INVESTIGATION OF MOLECULAR EFFECT OF PHENOLIC ACIDS ON METHICILLIN-RESISTANT AND METHICILLIN-SUSCEPTIBLE *Staphylococcus aureus* IN COMPARISON TO THEIR PHENOLIC ACID RESISTANT MUTANTS

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

DOCTOR OF PHYLOSOPHY

in Molecular Biology and Genetics

by
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December 2018
İZMİR

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ACKNOWLEDGMENTS

First of all, I would like to thank to my supervisor Assoc. Prof. Dr. Ferda Soyer for her guidance, understanding and support throughout this study. I am so grateful to her for the opportunity of studying under her supervision.

I would like to thank my committee members Prof. Dr. Anne Frary and Assist. Prof. Dr. Erdal Eroğlu for their advices during the progress of my thesis. Special thanks are extended to Prof. Dr. Talat Yalçın for not only being my committee member but also kindly providing all the required facility for proteomic experiments. I also would like to thank Prof. Dr. Rengin Eltem for accepting my committee member and for her valuable comments.

I am grateful to all my friends in the department of Molecular Biology and Genetics for their friendship and sincere help during my studies. I would like to state my thanks to Özgün Öykü Özdemir whom I worked with at the laboratory during almost ten years. She was not only a kind colleague sharing ideas but also a good friend to share the dreams and concerns.

I also would like to thank the members of the Mass Laboratory in Department of Chemistry, especially to Melike Dinç for her help during the proteomic experiments. I would like to thank 'İzmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center' for the facility support.

When it comes to my beloved family, it seems so hard to explain my gratitude. I would like to thank with all my heart to my mother Sabaat Keman and my father Özcan Keman for their endless love and support. Without their love, encouragement, advices and patience it was not possible to complete this thesis.

ABSTRACT

INVESTIGATION OF MOLECULAR EFFECT OF PHENOLIC ACIDS ON METHICILLIN-RESISTANT AND METHICILLIN-SUSCEPTIBLE *Staphylococcus aureus* IN COMPARISON TO THEIR PHENOLIC ACID RESISTANT MUTANTS

Staphylococcus aureus is a Gram-positive bacterium whose acquisition through an open wound results in various infections. Methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains are responsible for diseases ranging from soft tissue infections to fatal pneumonia which cannot be treated due to multiple drug-resistances of these strains. This situation increases the importance of searching for alternative antimicrobials worldwide. Having all these in mind, the capacity of phenolic acids cannot be denied to be used against pathogenic bacteria. Phenolic acids produced as plant secondary metabolites show antibacterial effects besides many beneficial properties for human health.

The aim of this study was to investigate the antibacterial action mechanisms of vanillic acid and 2-hydroxycinnamic acid on MRSA and MSSA. To achieve this, firstly the antibacterial effects of phenolic acids on both bacteria were investigated by determination of minimum inhibitory concentrations. Then, the resistance development ability of bacteria against phenolic acids was tested by continuous exposure to subinhibitory concentrations. Finally, the action mechanisms of phenolic acids on bacteria were elucidated using two different proteomic approaches. According to the results, bacteria were not able to develop resistance against phenolic acids. Proteomic studies displayed alterations in the protein profiles of phenolic acid treated bacteria and provided potential targets in the battle with pathogenic bacteria.

By showing the inability of MRSA and MSSA to develop resistance to phenolic acids and the important proteomic alterations that are induced by phenolic acid treatment, this study highlights the significance of phenolic acids to be used against antibiotic-resistant bacteria.

