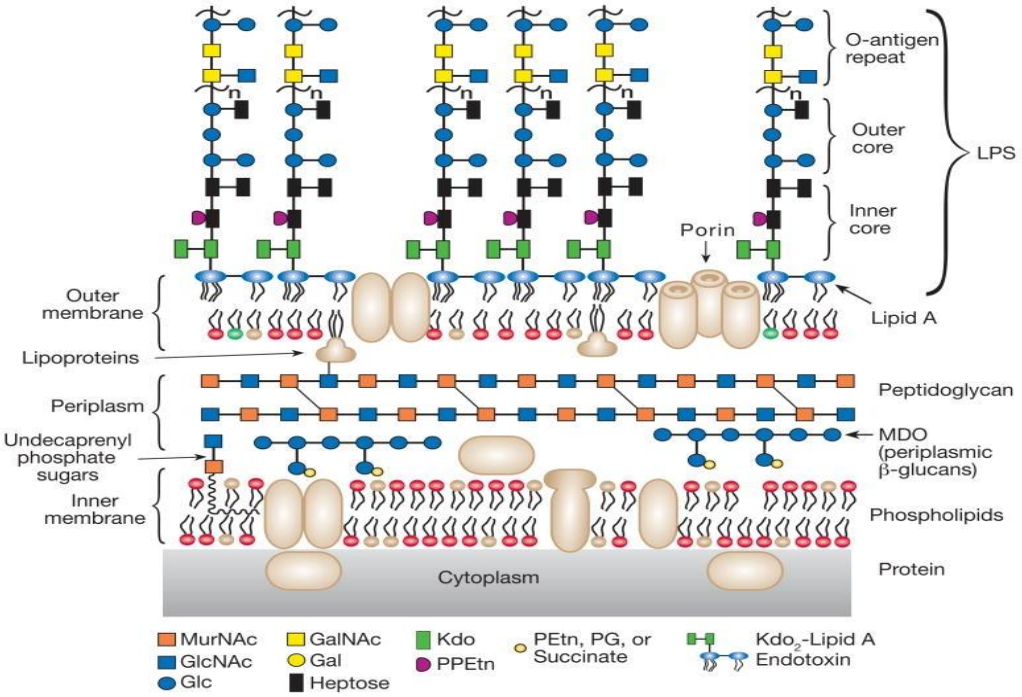


Figure 1.1. The Cell Wall of Gram Negative Bacteria

(Source: nbci, 2012)

1.3. Phenolic Compounds

Phenolic compounds are secondary metabolites that naturally found in almost all plant species. They are synthesized by plants via the shikimate and phenylpropanoid pathways by using the carbohydrates as precursors (Hurtado-Fernandez et al., 2010). They play important roles in plant processes like plant growth, lignification, pigmentation, pollination, protection against predators, environmental stresses and plant pathogens (Duthie et al., 2003). Phenolics are important part of the human diet and they have plenty of beneficial effects for human health. Besides their antimicrobial properties, they have antioxidant (Karaosmanoglu et al., 2010), antimutagenic, anticarcinogenic, cardioprotective (Andjelkovic et al., 2006) and antiallergenic effects (Hurtado-Fernandez et al., 2010).

Although there are 8000 different phenolic compound structures known, the general chemical structure for them is a benzene ring with one or more hydroxyl groups attached. They fall into classes related to their carbon skeleton nature; simple phenolic

compounds and polyphenolic compounds (Hurtado-Fernandez et al., 2010). Figure 1.2 shows the general structures of the phenolic compounds.

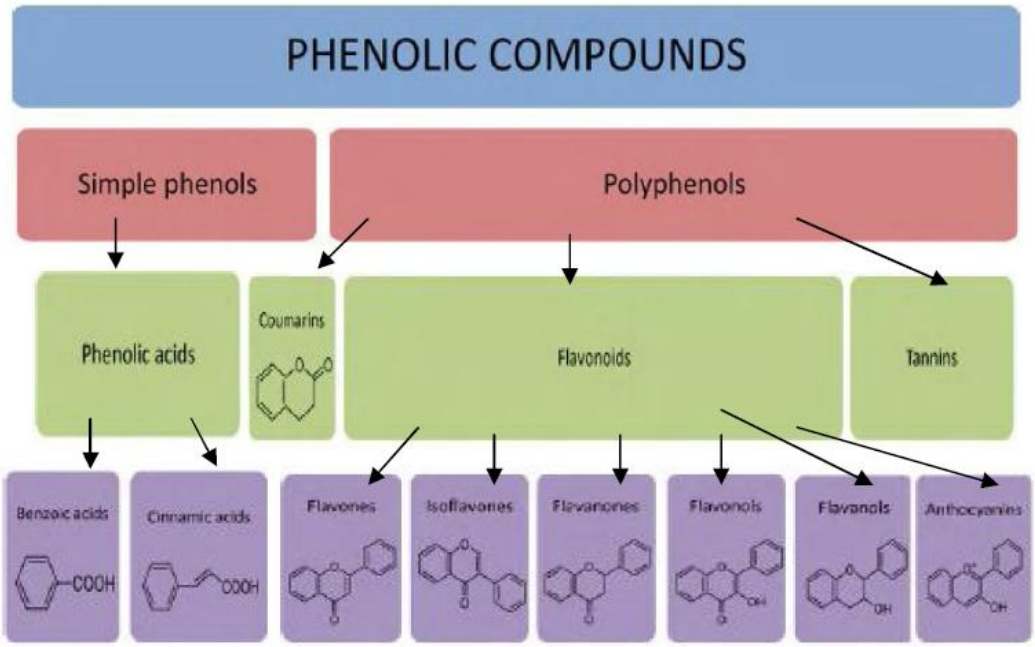


Figure 1.2. Categories and Representative Structures of the Phenolic Compounds
(Source: Hurtado-Fernandez et al., 2010)

Phenolic acids are structurally simple phenolic compounds and found in high amounts in nature. They have two main groups called hydroxybenzoic acids and hydroxycinnamic acids. Hydroxycinnamic acids have an ethylene group between carboxylic group and benzene ring while hydroxybenzoic acids have a directly attached carboxylic group to the benzene ring. Plants and plant derived foods contain phenolic acids in free or bound forms. Bound form is more common and found as esters, glycosides and bound complexes (Andjelkovic et al., 2006). Common chemical structure for hydroxybenzoic acids is C_6-C_1 while for hydroxycinnamic acids is C_6-C_3 (Tripoli et al., 2005).

The certain biological effects of phenolic compounds can be explained by the interaction of phenolics with membrane lipids and proteins. Their biological actions depend on the common chemical structure, phenol ring. After interacting with proteins, phenolics result in biological effects related to the function of the protein they were interacted (Fraga et al., 2010). Poncet-Legrand et al. reported that proline rich proteins

can be the targets for phenolic compounds to interact. Phenolic rings and proline residues have a hydrophobic association which results in protein precipitation *in vitro* (Poncet-Legrand et al., 2007). Many studies showed that cyclic hydrocarbons which include phenolic acids are toxic to the cells. It can be said that because these compounds are lipophilic (or hydrophobic), they interact with hydrophobic parts of the cell to display their toxic effects (Sikkema et al., 1995). Main targets of phenolic compounds as antimicrobial agents are thought to be the cell membranes for hydrophobicity of the phenolics is strongly related with their toxicity on bacterial cells. Phenolics are accepted as membrane active antimicrobial agents and they affect the cell membrane (Heipieper et al., 1991). Other parameters that important for antimicrobial activity of phenolic acids are number of hydroxyl groups attached to the aromatic ring because of the delocalized electrons on double bonds. Phenolics that have two hydroxyl groups (catechol) have more antimicrobial effect than the ones which have three hydroxyl groups (galloyl). The third hydroxyl group stabilizes the delocalization of electrons which results in decreased chelation of +2 charged ions by the phenolic compound, and causes reduced antimicrobial effect (Ultee et al., 2002). Puupponen-Pimia et al. used apigenin, caffeic acid, 3-coumaric acid, ferulic acid and *trans*-cinnamic acid on selected Gram-negative bacteria including *Salmonella.* They reported that these phenolic acids antimicrobially affected all tested Gram-negative bacteria, and discussed that antimicrobial activity of pure phenolic compounds might be affected from the degree of the hydroxyl groups attached on aromatic ring (Puupponen-Pimia et al., 2001).

1.3.1. Phenolic Acids in This Study

The phenolic acids that were used in this study named 3-Hydroxyphenylacetic acid (3-HPAA), cinnamic acid (CA) and *o*-coumaric acid are simple phenolics with a benzene ring in their structures.

3-HPAA with a molcular weight of 152.15 g/mole (Sigma-Aldrich) is a simple phenolic acid with one hydroxyl group attached on the benzene ring and has a 2-carbon side chain (C6-C2 structure) as it is seen in Figure 1.3. (Balasundrami et al., 2006). It is water soluable and found in olive oil waste water (OOWW) mostly (Zafra et al., 2006). OOWW is produced during the olive oil extraction during olive oil production process (Roig et al., 2006) and it contains high levels of polyphenols (Mulinacci et al., 2001).

Although 3-HPAA is found in olive oil waste water, in the study that was carried out in IZTECH, suprisingly, 3-HPAA was also present in olive oils which were produced in Burhaniye, Gomec and Altinoluk which are in North Aegean Region of Turkiye (Ocakoglu 2008).

Figure 1.3. The chemical structure of 3-HPAA
(Source: Del Rio et al., 2010)

Chemical structures of cinnamic acid and *o*-coumaric acid (or 2-hydroxycinnamic acid), are similar except the position of side chain and one hydroxyl group that *o*-coumaric acid possesses but cinnamic acid does not. Figure 1.4 demonstrates the chemical structures of these phenolic compounds. Molecular weights of cinnamic acid and *o*-coumaric acid are 148.16 g/mole and 164.16 g/mole respectively. Cinnamic acid can be dissolved in ethanol while *o*-coumaric acid in DMSO (Sigma-Aldrich, MSDS). They can be found in vegetables and fruits such as grapes, berries and products of plants as tea and wine (Puupponen-Pimiae et al., 2001).

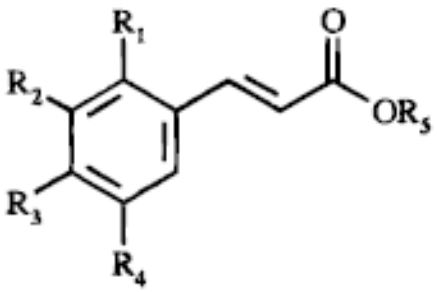


Figure 1.4. Chemical structures of cinnamic acid (All R are H) and *o*-coumaric acid (R1 is OH, other R are H) (Source: Foti et al., 1996)

1.4. Published Studies

There are various studies in the literature that show the effects of phenolic compounds on Gram-negative and Gram-positive bacteria. Karaosmanoglu et al. demonstrated the antimicrobial effects of phenolic compounds including cinnamic acid, ferulic acid, 4-hydroxybenzoic acid, syringic acid and vanillic acid, in the concentration that they found in olive oil, on *S. enterica*, *Escherichia coli* (*E. coli*) and *Listeria monocytogenes* (Karaosmanoglu et al., 2010).