ÖZET

METİSİLİNE DİRENÇLİ VE METİSİLİNE DUYARLI *Staphylococcus aureus*'LARDA FENOLİK ASİTLERİN MOLEKÜLER ETKİSİNİN FENOLİK ASİTLERE DİRENÇLİ MUTANTLARIYLA KARŞILAŞTIRILARAK ARAŞTIRILMASI

Staphylococcus aureus, açık yara yoluyla vücuda girmesi sonucu çeşitli enfeksiyonlara sebep olan Gram-pozitif bir bakteridir. Metisiline dirençli *S. aureus* (MRSA) ve metisiline duyarlı *S. aureus* (MSSA) suşları, birçok ilaca karşı dirençli olmalarından dolayı tedavi edilemeyen, yumuşak doku enfeksiyonlarından ölümcül zatürreye varan hastalıklardan sorumludurlar. Bu durum, tüm dünyada, alternatif antimikrobiyal arayışının önemini arttırmaktadır. Tüm bunlar düşünüldüğünde, fenolik asitlerin hastalık yapan bakterilere karşı kullanılma potansiyeli yadsınamamaktadır. Bitki ikincil metabolitleri olarak üretilen fenolik asitler, antibakteriyel etkilerinin yanında insan sağlığına yararlı özellikler göstermektedirler.

Bu çalışmanın amacı, vanilik asit ve 2-hidroksisinamik asidin MRSA ve MSSA üzerine antibakteriyel etki mekanizmasını araştırmaktır. Bu amaçla öncelikle, fenolik asitlerin her iki bakteri üzerine antibakteriyel etkileri, minimum inhibe edici konsantrasyonlarının belirlenmesi yoluyla incelenmiştir. Daha sonra, bakterilerin fenolik asitlere karşı direnç geliştirme kabiliyeti, sürekli olarak inhibe edici olmayan konsantrasyonlara maruz bırakılmaları yoluyla test edilmiştir. Son olarak, fenolik asitlerin bakteriler üzerindeki etki mekanizmaları iki farklı proteomik yaklaşım kullanılarak değerlendirilmiştir. Sonuçlara göre, bakteriler fenolik asitlere karşı direnç geliştirememiştir. Proteomik çalışmalar, fenolik asitlerle muamele edilen bakterilerin protein profillerinde, hastalık yapan bakterilerle mücadelede potansiyel hedef olabilecek değişiklikleri ortaya çıkarmıştır.

Bu çalışma, MRSA ve MSSA'nın fenolik asitlere karşı direnç geliştirememesi ve fenolik asit uygulaması ile tetiklenen önemli proteomik değişiklikleri göstererek, fenolik asitlerin antibiyotiklere dirençli bakterilere karşı kullanılabilmelerindeki önemini vurgulamaktadır.

Dedicated to my beloved family who are always besides me and teach me what life is really about…

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2-HCA	2-Hydroxycinnamic Acid
ABC	Ammonium Bicarbonate
APS	Ammonium per Sulfate
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CHAPS	3-((3-chloamidopropoyl)dimethylammonio)-1-propanesulfonate
CHCA	alpha-Cyano-4-Hydroxycinnamic Acid
cfu	Colony Forming Unit
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
ESI	Electrospray Ionization
FA	Formic Acid
FDA	Food and Drug Administration
g	Gram
g	Gravity
h	Hour
IPG	Immunomobilized pH Gradient
IEF	Isoelectric Focusing
LC	Liquid Chromatography
l	Liter
MALDI	Matrix-Assisted Laser Desorption Ionization
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MSSA	Methicillin-Susceptible *Staphylococcus aureus*
min	Minute
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
ml	Milliliter
mg	Milligram

μg	Microgram
μl	Microliter
nm	Nanometer
TFA	Trifluoroacetic Acid
2D-PAGE	Two-Dimensional Polyacrylamide Gel Electrophoresis
OD	Optical Density
PBP	Penicillin Binding Protein
PVL	Panton-Valentine Leukocidin
rpm	Revolutions per Minute
RT-PCR	Real Time-Polymerase Chain Reaction
SCC	Staphylococcal Cassette Chromosome
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
TOF	Time of Flight
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VA	Vanillic Acid

CHAPTER 1

LITERATURE REVIEW

1.1. *Staphylococcus aureus*

Staphylococci are Gram-positive bacteria that grow by forming grape-like clusters. Their first classification was made as *Staphylococcus aureus* and *Staphylococcus albus* in 1884 depending on the colony morphology and the ability of the organism to clot blood. While coagulase positive *S. aureus* forms yellow colonies on blood agar, coagulase negative *S. albus* forms white colonies (Patel et al., 2008).

Staphylococcus aureus is one of the major causes of bacterial infections in humans. Diversity and intensity of diseases caused by this bacterium vary from skin infections to fatal pneumonia (Deurenberg et al., 2007; DeLeo et al., 2010). This bacterium lives commensally in the nostrils of about 30% of healthy people who do not show symptoms of *S. aureus* infections (DeLeo et al., 2010). Although they are localized to the mouth, sinus, ears and throat, following colonization they do not always result in infection of these parts. According to studies of regulation of *S. aureus*, virulence genes such as *agr* (accessory gene regulator) are down-regulated during colonization. This gene is localized in a quorum sensing locus and responsible for the control of genes encoding virulence (Liu, 2009).

When *S. aureus* is acquired, it can lead to infections in an open wound. Colonization of bacteria commonly occurs on skin or mucosal surfaces such as nose, throat, vaginal wall and gastro-intestinal tract of the infected person. Among these body parts, nasal carriage results in the most common spread of bacteria to other body parts and people. Especially for the colonization on the nose, bacteria need to adhere to the epithelial cells and fight against host defense system and the normal flora. Adhesion to the epithelial cells is provided by the molecules which are termed as 'microbial surface components recognizing adhesive matrix molecules (MSCRAMM)' (Liu, 2009). After *S. aureus* is localized into an open wound, neutrophils release antimicrobial peptides, reactive oxygen species (ROS), reactive nitrogen species, proteases and lysozyme. Disposal of ROS and reactive nitrogen species in *S. aureus* is provided by antioxidant

enzymes such as catalase and superoxide dismutase. *S. aureus* are able to change surface charges of bacterial membrane to protect themselves from the effect of the antimicrobial peptides as well as degradation and neutralization of these peptides (Liu, 2009).

S. aureus strains are transmitted as a result of direct contact with the microorganism. Skin to skin contact with a person who is infected with bacteria usually (90%) results in skin and soft-tissue infections (DeLeo et al., 2010).

1.1.1. Antibiotic Resistance of *Staphylococcus aureus*

Before the introduction of penicillin, *S. aureus* infections had an 80% mortality rate in the 1940s. However, it did not take long time for bacteria to get resistance against penicillin (Deurenberg et al., 2007). Penicillin acts during the formation of peptidoglycan cell wall by preventing the cross-linking of peptide chains. This action mechanism results in disruption of the cell wall rigidity of newly formed bacteria while formerly produced cells are unaffected. Overuse of penicillin resulted in development of resistance in *S. aureus* against penicillin by penicillinase. This enzyme is responsible for the cleavage of the beta-lactam ring of penicillin (Patel et al., 2008).

In the 1960s, a new class of penicillins was developed to fight against the resistance. Methicillin belongs to semisynthetic class of penicillins. When compared to penicillin, methicillin had one more acyl group on the beta-lactam ring. This additional group provided higher degree of resistance. However, *S. aureus* developed resistance against methicillin by expressing an alternative penicillin binding protein, PBP2a after two years of usage of this antibiotic for treatment of penicillin-resistant strains. This protein is encoded by *mecA* methicillin resistance gene that located on the staphylococcal cassette chromosome (SCC) (Patel et al., 2008). Presence of the 2.1 Kb *mecA* gene on the chromosomal DNA confers resistance to beta-lactam antibiotics making *S. aureus* a methicillin-resistant *Staphylococcus aureus* (MRSA). *mec* stands for methicillin resistance and it is present in the chromosomal DNA of MRSA but not in methicillin-susceptible *S. aureus* (MSSA). MecA protein is a 78-kDa penicillin binding protein also called PBP2' or PBP2a (Hiramatsu et al., 2013; Deurenberg et al., 2007). Normally, penicillin binding proteins (PBPs) in the cell walls of bacteria are the places where beta-lactam antibiotics bind which then leads to peptidoglycan layer disruption.