Puupponen-Pimia et al. demonstrated the effect of some phenolic compounds including phenolic acids apigenin, caffeic acid, chlorogenic acid, 3-coumaric acid, ferulic acid and *trans*-cinnamic acid on several bacteria including *E. coli* and *S. enterica*. They found that the DNA repair mutant *E.coli* was the most effected bacterial strain among Gram-negative bacteria tested, so they hypothesized that the mechanism of antimicrobial effect of the phenolic acids may depend upon their interaction with DNA. However, relying on the results of the antimicrobial activities on wild type *E. coli* and *S. enterica*, they concluded that there could be another explanation about the mechanism such as outer membrane disruption effect of the phenolic acids (Puupponen-Pimia et al., 2001).

The binding properties of phenolics to proteins was demonstrated by Rawel et al. Phenolic compounds involving chlorogenic acid, ferulic acid and gallic acid bind non-covalently to albumin and lysozyme at neutral and low pH levels *in vitro* (Rawel et al., 2005).

Yi et al. studied the effects of tea polyphenols especially catechins toward Gram-negative bacteria *Pseudomonas*. They reported that according to leakage of cell constituents and transmission electron microscopy images of the tea polyphenol exposed bacteria, tea polyphenols affect the outer membrane by alterating its integrity and these compounds have disruptive effects on the outer membrane. Also in proteomic assays, they found that the membrane proteins of polyphenol-treated bacteria increased compared to control group (Yi et al., 2010).

3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxycinnamic acid, 3-propionic acid and 3-phenylpropionic acid were examined for their effects on *S. enterica* strains by Alakomi et al. They discussed that these phenolic acids destabilized the *Salmonella* strains tested. They also announced that in

their previous study gallic acid disintegrated the outer membrane of *Salmonella* (Alakomi et al., 2007).

In the study of Cueva et al. benzoic acids, phenylacetic acids and phenylpropanoid acids were shown to have antimicrobial effect on bacteria involving *E. coli*. They showed that the effect of phenolic compounds on bacteria is specific to strain and it depends on concentration and structure. They announced that although Gram-negative bacteria have the outer membrane which protect the Gram-negative bacteria as a hydrophobic layer against large hydrophobic compounds, phenolic acids may cross through the outer membrane and show antimicrobial effect because of their small molecular weights (Cueva et al., 2010).

Phenol was used for exposing on *E. coli* K-12 strain by Zhang et al. After treating the bacteria with phenol, they isolated the outer membrane proteins and seperated them by 2-D PAGE analysis. 50 protein spots were perceived and 11 of these were altered. After MALDI-TOF/MS analysis, it was found that OmpT which is a protease, LamB which is a porin responsible for diffusion of maltose and FadL which is a protein binding long chain fatty acid that helps membrane permeability were upregulated after phenol treatment while two OmpA which are key outer membrane proteins responsible for preserving the integrity of cell surface were downregulated. It was discussed that increasing of FadL shows that the bacteria attempt to maintain the fluidity of the membrane that was decreased by downregulation of OmpA. For LamB is a porin, increasing amount of LamB helped to limit the entry of phenol into the cell. The main source of this proteolitic reaction was OmpT which had a decreased amount after exposure of phenol, for providing permeability decline of outer membrane to decrease the enterance of phenol into the cell (Zhang et al., 2010).

Salmonella enterica serovar Thompson was exposed to sublethal concentrations of thymol in the study of Pasqua et al. Total protein extraction of bacteria was carried out after treatment with thymol for performing 2 dimensional PAGE. After MALDI-TOF mass spectrometer analysis it was displayed that 44 proteins were altered which seven of them were outer membrane proteins. OmpX and two OmpA were upregulated in thymol exposed cells. The outer membrane chanel protein TolC started to be expressed in treated cells. AcrB which is a transporter protein and TolC form the efflux system together. Because two multidrug-resistant *Salmonella* strains use efflux system as a protection from detergents and bile salts, Pasqua et al. hypothesized that the

resistance of *Salmonella* from thymol treatment is related to efflux system. With all the altered proteins found, it was concluded that thymol is a molecule that causes *Salmonella* to act as if there were different types of stresses in the environment (Pasqua et al., 2010).

CHAPTER 2

INTRODUCTION

Salmonella Enteritidis is one of the major foodborne pathogen bacterium which spreads worldwide, causes plenty of salmonellosis outbreaks all over the world and can be lethal in elderly, very young or immunosupressed human. For *Salmonella* isolates from human are considered as antibiotic resistant, it is necessary to search for new and effective antimicrobial agents which are not harmful to human health. Phenolic compounds which are naturally found in almost all plants are important in several plant processes and they protect the plant from microorganisms, thus they can be promising antimicrobial agents against bacteria.

Outer membrane proteins are the first barriers that come face to face with environmental changes. For these surface exposed proteins are responsible for transportation of nutrients and macromolecules, it is not surprising to think them as the first targets of antimicrobial agents.

In this study, the antimicrobial effect of 3-HPAA, *o*-coumaric acid and cinnamic acid on *Salmonella* Enteritidis was investigated and their effects on the bacterial shape and outer membrane protein profiles were elucidated.

CHAPTER 3

MATERIALS AND METHODS

3.1. Bacterial Culture

Salmonella enterica subspecies *enterica* serovar Enteritidis was purchased from National Culture Type of Collection (NCTC, United Kingdom). Tryptic soy broth (Fluka) and tryptic soy agar (Agar Agar, Merck) were used for cultivation of the bacteria. The bacterial culture was maintained in TSB medium containing 20% glycerol at -80 ^{o}C and transferred to TSA plates as needed. They were subcultured every week by using TSA plates and/or slant agars, and kept at 4 ^{o}C for short term storage.

3.2. Phenolic Acids

3-HPAA, *o*-coumaric acid and cinnamic acid were supplied commercially (Sigma Aldrich). Solvents used for dissolving phenolic acids were chosen according to these parameters: 1. maximum dissociation properties of the phenolic acids and 2. not affecting the bacterial growth. Sterile ultrapure water for 3-HPAA, DMSO (3% in final volume) for *o*-coumaric acid, and ethanol (0.1% in final volume) for cinnamic acid were used as solvents in all experiments. All the phenolic acid solutions were prepared at the time of the experiment.

3.3. Determination of Antimicrobial Activities of the Phenolic Acids

3.3.1. Preparation of the Bacterial Culture

A single colony of *Salmonella* Enteritidis was inoculated into four ml of TSB and incubated at 37^{o}C for approximately 17 hours without shaking. Then, optical density (OD) of the culture was adjusted with TSB to the value which was determined

in previous studies at 600 nm wavelenght in which the culture has a $1x10^8$ cfu/ml bacterial load. Dilution of the bacterial culture with TSB (1:9, v/v) was carried out to 10^{-6} dilution. The dilution that provided $1x10^4$ cfu/ml was used for 96-well microtiter plate assay. For confirmation, tubes that contained 10^{-5} and 10^{-6} dilutions were used for enumeration of the bacterial culture.

3.3.2. Preparation of the Phenolic Acids

The concentration ranges of 3-HPAA, *o*-coumaric acid and cinnamic acid used in this study were shown in Table.1. Firstly, the maximum concentration of each phenolic acids was prepared by dissolving the required amount in the appropriate solvent and TSB medium (see section 3.2). Then, the lower concentrations were achieved by diluting from the maximum concentration by adding TSB medium.

Table.3.1. The prepared concentrations of phenolic acids that were prepared in test tubes for microtiter plate assay

Phenolic Acids	3-HPAA	Cinnamic Acid	*o*-Coumaric Acid
Concentrations (mM)	80	30	30
	70	25	25
	60	20	20
	50	15	15
	40	10	
	20	5	

3.3.3. 96-Well Microtiter Plate Assay

Assay which was described by Karaosmanoglu et al. (2010) was carried out for determination of antimicrobial effects of phenolic acids on *Salmonella* Enteritidis. After pouring 100 µl of phenolic acid solution into a well of flat bottom 96-well microtiter plate (Bio-Grainer, Germany), 100 µl of the bacterial culture in logarithmic growth phase with $1x10^4$ cfu/ml were added into the same well. 100 µl of phenolic acid solution

and the same volume of TSB without bacteria were dispensed into a well for using as blank. These processes were applied for each concentration of the phenolic acid tested, and to have the concentrations that were shown in table 3.1, two-fold of each concentration was prepared in test tubes. For control, the same ratio (v/v) of the bacterial culture and TSB were filled in a well with a final volume of 200 µl and 200 µl of TSB were also poured into another well. Four wells were used for control to have the average value. Absorbance was measured by spectrophotometer (Thermo Multiscan Spectro Reader, Finland) at 600 nm in every 3 hours for 24 hours during incubation at 37^{o}C. After bacterial dilution with TSB (1:9, v/v) was done until 10^{-6} dilution, 10^{-5} and 10^{-6} diluted bacterial cultures were inoculated onto TSA plates by spread plating and they were allowed to incubate overnight at 37^{o}C. Experiments for each phenolic acid were carried out four times.

3.4 Scanning Electron Microscopy

3.4.1. Preparation of the Bacterial Preparates

Two mililiters of 50 mM 3HPAA, 20 mM cinnamic acid and 30 mM *o*-coumaric acid were prepared in sterile glass tubes in appropriate solvents (see section 3.2). Two ml of TSB were added to phenolic acid solutions to have the half of these concentrations in four ml of solutions. A single colony of *Salmonella* Enteritidis was inoculated into each phenolic acid solution and into four ml TSB for control group. Inoculated solutions were placed in incubator at 37^{o}C for 18 hours.

Cultures were transferred into sterile 1.5 ml centrifuge tubes and cells were harvested by centrifugation at 8000 g for 10 minutes. Cells were washed with 0.1% peptone for three times at 8000 g for 10 minutes in each washing step. Washed bacteria were dissolved in 50-100 µl of 0.1% peptone and 10-20 µl of the cultures were poured onto a piece of aluminium foil and spreaded by loops. Foils were allowed to dry for approximately 15-20 minutes. After drying, they were cut into small pieces by scissors and put into petri dishes. Preparates were kept in petri dishes in dessicator for maximum one night.

3.4.2. Visualizing the Preparates by SEM

Preparates were placed onto small metal grid which were applied by small pieces of carbon band. The preparates were covered by gold particles for one minute and placed in scanning electron microscopy (Philips XL 30). Photographs of the preparates with different magnifications were taken.

3.5. Analysis of OMPs of Bacteria in the Presence of Phenolic Acids

3.5.1. Isolation of OMPs

Isolation of OMPs of *Salmonella* Enteritidis was carried out according to protocol explained in Lin et al. (2008) with modifications after phenolic acid exposure to bacteria with higher volume and the same ratio used in 96-well microtiter plate assay described previously (see section 3.3.3).

3.5.1.1. Preparation of Bacterial Culture

Bacterial culture was prepared in the same way which was stated in section 3.3.1. without bacterial dilution. *Salmonella* Enteritidis culture of which OD was adjusted to required value was used in the experiment. Bacterial dilution (1:9, v/v) was done for cfu/ml calculations by spread plating of appropriately diluted cultures on TSA plates with overnight incubation at 37^{o}C.

3.5.1.2. Preparation of Phenolic Acids

The concentration which caused retarded bacterial growth at 18^{th} hour of the incubation in antimicrobial experiments was chosen for phenolic acid treatment that was done before isolation of OMPs. Chosen concentration of the phenolic acid was prepared in 101 ml volume by using the twice of the required amount of phenolic acid in TSB and appropriate amount of solvents which were stated in section 3.2. Prepared phenolic

acid solution (101 ml) and the same volume of TSB were mixed. Four ml of this solution was taken out and poured into a glass tube for using as blank.