Inability of beta-lactam antibiotics to bind PBP2a provides maintenance of the cell wall synthesis. MecI repressor and MecRI transmembrane beta-lactam–sensing signal transducer proteins are responsible for regulation of the *mecA* gene. When there is no beta-lactam antibiotic, transcription of *mecA* and *mecRI* is repressed by MecI protein. However, presence of the beta-lactam antibiotic induces autocatalytic cleavage of MecRI. Activation of MecRI leads to the separation of MecI repressor from the operator of *mecA* and allows PBP2a expression (Deurenberg et al., 2007).

Genomic islands (GIs) on the chromosome possess the genes responsible for virulence or drug resistance as well as the integrated prophages, *oriC* (origin of replication) and vSa islands (have roles in movement of the genes). *oriC* includes conserved *orfs* (open reading frames) sparsely which determines the presence of genes specific to each species. However, orfX located near the *oriC* provides a place for the attachment of staphylococcal cassette chromosome (Hiramatsu et al., 2013). Within this 32 to 60 Kb long chromosomal DNA region, two important gene clusters are localized: *mec* gene complex that encodes for the resistance to methicillin and, cassette chromosome recombinase (ccr) gene complex that encodes for site specific recombinases to move the element from one site to the other (Hiramatsu et al., 2013; Deurenberg et al., 2007). Among staphylococci, SCC is responsible for horizontal gene transfer. As mobile genetic elements, insertion sequences (ISs) and transposons are included in the *S. aureus* genome. Ability of ISs to inactivate a gene function (as a result of insertion to the gene proximity) or up-regulate transcription (as a result of integration to the upstream of the gene) provides advantage for survival in challenging environments. When SCC*mec* is integrated into the *oriC* of MSSA, MRSA is generated (Hiramatsu et al., 2013).

Different types of SCC provide the variability in resistance to antibiotics (Patel et al., 2008). SCC*mec* types I to V range between 20.9 to 66.9 Kb. While encoding SCC*mec* type I, IV and V provide resistance to beta-lactam antibiotics, type II and III provide multi-resistance due to presence of the genes on integrated plasmids such as pUB110, pI258, pT181 and a transposon Tn554 encoding additional drug resistance genes as well as carrying insertion sequences on chromosomal DNA (Deurenberg et al., 2007).

Biofilm formation ability of MRSA causes tendency to infections in implants and provide about 100 times more resistance to antibiotics. When bacteria adhere to the implant, they reduce metabolic activity and secrete glycocalix layer. This special layer

provides the protection against antibiotics and phagocytosis. Adhesion to surfaces is provided by presence of many surface proteins in MRSA. By quorum sensing ability which is provided by interactions between cells, bacterial reproduction is inhibited to keep ideal colony number stable (Patel et al., 2008).

1.1.2. Methicillin-Resistant *Staphylococcus aureus* (MRSA)

According to the data obtained in 2005 from the United States (US) Centers for Disease Control and Prevention (CDC), MRSA infections resulted in death of 18,650 patients in the US in 2005 which is more than the number of deaths due to AIDS (Rehm and Tice, 2010). More recent data estimated 80,461 MRSA infections in the US in 2011 which reported approximately 30,800 fewer infections than of infections in 2005 (Dantes et al., 2013).

Use of beta–lactam and quinolone antibiotics is related with the overgrowth of MRSA by allowing proliferation of the organism rapidly. Also, they increase pathogenicity of the organism by changing the molecular mechanisms such as adhesion, quorum sensing, biofilm formation and production of exotoxin. These molecular changes result in more severe infections (Rehm and Tice, 2010).

When it was first reported in 1961, MRSA was admitted as nosocomial pathogen. However, prevalence of community-acquired-MRSA infections broke this assumption and lead to requirement for a sub-classification since 1990s (DeLeo et al., 2010). Sub-classification of MRSA was done based on pathogenicity, virulence, resistance against antibiotics and the patients affected by those strains. According to those differences, MRSA is sub-classified as community-acquired (CA) and hospital-acquired (health-care acquired) (HA) MRSA (Patel et al., 2008; Liu, 2009). In general, standard antimicrobial susceptibility tests for susceptibility to non-beta-lactam antibiotics, a latex agglutination test for detection of penicillin–binding protein PBP2a, PCR for detection of genes such as *mecA* or *pbp2a* can be used for identification of MRSA. However, Community-acquired MRSA (CA-MRSA) strains and Hospital-acquired MRSA (HA-MRSA) strains cannot be distinguished from each other by susceptibility tests. For discrimination of these strains, PFGE, *spa*, MLST or SCC*mec* genotyping can be used as well as control of existence of Panton-Valentine leukocidin (PVL) genes (DeLeo et al., 2010).

According to definition of CDC, CA-MRSA is a methicillin-resistant *S. aureus* strain isolated from a person within the 48 hours following admission (Patel et al., 2008; Deurenberg et al., 2007). CA-MRSA is more susceptible to beta lactam antibiotics, erythromycin and quinolones than hospital acquired ones. Affected population includes young healthy people and, the skin and the lungs as the mostly infected area (Patel et al., 2008; Rehm and Tice, 2010). PVL gene and *mecA* locus in SCC type IV and V mobile elements are the genetic characteristics for CA-MRSA (Hiramatsu et al., 2013; DeLeo et al., 2010; Rehm and Tice, 2010; Patel et al., 2008). The presence of the genes such as *lukS-PV* and *lukF-PV* encoding PVL is the reason for the high virulence of CA-MRSA compared to HA-MRSA (Rehm and Tice, 2010). Almost all CA-MRSA strains express *pvl* gene, however, only 2% of HA-MRSA strains are found to be expressing it. Product of *pvl* gene called leukotoxin lyses neutrophils resulting in soft tissue infections and necrotizing pneumonia by both MSSA and CA-MRSA strains (Rehm and Tice, 2010). Having SCC types IV and V among other SCC elements provides resistance to beta-lactam antibiotics (Hiramatsu et al., 2013; DeLeo et al., 2010; Rehm and Tice, 2010). Due to requirement of hospital association to be infected by HA-MRSA; CA-MRSA is accepted to have more virulence because it can lead to infections in healthy people (DeLeo et al., 2010).

HA-MRSA has multiple drug resistance due to presence of SCC type I, II, and III mobile genetic elements which are the characteristics of HA-MRSA (Patel et al., 2008; Liu, 2009; Hiramatsu et al., 2013). Generally, hospitalized patients, hemodialysis patients, HIV-infected patients, institutional residents and elderly people belong to affected population with variation in infection area (Patel et al., 2008).

1.1.3. Methicillin-Susceptible *Staphylococcus aureus* (MSSA)

Lack of allelic equivalent of *mecA* gene in methicillin-susceptible *S. aureus* (MSSA) makes these bacteria sensitive against methicillin. Thus, main difference between MRSA and MSSA is the presence of *mecA* gene and its product PBP2a in MRSA responsible for resistance. Infections caused by MSSA show similarity with CA-MRSA infections with decreased severity (DeLeo et al., 2010).

1.1.4. Antibiotics Used for Treatments of *Staphylococcus aureus* Infections

Many types of antibiotics are used for the treatment of MRSA infections. An antibiotic called mupirocin is produced by *Pseudomonas fluorescens* and acts by binding to bacterial isoleucyl-tRNA synthetase, irreversibly. This enzyme is responsible for converting isoleucine-tRNA to isoleucyl-tRNA and its absence inhibits RNA and protein synthesis. In Europe and the United States, intravenous daptomycin usage became common to combat with MRSA by means of its concentration dependent bactericidal and broad-spectrum activity (Patel et al., 2008). For treatment of CA-MRSA infections, antibiotics such as clindamycin, trimoxazole, long-acting tetracyclines, rifampicin and fusidic acid are commonly used (DeLeo et al., 2010). Linezolid is another antibiotic which is recommended by the Food and Drug Administration (FDA) in the USA used for the treatment of pneumonia, skin and soft tissue infections (DeLeo et al., 2010; Patel et al., 2008).