.3.5.1.3. Treatment of Bacteria with Phenolic Acids

Two ml of OD-adjusted bacterial culture was inoculated into the solution which includes the same volume of TSB and phenolic acid with a final volume of 198 ml. The same process was applied for 198 ml of TSB as control. These two inoculates were allowed to incubate at 37^{o}C for 18 hours to make the bacteria reach to stationary phase of the growth. Bacterial dilution until 10^{-6} was done (9:1, v/v) and the tubes provided 10^{-5} and 10^{-6} diluted bacterial cultures were used for cfu/ml calculation. They were incubated at 37^{o}C in overnight incubation. The OD measurement at 600 nm was done at 18th hour of incubation to check the bacterial growth and inhibition of bacterial growth in phenolic acid treated culture which were used in OMP isolation.

3.5.1.4. Protein Isolation

The method which was described by Lin et al. (2008) was used for OMP isolation with some modifications. Centrifugation was carried out at 4000 g for 20 minutes at 4^{o}C to harvest the cells. After removal of the supernatant, pellet was washed twice with sterile saline (0.85%, w/v). Washed pellets were kept at -80^{o}C for 90 minutes before resuspending in equal volumes of sterile ultrapure water and 100 mM Tris.HCl (pH:7.4). Sonication was performed by 85% power with eight cycle for phenolic acid treated bacteria and 10 cycle for control bacteria when one cycle of sonication includes 30 seconds of sonication with nine second of intervals. Sonication process was done on ice in all cycles. After sonication, centrifugation at 6000 g for 20 minutes at 4^{o}C was carried out and supernatant was collected. Ultracentrifugation of the supernatant was performed at 100,000 g for 45 minutes at 4^{o}C. Supernatant was removed and pellet was resuspended in 50 mM Tris containing 2% *N*-lauroyl sarcosine. Resuspended pellet was incubated at 25 ^{o}C for 20 minutes and ultracentrifuged at 100,000 g for 45 minutes at 4^{o}C. After the removal of the supernatant pellet was resuspended in 50 mM Tris.HCl

(pH: 7.4) and transferred to 1.5 ml centrifuge tubes (Protein LoBind Tubes, Eppendorf). Proteins were kept at -80 ^{o}C until usage.

3.5.2. Seperation of the Proteins

3.5.2.1. Denaturating SDS PAGE of OMPs

3.5.2.1.1. Preparation of Gels and Running Buffer

Stacking and seperating gels were prepared with ingredients of acrylamide/bisacrylamide solution, Tris.HCl (1.5 M, pH: 8.8 for seperating gel and 0.5 M, pH: 6.8 for stacking gel), SDS (10%, w/v), ultrapure water, APS (10%, w/v) and TEMED with required amounts. Running buffer was prepared in 5X concentration and was diluted to 1X with distilled water. It includes 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS. The amounts of the reagents for preparing the seperating and stacking gels in SDS PAGE are shown in Table 3.2.

Table 3.2. The amounts of the reagents for polyacrylamide gels

Concentration of the gel	12%	4%
Acrylamide/Bisacrylamide	4ml	0.65 ml
Ultrapure Water	3.35 ml	3.05 ml
1.5 M Tris.HCl (pH 8.8)	2.5 ml	-
0.5 M Tris.HCl (pH 6.8)	-	1.25 ml
10% SDS	0.1 ml	0.05 ml
10% APS	0.05 ml	0.025 ml
TEMED	0.005 ml	0.005 ml
Total Volume of the Solution	10 ml	5 ml

3.5.2.1.2. Loading of Proteins into the Gel and Running Conditions

Concentrations of the proteins were measured by Bradford assay (Figure A.1.) and adjusted to concentration that was loaded into gel by diluting with 50 mM Tris.HCl

(pH:7.4). Onto each concentration-adjusted protein, the same volume of sample buffer that consists of 62.5 mM Tris.HCl (pH 6.8), 20% glycerol (v/v), 2% SDS (w/v), 5% β-mercaptoethanol (v/v) was added. Protein-sample buffer mixtures were heated for 10 minutes at 100^{o}C for denaturation step. The same amount of proteins were poured into wells for each concentration of both OMPs from phenolic acid treated bacteria and OMPs for control bacteriaThe protein marker (Fermentas SM0671, Fermentas SM0431, Thermo 26616) that was denaturated at 100 ^{o}C for 10 minutes at the same time with proteins (except Thermo protein marker which was ready-to-use) was also poured into a well. Gel was run at 100V for 110 minutes with BioRad mini gel system.

3.5.2.1.3. Staining and Visualising the Protein Bands

Silver staining of the gels were carried out with using following solutions. Fixer solution (For 300 ml total volume, 150 ml methanol, 36 ml acetic acid, 150 µl 37% formaldehyde, distilled water), pretreatment solution (For 400 ml final volume, 0.08 g sodium thiosulfate pentahydrate, distilled water), silver nitrate solution (For 400 ml final volume, 0.8 g silver nitrate, 300 µl 37% formaldehyde, distilled water), developing solution (For 400 ml total volume, 9 g potassium carbonate, 300 µl 37% formaldehyde, 8 ml pretreatment solution) and stop solution (For 400 ml final volume, 200 ml methanol, 48 ml acetic acid, distilled water). After removal of the fixation buffer gels were washed with 50% ethanol for 20 minutes for three times on the orbital shaker. Pretreatment solution were applied on the gels for one minute slowly by manually shaking. Gels were rinsed with distilled water for 20 minutes for three times. Silver nitrate solution was applied on the gels for 20 minutes and distilled water was used for rinse the gels for two times for 20 seconds. Gels were placed in developing solution about one minute until the bands were become visible. Distilled water was used for slowing the reaction. After all bands become visible, gels were put in stop solution. Photographs of the gels were taken with VersaDoc system.

3.5.2.2. 2-Dimensional PAGE of OMPs

3.5.2.2.1. First Dimension of 2-D PAGE

Measurements of protein concentrations of OMPs from both phenolic acid treated and control bacteria were performed by Bradford assay. Their concentrations were adjusted to 100 μg/ml protein by diluting with 50 mM Tris.HCl or by condensing with speed vacuum condensator. Proteins were cleaned up with 2D clean up kit (GE Health Care, Bio-Sciences Corp. 2D Clean Up Kit) according to manual's instructions. Rehydration buffer that consists of urea, chaps, DTT, ampholites (pH 3-10) and a trace of bromophenol blue were used in a volume of 350 μl for mixing with protein solution. 100 μg/μl protein in 350 ml rehydration buffer were poured on a tray and strips (Bio-Rad, 17 cm, linear for 3-HPAA experiment and non-linear for cinnamic acid and *o*-coumaric acid experiments, pH 3-10 IPG strip) were placed on the protein solutions. After keeping at room temperature for one hour, two ml of mineral oil were poured on each strip and they were allowed to absorb this solution at 20^{o}C in overnight passive rehydration. After the rehydration step, isoelectric focusing (IEF) of the proteins was performed with Bio-Rad IEF system with ascending voltages and volt-hours. The conditions of IEF steps are shown in Table 3.3.

Table 3.3. The conditions of IEF steps

Step	Voltage	Volt-hours	Angle of Current
1	200	300	Linear
2	500	500	Linear
3	1000	1000	Linear
4	4000	4000	Linear
5	8000	24000	Rapid
6	8000	30000	Rapid

3.5.2.2.2. Second Dimension of 2-D PAGE

12% polyacrylamide gel was prepared as stated in Table 3.2. in higher volume. Before loading into the gel, strips were exposed to equilibration buffers one and two for 10 minutes for each one. Reagents which both buffers include are urea, Tris.HCl, glycerol, SDS and distilled water. Different from each other, buffer one includes DTT while buffer two includes iodoacetamide. Buffers were kept at $+4^{o}C$, DTT and iodoacetamide were added at the time of usage into buffer one and two respectively. After equilibration step, strips were placed onto the gel and covered with overlay agarose (0.5% low melting agarose in 25 mM Tris.HCl, 192 mM glycine, 0.1% SDS and a drop of bromophenol blue). Gels were run at 32 mA for two gels for the first 30 minutes and 50 mA for two gels for five hours. Running buffer was used that was stated in section 3.4.2.1.1.

3.5.2.2.3. Staining the Gel and Visualising the Protein Spots

Gels were stained with silver staining (see section 3.5.2.1.3.) for visualising the spots. The gel was photographed with Versa Doc system.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Antimicrobial Activities of the Phenolic Acids

The graphs which show the bacterial growth in every three h during 24 h after exposure of each phenolic acid; 3-HPAA, cinnamic acid and *o*-coumaric acid in 96-well microtiter plate assays were plotted as optical density (600 nm) versus time (h). Figure 4.1, Figure 4.2 and Figure 4.3 show the growth of *Salmonella* Enteritidis in the presence of 3-HPAA, cinnamic acid and *o*-coumaric acid respectively. Percentage of the bacterial growth inhibitions of 3-HPAA, cinnamic acid and *o*-coumaric acid at the 18th hour and 24th hour of incubation are given in Table 4.1.