1.2. Action Mechanisms of Antibiotics on Pathogenic Bacteria

Antibiotics are the molecules mainly produced as metabolites of fungi and actinomycetes (Such as *Streptomyces*, *Bacillus*, *Myxobacteria*, etc.). These low molecular weight metabolites are commonly found in the environment. Their natural roles include protection of the strain from the other organisms and acting as signaling molecules to induce differentiation into a distinct phenotype (Laureti et al., 2013). Their classification can be based on their structure, their biological effect or the action mechanism on bacteria. Structure-based classes of antibiotics include beta-lactams, quinolones, aminoglycosides, tetracyclines, etc. When their biological effect is considered, they are classified as bactericidal or bacteriostatic. While bactericidal antibiotics kill the bacteria, bacteriostatic antibiotics inhibit the bacterial growth. When the mode of actions of main antibiotics on bacteria are considered, a few targets in the cell are provided: Inhibition of biosynthesis of cell wall, inhibition of nucleic acid synthesis, inhibition of proteins synthesis (Laureti et al., 2013; Walsh, 2000), damaging structural components of cell membrane, prevention of metabolic pathways (Etebu and Arikekpar, 2016) and increasing production of reactive oxygen species (ROS) to

damage molecules such as lipids, proteins and DNA (Laureti et al., 2013). Antibiotics inhibiting the cell wall biosynthesis hinder the peptide chains from crosslinking to form peptidoglycan layer (Walsh, 2000). Mode of action of antibiotics on nucleic acid biosynthesis compromise the DNA replication and repair by targeting enzymes like gyrases, topoisomerases. For inhibition of protein biosynthesis, antibiotics act on subunits of the ribosome and prevent different binding and catalysis steps during initiation, elongation and termination phases of synthesis (Etebu and Arikekpar, 2016; Walsh, 2000). Antibiotics damaging the cell membrane mostly interact with the lipid moieties of the membranes and disrupt their structure. A few antibiotics display their inhibitory effect by blocking metabolic pathways through mimicking essential substrates of the metabolism (Etebu and Arikekpar, 2016).

In developing countries most of the deaths are due to the infectious diseases. Overuse and misuse of drugs result in the gain of resistance in the bacteria. These resistant microorganisms are able to live in the presence of drugs and responsible for the serious diseases such as tuberculosis, pneumonia, and diarrhea. Nevertheless, therapeutic applications to fight against the microorganisms, resistant to penicillin, methicillin and vancomycin are limited as well as the development of new generation antimicrobials (Daglia, 2012).

1.3. Antibiotic Resistance of Bacteria

Although antibiotics have several action mechanisms to inhibit or kill bacteria, these microorganisms develop several strategies to fight against them. These survival strategies result in development of antibiotic resistance. Prevention of the antibiotic to enter the cell or pumping it out through the efflux pumps is one of the mechanisms of bacteria to combat with antibiotics. The alteration of the target site with some enzymatic modifications also prevents antibiotics to complex with their targets to inhibit bacteria. Another mechanism involves modification of the antibiotic in which the responsible chemical moiety that pair with the target site is disrupted (Hawkey, 1998; Walsh, 2000). Another strategy for resistance is producing a different target while the original one is still present in the cell. The genes responsible for resistance mechanisms can already be encoded within the bacterial genome and their presence leads to intrinsic resistance.

Otherwise, resistance can be originated through mutations or transferred from one species to the other by horizontal gene transfer (Hawkey, 1998).