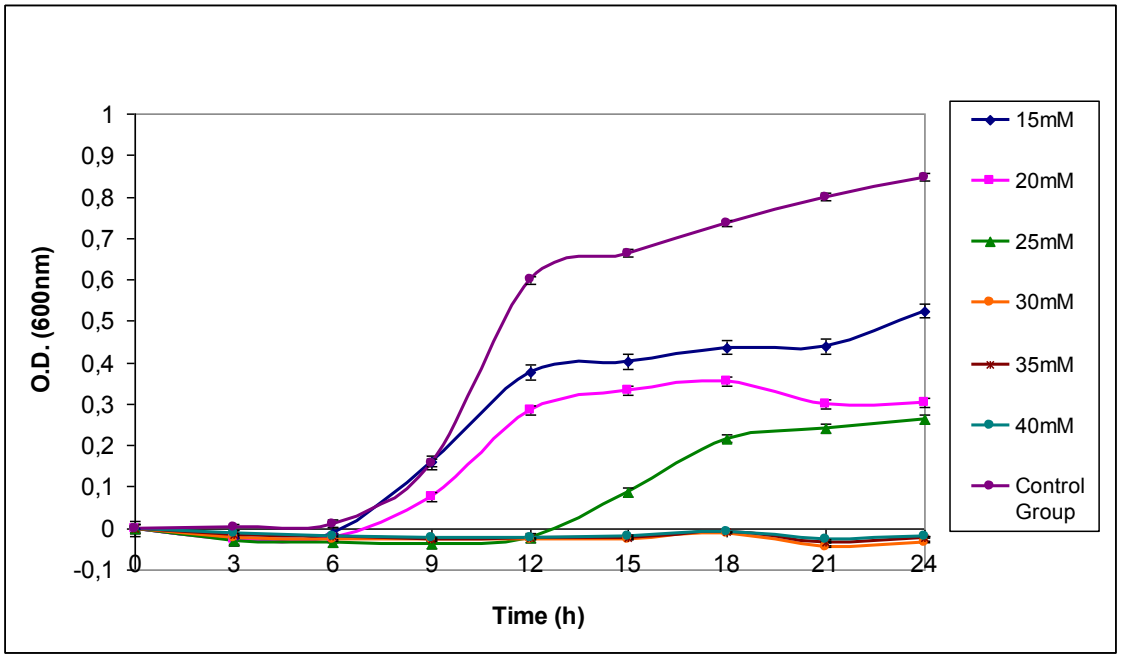


Figure 4.1 The Growth of *Salmonella* Enteritidis in the presence of 3-HPAA

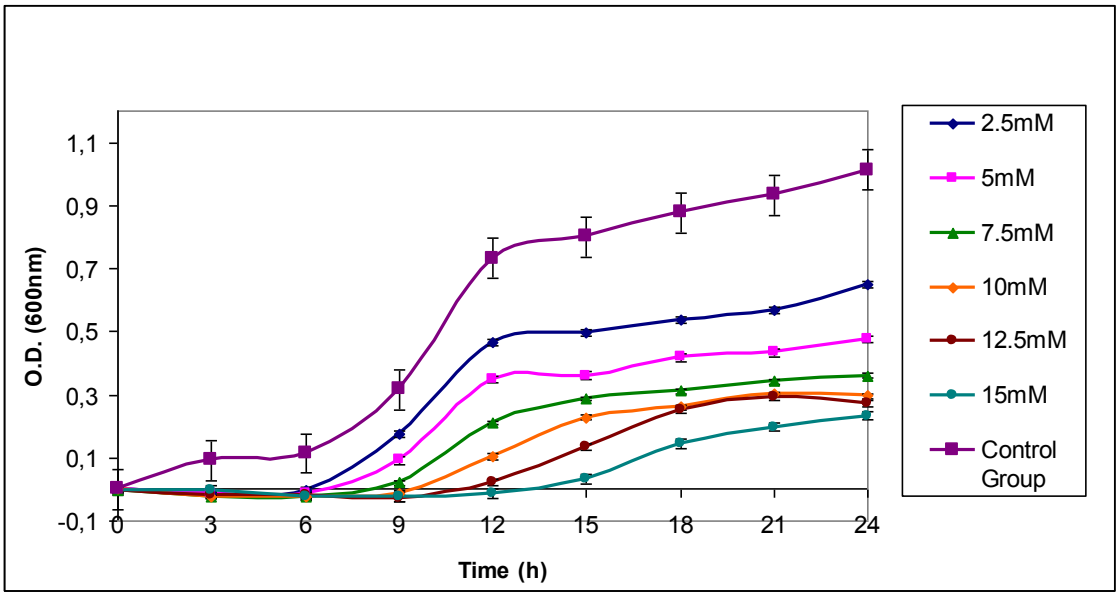


Figure 4.2. The growth of *Salmonella* Enteritidis in the presence of cinnamic acid

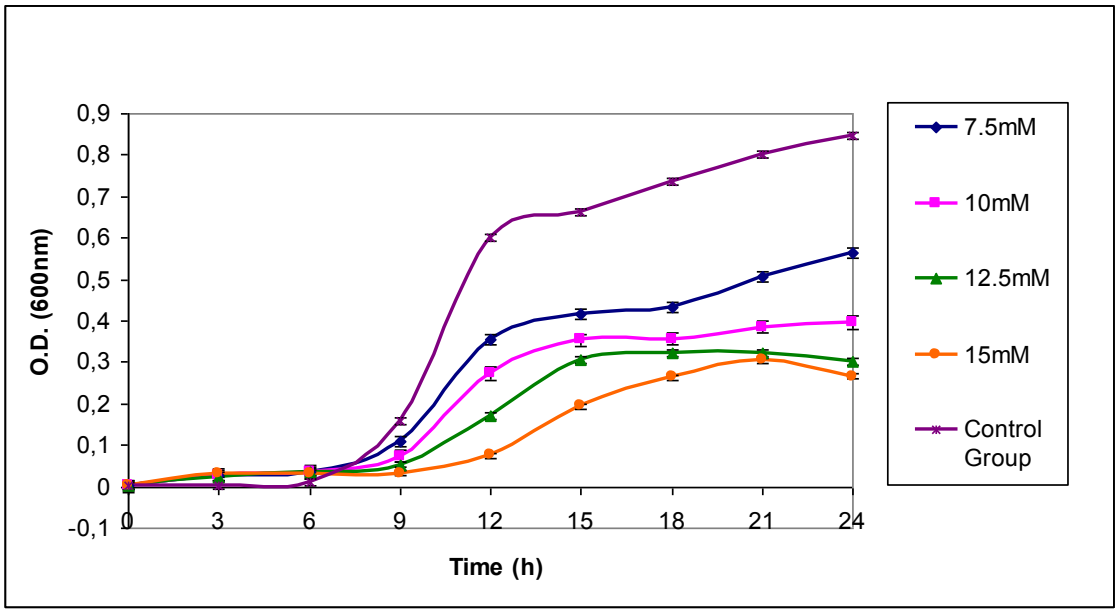


Figure 4.3. The growth of *Salmonella* Enteritidis in the presence of *o*-coumaric acid

Table 4.1. Percent inhibitions at the 18th and 24th hours of incubation

Phenolic Acid	Concentrations in wells	% Inhibition at 18th h	% Inhibition at 24th h
3-HPAA	15	40.59	38.04
	20	51.86	64.40
	25	70.49	68.99
	30	101.48	103.91
	35	101.14	102.55
	40	100.99	102.29
Cinnamic Acid	2.5	38.89	35.82
	5	52.25	52.96
	7.5	76.87	64.37
	10	70.33	70.76
	12.5	71.22	73.12
	15	83.56	77.00
o-Coumaric Acid	7.5	41.13	33.47
	10	51.50	53.21
	12.5	56.33	64.36
	15	64.11	68.66

According to these results, all these three phenolic acids have antimicrobial effect on *Salmonella* Enteritidis. Graphics show that cinnamic acid and *o*-coumaric acid showed retardation on bacterial growth in the tested range. However 3-HPAA caused inhibition of bacterial growth and 30 mM is the obtained minimum inhibitory concentration (MIC) of 3-HPAA on *Salmonella* Enteritidis upon $1x10^3$ bacterial load. MICs of other phenolic acids could not be found. Because ethanol and DMSO concentrations more than 0.1% and 3% respectively in the media have bacteriocidal effects on the tested bacteria, higher amounts of cinnamic acid and *o*-coumaric acid could not be dissolved. The concentrations shown in Table 4.1 are the maximum soluble concentrations of these two phenolic acids for exposing *Salmonella* Enteritidis.

20 mM concentration of 3-HPAA, five mM concentration of cinnamic acid and 10 mM concentration of *o*-coumaric acid were the concentrations which retarded the bacterial growth at the 18th hour of incubation in antimicrobial experiments and these concentrations were used in OMP isolation for they are the sublethal concentrations. 18th hour was chosen because of being the time that the bacteria tested are in the late-exponential phases of their growth, so that they had produced all the proteins for the survival in the environment containing phenolic acids.

4.2. Scanning Electron Microscopy Results

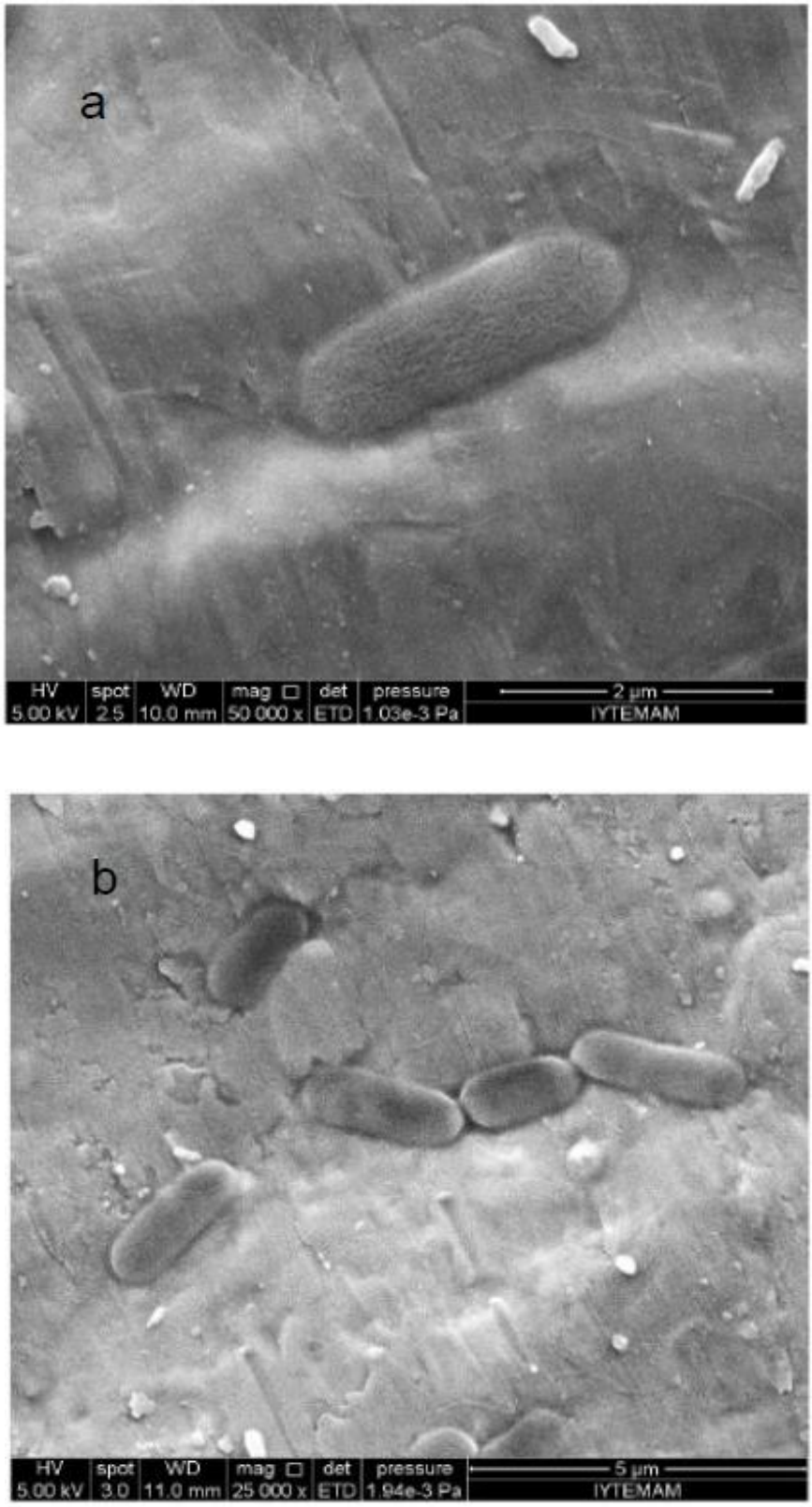


Figure 4.4. The SEM images of control bacteria (Sections a and b)

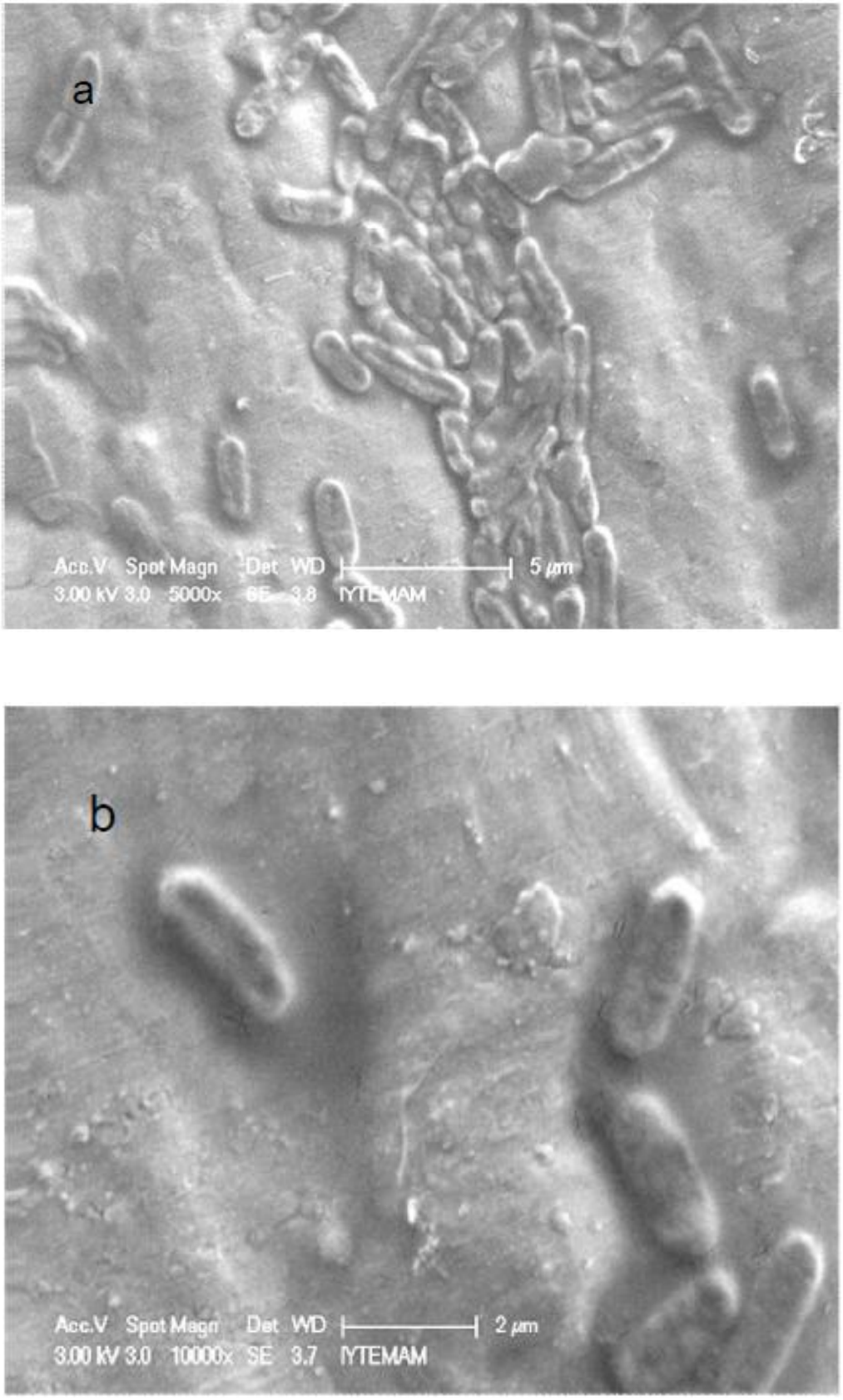
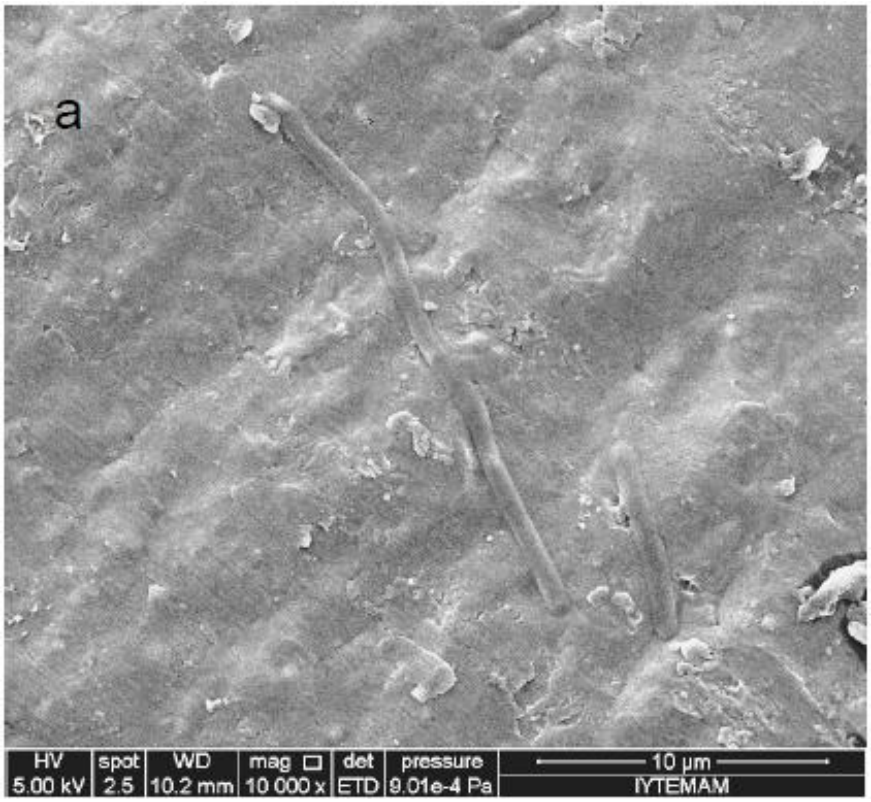


Figure 4.5. The SEM images of 25 mM 3HPAA treated bacteria (Sections a and b)



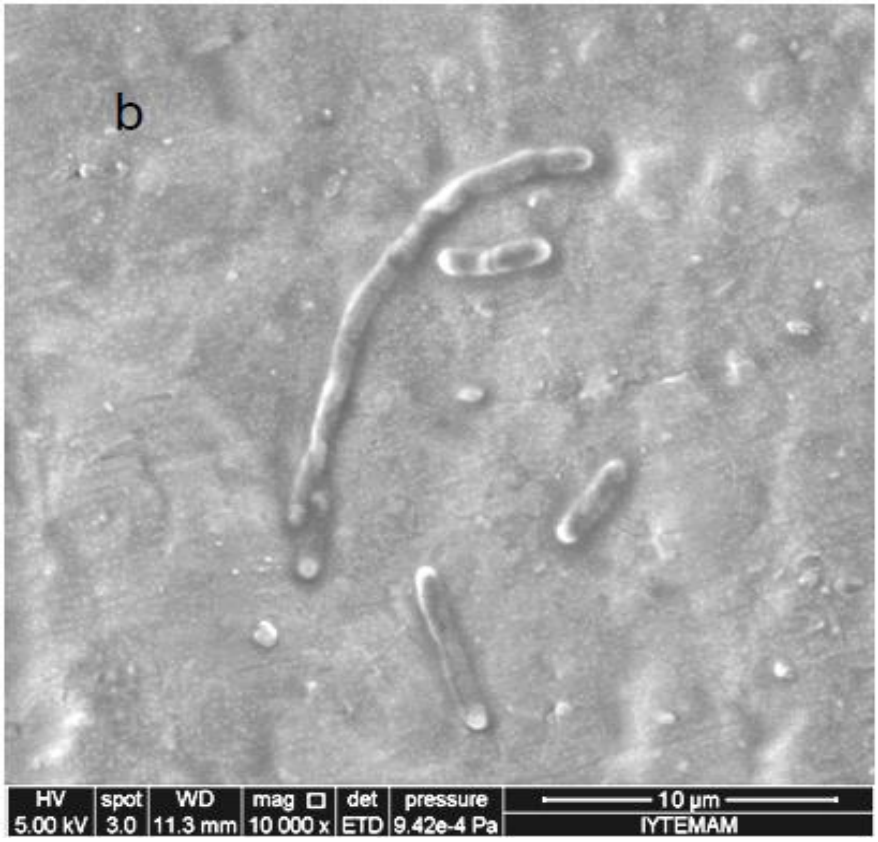


Figure 4.6. The SEM images of 10 mM cinnamic acid treated bacteria (Sections a and b)

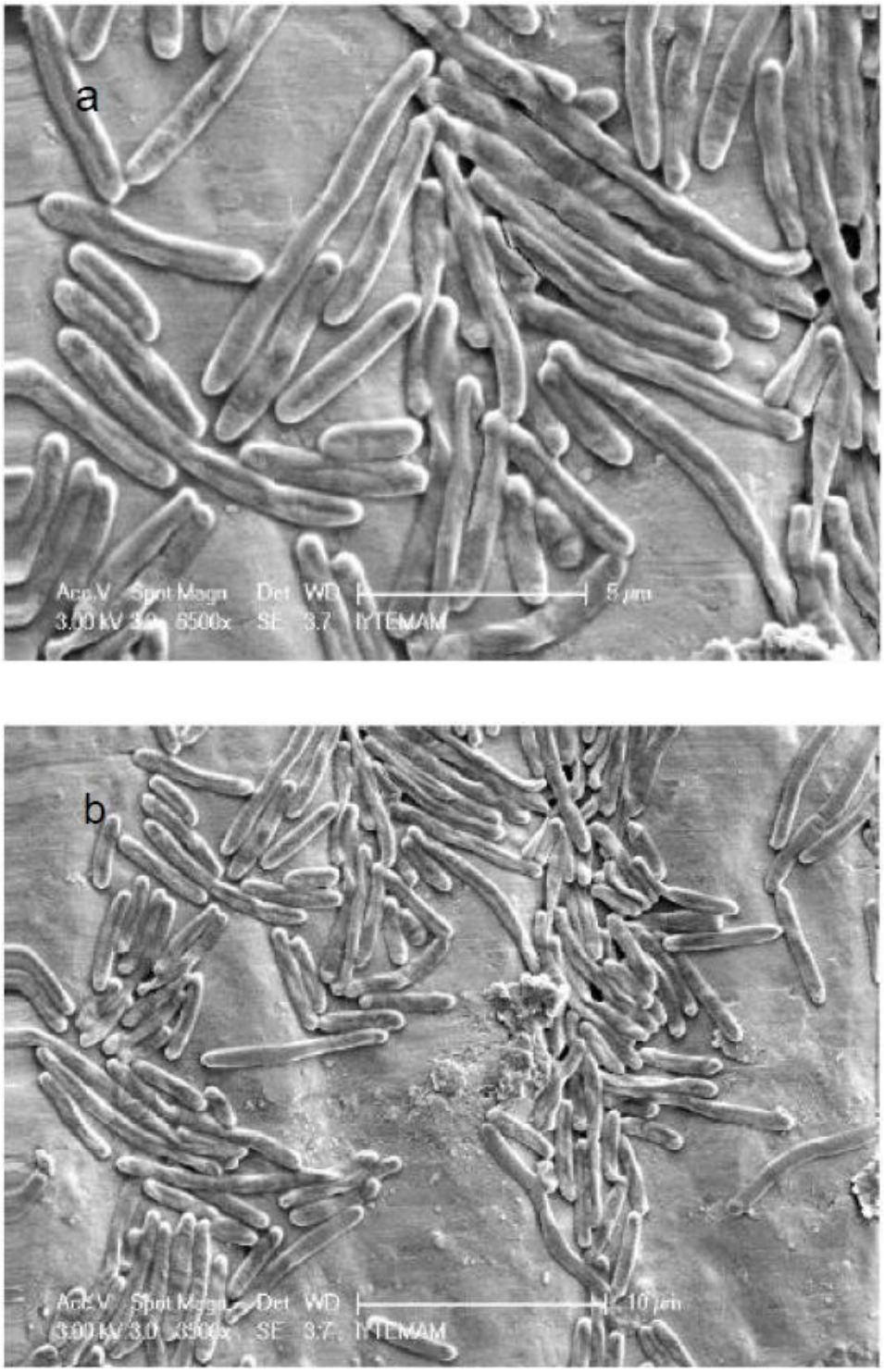


Figure 4.7. The SEM images of 15 mM *o*-coumaric acid treated bacteria (Sections a and b)

Figure 4.4 shows the SEM images of the control bacteria. As it is seen in the images, *Salmonella* Enteritidis are rod-shaped bacteria. Figure 4.5, 4.6 and 4.7 demonstrates the SEM images of 25 mM 3HPAA, 10 mM cinnamic acid and 15 mM *o*-

coumaric acid treated bacteria, respectively. In Figure 4.5 bacterial cells with cavities are seen which indicates that the membrane of the cells were affected. In Figures 4.6 and 4.7, which is obvious in Figure 4.6, bacteria elongated and it is understood that the cells have problems in cell dividing. Because bacteria can not divide, bacterial growth was supressed. These results may be related with the affected membrane bounded proteins such as FtsZ. With regards to phenolic acids' chemical structures, it is seen that similar structured phenolics cinnamic acid and *o*-coumaric acid have similar effect on bacteria while 3HPAA, which has different chemical structure from these two phenolic acis, had a different effect.