In the literature there are several studies indicating the gain of resistance ability of bacteria against antibiotics which they were susceptible prior to exposure. Kohanski and his colleagues (2010) showed that exposure of bacteria with sublethal concentrations of antibiotics resulted in the development of multidrug resistance. In this study, they applied low levels of the norfloxacin, ampicillin and kanamycin to *E. coli* strain MG1655. While growth in the presence of the antibiotics increased the MIC for some of the drugs in different levels, growth in the absence of the antibiotics did not resulted in change in the MIC values (Kohanski et al., 2010).

In another study, transcriptome-level effect of increased MIC of an antibiotic was examined. Mongodin and his colleagues (2003) investigated the transcriptomes of clinical isolates of two different vancomycin intermediate resistant *S. aureus* (VISA) strains. Within three vancomycin passages obtained by subculturing bacteria into increasing concentrations of vancomycin, they observed stable increase in MIC. While the initial MIC for clinical isolates of VISA was 8 μg/ml, at the end of the process MIC was increased to 32 μg/ml. Transcriptional profiling of the isolates with increased MIC values revealed that expression of 35 genes present in both strains were increased and the expression of 16 genes were decreased (Mongodin et al., 2003).

The ability to gain resistance against almost all the antibiotics in use is one of the main reasons that make treatment of *S. aureus* infections more difficult. This resistance then leads to increase in the cost of the treatments and the loss of labor. As noted in the literature, investigation of alternative antimicrobials is essential to fight against antibiotic-resistant bacteria.

1.4. Phenolic Acids

Phenolic compounds are secondary metabolites that are found in most plants. Even though they are not required in processes related with growth, development and reproduction, they have functions in providing interaction to the plant and its environment. Being part of the human diet is another importance of the phenolics because of their antioxidant, anti-carcinogen (Khadem and Marles, 2010), anti-inflammatory and antimicrobial properties. Ability of polyphenols to inhibit bacterial

replication enzymes, induce apoptosis, stimulate macrophages and neutrophils help them in preventing diseases. Among their antimicrobial activities, inactivation of bacterial toxins makes them candidates as new antimicrobials to fight against antibiotic-resistant bacteria. Moreover, when bacteria are stressed with polyphenols their quorum sensing mechanism is affected and this influences the pathogenicity of the organisms (Ferrazzano et al., 2011).

Phenolic compounds are made of an aromatic ring that carries one or more hydroxyl groups. Carrying one hydroxyl group in chemical structure makes them phenols and having more than one phenolic group makes them polyphenols regardless of the number of the hydroxyl groups. Basic classification can be done by considering the phenol ring number and subgroups which are attached to these rings (Ferrazzano et al., 2011; Khadem and Marles, 2010). Flavonoids with C6-C3-C6 structure and non-flavonoids are the two main classes of the phenolic compounds. Polyphenols of non-flavonoid group are grouped according to their carbon skeleton (Khadem and Marles, 2010). All vegetative organs, flowers and fruits of the flowering plants are the most common reservoirs of the polyphenols (Ferrazzano et al., 2011). Phenolic acids belong to non-flavonoid group. Their chemical structures contain a carboxyl group attached to a benzene ring. Phenolic acids can be derivatives of either benzoic acids or cinnamic acids. While hydroxybenzoic acids have C6-C1 structures, hydroxycinnamic acids have C6-C3 structures as shown in Figure 1.1 (Khadem and Marles, 2010).

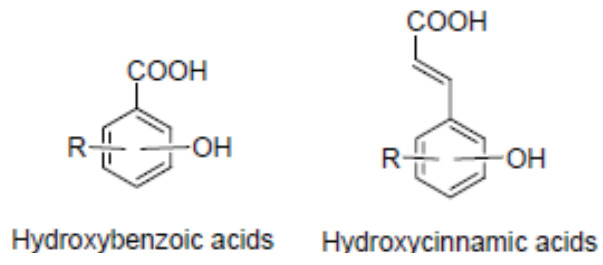

Figure 1.1. Classes of phenolic acids (Khadem and Marles, 2010).