4.3. SDS PAGE Results

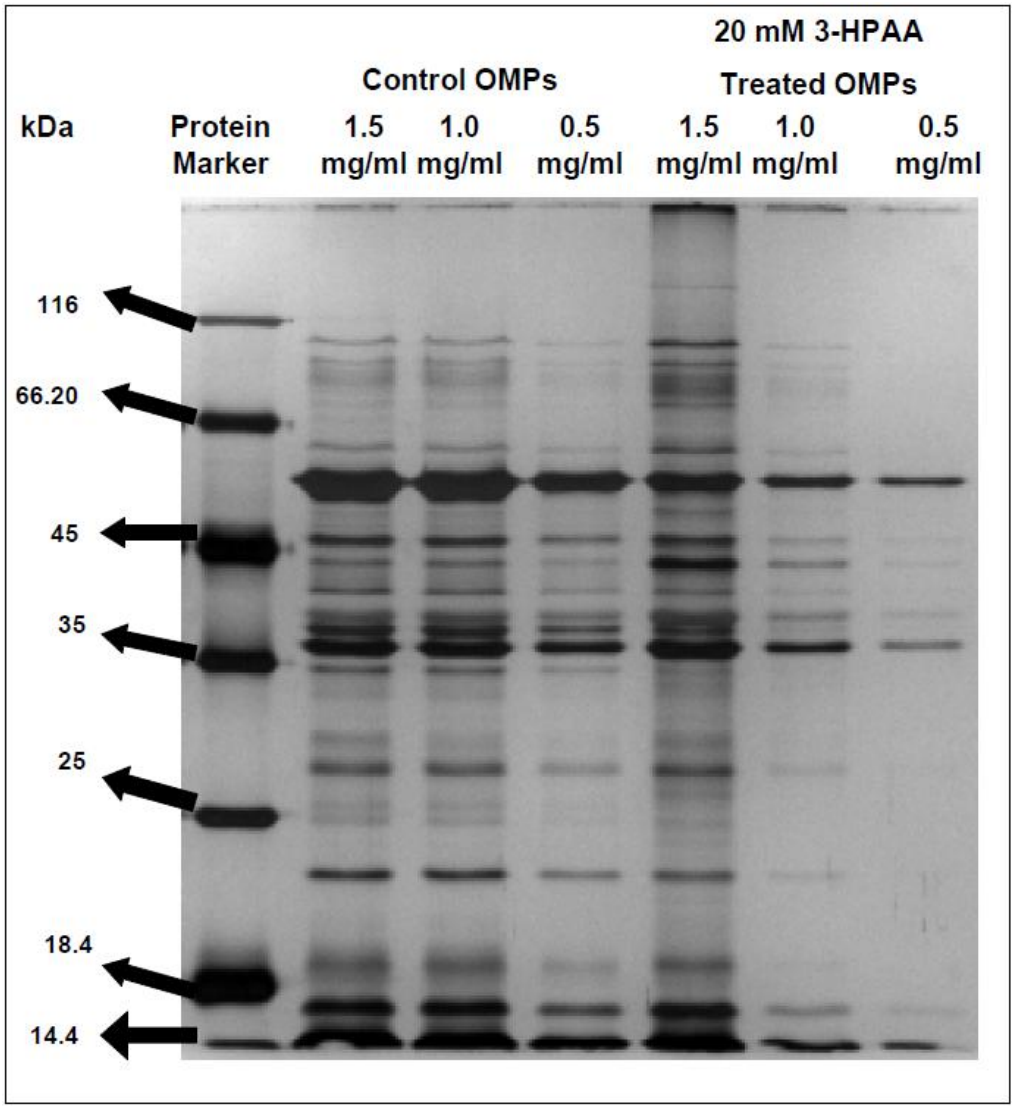


Figure 4.8. SDS PAGE image of OMPs treated with 20 mM 3-HPAA (Lane one contains the protein marker. Lanes two, three and four contain OMPs of control bacteria with 1.5 mg/ml, 1 mg/ml and 0.5 mg/ml respectively. Lanes five, six and seven contain OMPs from 3-HPAA treated bacteria with the same protein concentrations in lanes two, three and four respectively.

In Figure 4.8, protein marker is loaded in the first well, 2nd, 3rd and 4th wells are loaded with 1.5 mg/ml, 1 mg/ml and 0.5 mg/ml of OMPs from control bacteria, respectively, while last three wells contain the same amounts of OMPs from 20 mM 3-HPAA treated bacteria. The protein marker is Fermentas unstained protein molecular weight marker SM0431 which have protein bands with molecular weights

of 116 kDa, 66.20 kDa, 45 kDa, 35 kDa, 25 kDa, 18.4 kDa and 14.4 kDa in descending order. In the OMP profile of 3-HPAA treated bacteria a decrease in the bands that have nearly 55 kDa and 34 kDa molecular weights was detected while the increase was seen in the bands with molecular weight of nearly 43 kDa. The other changes between protein profiles which are seen in Figure 4.8 are negligible.

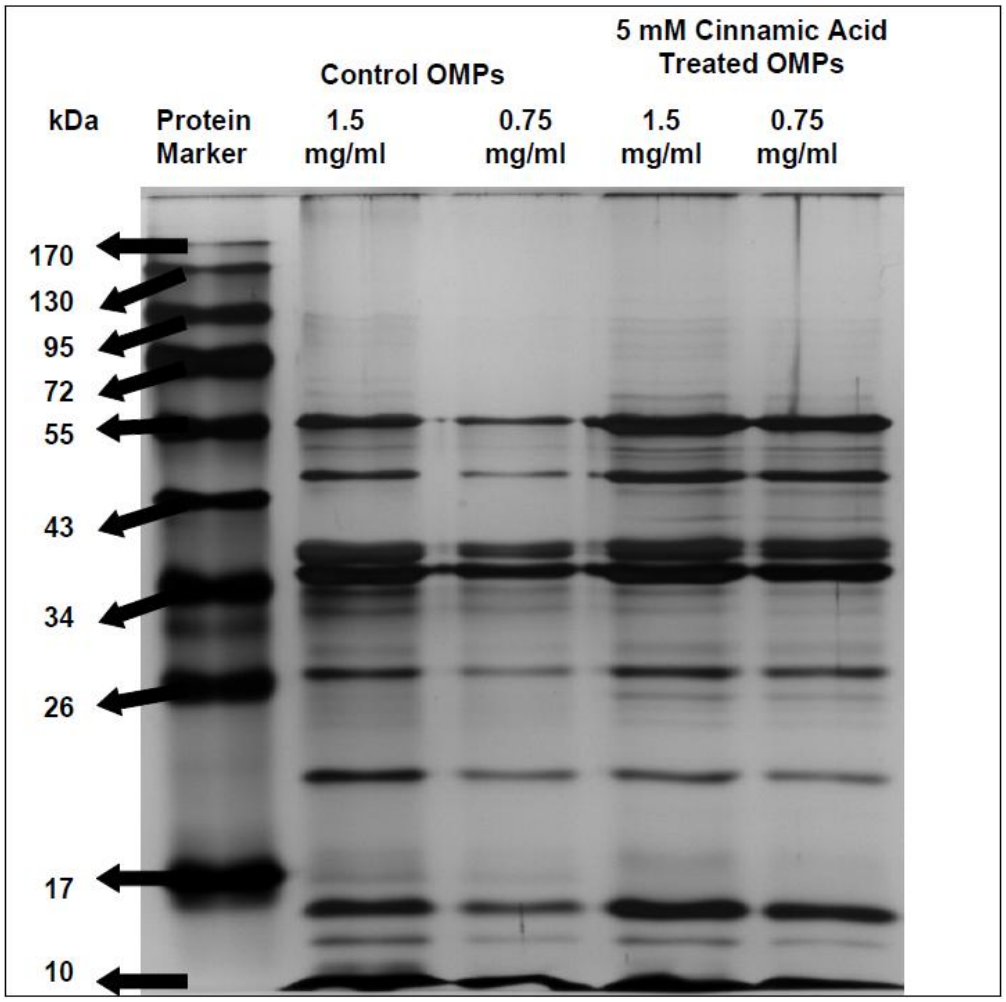


Figure 4.9. SDS PAGE image of OMPs treated with 5 mM cinnamic acid (Lane one contains the protein marker. Lanes two and three contain OMPs of control bacteria with 1.5 mg/ml and 0.75 mg/ml respectively. Lanes four and five contain OMPs from cinnamic acid treated bacteria with the same protein concentrations in lanes two and three respectively).

Figure 4.9 shows the SDS image of the OMPs in the presence of 5 mM cinnamic acid. The 1st well contains protein marker, next two wells following the marker well contains 1.5 mg/ml and 0.75 mg/ml OMPs from control bacteria respectively and last two wells have the same amount of OMPs from cinnamic acid treated bacteria.

Fermentas prestained protein ladder SM0671 was used as protein marker and the molecular weights of its protein bands are 170 kDa, 130 kDa, 95 kDa, 72 kDa, 55 kDa, 43 kDa, 34 kDa, 26 kDa, 17 kDa and 10 kDa in descending order. The protein amount around 56 kDa and 46 kDa was seen as increased in cinnamic acid treated bacterial OMPs compared to control bacterial OMPs. The proteins in these bands can be thought as the stress proteins that bacteria expressed to adjust itself when growing in the environment with 5 mM cinnamic acid. A negligible decrease in the bands of OMPs of treated bacteria with a molecular weight of about 33 kDa was seen. No other significant changes were observed in SDS PAGE of OMPs of cinnamic acid exposed bacteria.

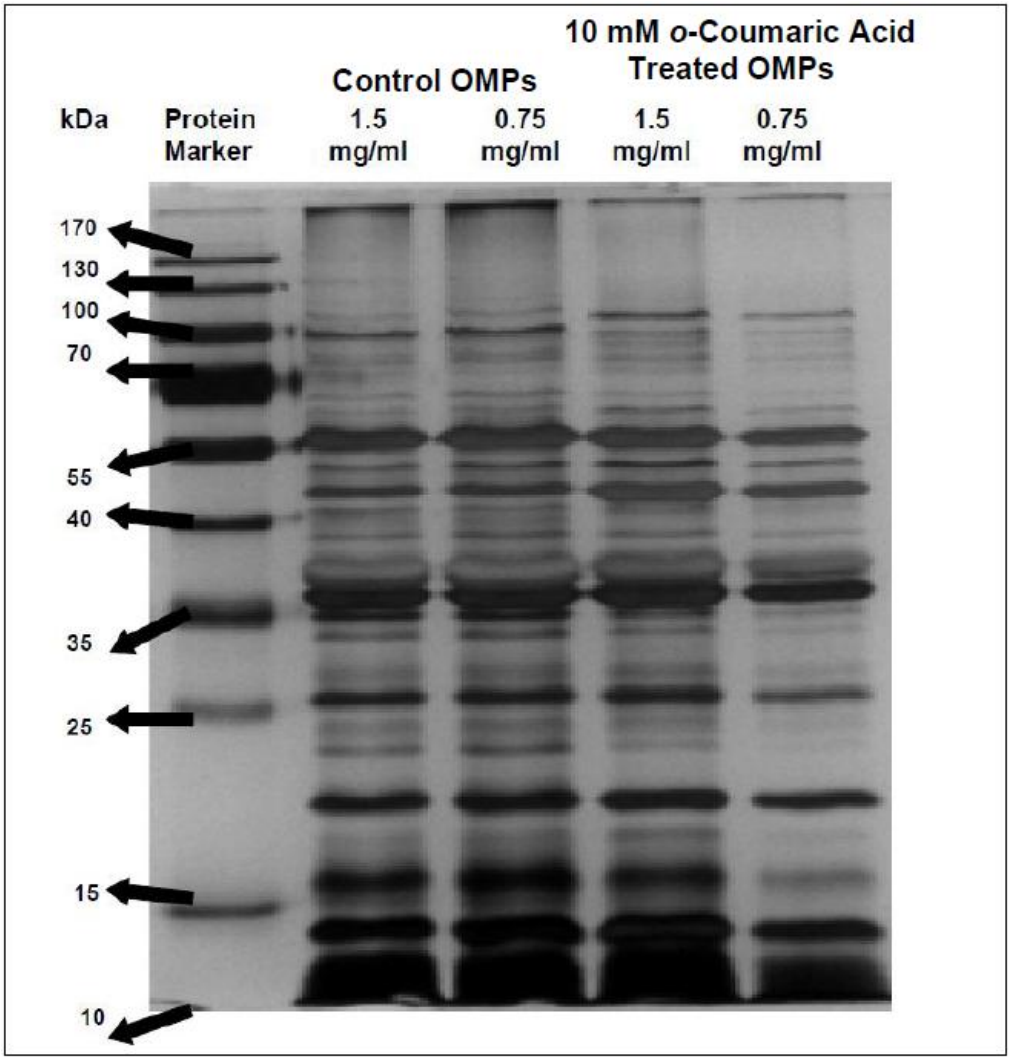


Figure 4.10. SDS PAGE image of OMPs treated with 10 mM *o*-coumaric acid. (Lane one contains the protein marker. Lanes two and three contain OMPs of control bacteria with 1.5 mg/ml and 0.75 mg/ml respectively. Lanes four and five contain OMPs from *o*-coumaric acid treated bacteria with the same protein concentrations in lanes two and three respectively).

The 1st well in Figure 4.10, which shows the SDS image of OMPs in the presence of 8 mM *o*-coumaric acid, contains the protein marker. Second and 3rd wells have 1.5 mg/ml and 0.75 mg/ml OMPs of control bacteria respectively while 4th and 5th ones have the same amount of OMPs from 10 mM *o*-coumaric acid treated bacteria. Thermo 26616 PageRuler prestained protein ladder was loaded in the first well. Its bands have 170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa, 15 kDa and 10 kDa -which can not seen in Figure 4.10- molecular weights. The protein amount of the bands with nearly 112 kDa and 46 kDa showed increase OMPs of treated bacteria, however, the bands with about 35 kDa and 23 kDa molecular weights showed decrease. No other changes were observed in SDS PAGE results between the OMP profiles of control bacteria and 10 mM *o*-coumaric acid treated bacteria.

4.4. 2-D PAGE Results

The Figure 4.11 shows the 2-D PAGE gel images of OMPs of control (Fig. 4.11-A) and 20 mM 3HPAA treated (Fig. 4.11-B) *Salmonella* Enteritidis. Figure 4.12 and 4.13 demonstrate the comparison of 2-D PAGE images of the control bacteria (Fig.4.12-A and Fig. 4.13-A) OMPs of 5 mM cinnamic acid treated and 10 mM *o*-coumaric acid treated bacteria (Fig. 4.12-B and Fig. 4.13-B) respectively. It is known that the bacteria change their outer membrane protein profiles to maintain their cell integrity and membrane fluidity for survival. The images show that there are some differences in expression of some protein spots. For instance, in the images of phenolic acid treated OMPs, increases in spot dimensions are seen. These proteins may be the proteins which are expressed in stress conditions because of the addition of the phenolic acids into growth environment of the bacteria.

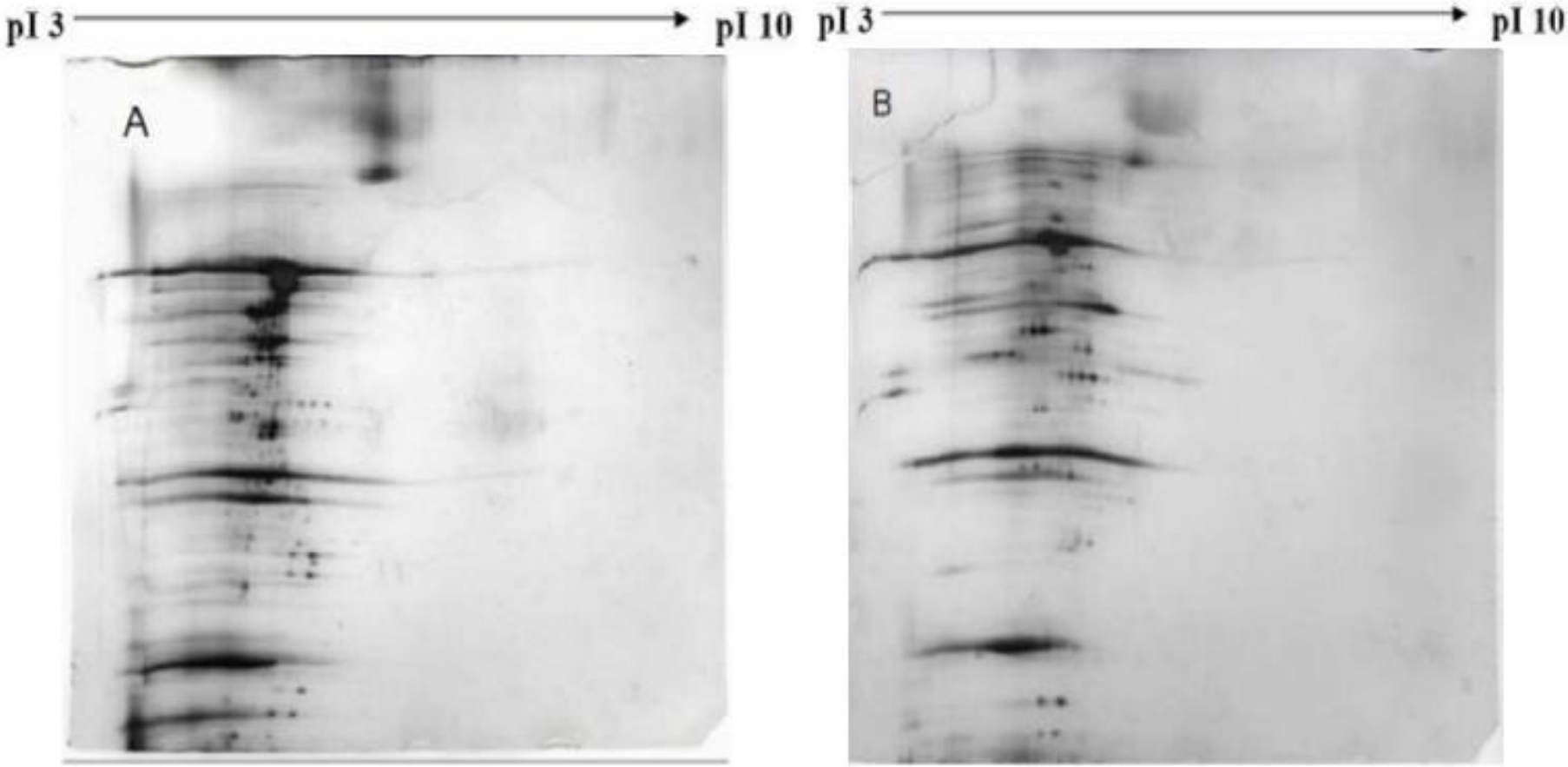


Figure 4.11. The 2-D PAGE images of OMPs of control bacteria (Section A) and OMPs of 20 mM 3-HPAA treated bacteria (Section B). (Bio-Rad pH 3-10, Linear IPG Strip; 100 μg protein)

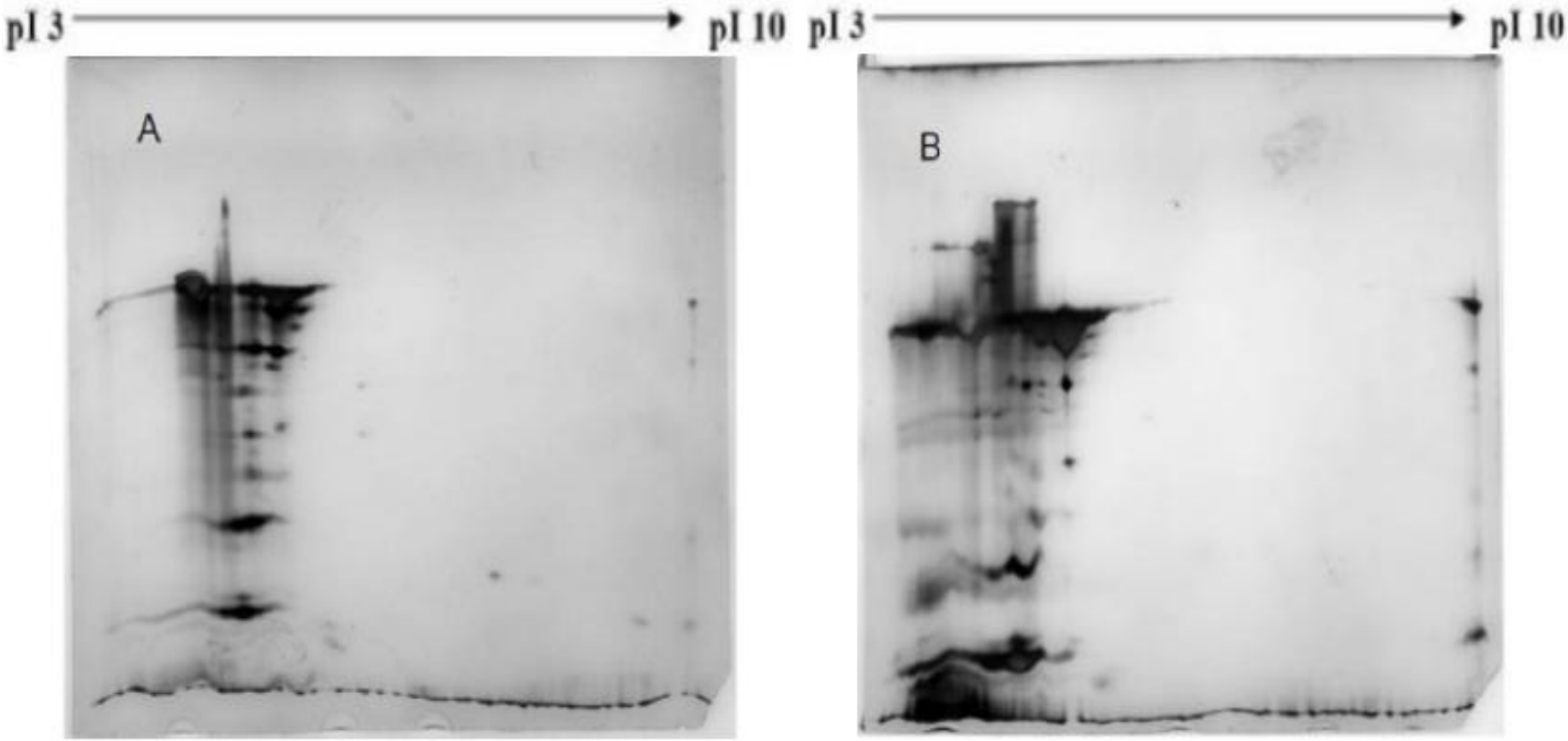


Figure 4.12. The 2-D PAGE images of OMPs of control bacteria (Section A) and OMPs of 5 mM cinnamic acid treated bacteria (Section B) (Bio-Rad, pH 3-10, Non-linear IPG strip; 100 µg protein)

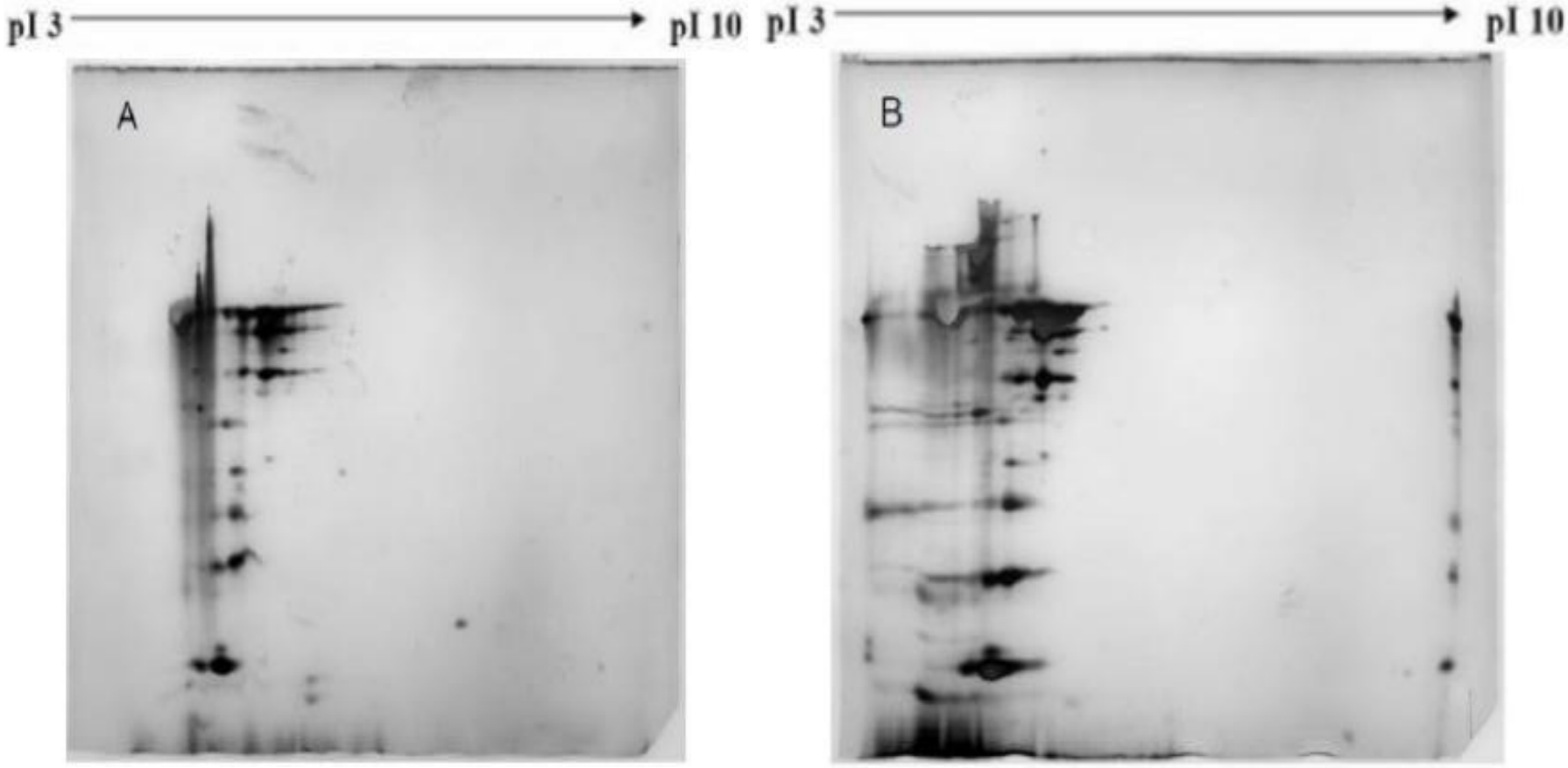


Figure 4.13. The 2-D PAGE images of OMPs of control bacteria (Section A) and OMPs of 10 mM *o*-coumaric acid treated bacteria (Section B) (Bio-Rad, pH 3-10, Non-linear IPG strip; 100 µg protein)

CHAPTER 5

CONCLUSION

In this study it was aimed to investigate the antimicrobial activities of three phenolic acids, 3-HPAA, cinnamic acid and *o*-coumaric acid on *S. enterica ser*. Enteritidis and the effects of these phenolics on bacterial shapes and on the outer membrane protein profile of the bacteria. For this purpose a range of concentrations of the phenolic acids were exposed to the bacteria and the growth curves of the cultures in the presence of phenolics were plotted. Among these three phenolic acids, 3-HPAA was found to have bacteriocidal effect with 30 mM MIC value. Cinnamic acid and *o*-coumaric acid have bacteriostatic effects.

By using SEM, the effects of the phenolics on bacterial shape were observed. It was demonstrated that in the presence of 3-HPAA the bacteria had collapses on their surfaces while in the presence of cinnamic acid and *o*-coumaric acid the cells became longer which show that the bacteria have some problems in cell division.

To investigate the effects of the phenolics on bacteria in the proteomic way, displaying outer membrane protein profile changes of the bacteria in the presence of phenolics were chosen as a first step. To achieve it, bacteria were grown in the media with and without phenolic acids. Concentrations were chosen by examining the antimicrobial results and the concentrations that have bacteriostatic effect were chosen to apply. For the bacteria passed to the late exponential phase of the growth at the 18^{th} h, it was accepted that they produced all the required proteins. Therefore, the outer membrane protein isolation was carried out at the 18^{th} h of bacterial growth. *N*-lauroyl sarcosine method was performed for isolating the outer membrane proteins. This is an anionic detergent and it solubilizes the cytoplasmic proteins. After the ultracentrifugation following N-louroyl sarcosine exposure, pellets include the most of the outer membrane proteins.

To seperate the proteins SDS-PAGE and 2-D PAGE analysis were performed. In the gel images of SDS-PAGE and 2-D PAGE, some modifications were seen in protein bands and protein spots respectively. They were stained with silver staining so they are not competible with protein identification techniques.

In further studies the experiments can be repeated for staining the gels with a stain which is compatible with protein identification techniques such as mass spectrometry for altered spots to be identified.

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APPENDIX A

STANDART CURVE FOR BOVINE SERUM ALBUMIN

Standart curve for determination of protein concentrations by using bovine serum albumin (BSA) was plotted. 5X Bradford reagent with a total of 250 ml volume containing 0.1 g Coomassie Brilliant Blue G-250, 50 ml absolute ethanol, 85% phosphoric acid and ultrapure water was prepared and dissolved five times before use. 5, 10, 25, 50, 75 and 100 µg/ml concentrations of BSA which were prepared from stock with the concentration of 1 mg/ml were used. By using 9:1 ratio (v/v) of bradford reagent and BSA respectively, each BSA concentration and reagent were mixed in 96 well-plate. After keeping the plate at dark for 10 minutes, OD was measured by spectrophotometer at 595 nm and standart curve was plotted (Figure A.1).

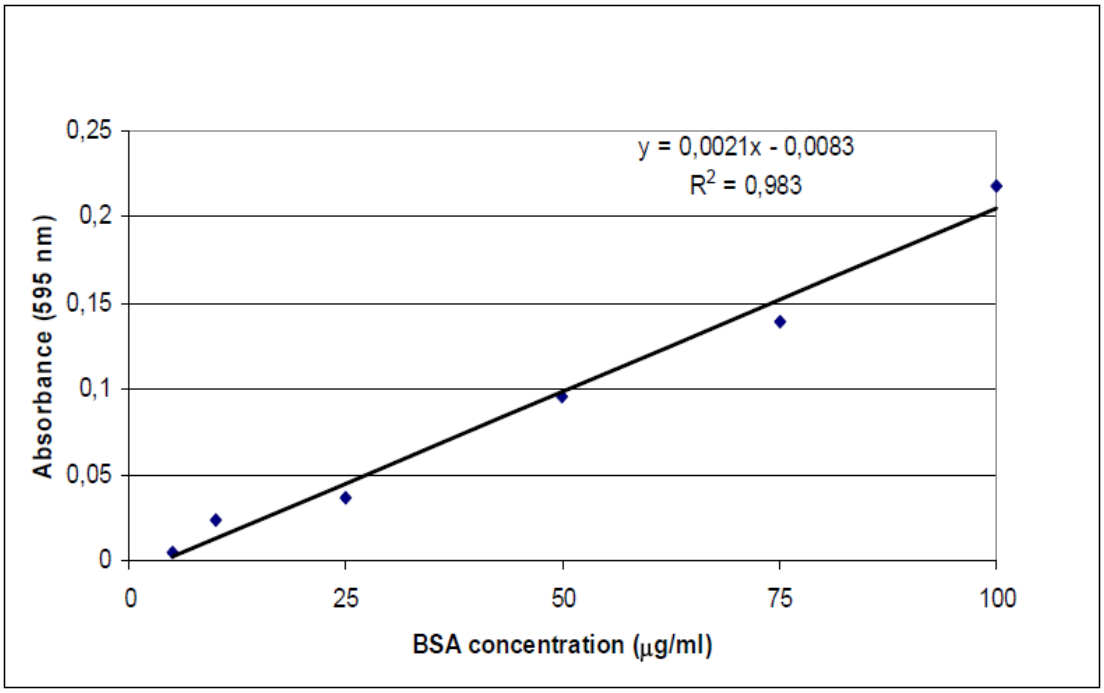

Figure A.1. Standart Curve for Bradford Protein Assay