The number and the position of the hydroxyl group as well as the presence of other substituents determine the effect and the properties of the phenolic acids. Increased number of hydroxyl groups increases the toxicity of the compound. The

reason of hydroxycinnamic acids having more antioxidant activity than hydroxybenzoic acids is the presence of CH=CH-COOH group in hydroxycinnamic acids whereas the COOH group in hydroxybenzoic acids. Higher antioxidant activity can be attributed to higher capability of H-donation and radical stabilization in hydroxycinnamic acids (Borges et al., 2013).

Cueva and his colleagues (2010) reported a study concluding the number and the position of benzene ring moieties affected the antimicrobial properties of phenolic acids. In this study, thirteen phenolic acids, including benzoic acids (e.g. vanillic acid), phenylacetic acids and phenylpropionic acids displayed antibacterial effect on bacteria such as *Escherichia coli*, *Lactobacillus* spp. and *Staphylococcus aureus* (MSSA) EP167. The growth of *S. aureus* EP167 was inhibited by all of the tested phenolic acids in a concentration dependent manner. For example, vanillic acid at the concentration of 1000 μg/mL inhibited the growth of *S. aureus* EP167 at the percentage of 71.89%; and benzoic acid at the concentration of 1000 μg/mL inhibited 94.35%. In this publication, it was also concluded that the number and position of benzene ring moieties affected the antimicrobial properties of the phenolic acids depending on the membrane structure of bacteria. Gram positive *S. aureus* lacking an outer membrane was found to be the most susceptible strain among all tested bacteria (Cueva et al., 2010).

In a similar study in 2013 (Borges et al., 2013) gallic acid and ferulic acid, a trihydroxybenzoic acid and a hydroxycinnamic acid respectively, were tested on some pathogenic bacteria including *S. aureus*. Their aim was to search for new chemicals with antimicrobial properties to combat with antibiotic-resistant bacteria. Action mechanisms of gallic acid and ferulic acid were studied to find minimum inhibitory concentration, minimum bactericidal concentration, permeability of membrane, release of potassium and change in the surface charge. Minimum inhibitory concentration of ferulic acid and gallic acid for *S. aureus* was found to be 1750 μg/mL and 1100 μg/mL, respectively. Minimum bactericidal concentration for *S. aureus* was 5500 μg/mL for ferulic acid and 5250 μg/mL for gallic acid. These phenolic acids also affected the membrane properties by changing hydrophobicity and leading to formation of pores which then resulted in intracellular leakage (Borges et al., 2013). These studies highlighted the higher inhibitory effect of benzoic acids on bacteria in comparison to cinnamic acids when they are applied to same bacterial strain.

1.4.1. 2-Hydroxycinnamic Acid (*o*-coumaric acid)

2-hydroxycinnamic acid is one of the phenolic acids that were tested in this study and it is a derivative of cinnamic acid (Borges et al., 2013). Its name origins from the presence of one hydroxyl group at the second carbon (Figure 1.2). This phenolic acid is also called *o*-coumaric acid.

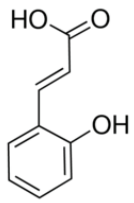

Figure 1.2. Structure of 2-hydroxycinnamic acid (Sigma-Aldrich).

1.4.2. Vanillic Acid

Vanillic acid (Figure 1.3) is another phenolic acid that was tested in this study. It is a hydroxybenzoic acid carrying one methoxy and one hydroxyl group. Vanillic acid is also called 4-hydroxy-3-methoxybenzoic acid. It is found in many plants such as Japanese alder, cotton, ginseng, red sandalwood and some of the mushroom species. Vanillic acid has antisickling and anthelmintic activities besides its antioxidant and antimicrobial activities. Suppression of hepatic fibrosis and inhibiting snake venom 5-nucleotidase are its other properties (Khadem and Marles, 2010).

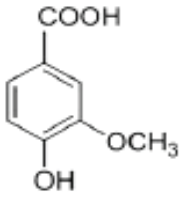

Figure 1.3. Structure of vanillic acid (Khadem and Marles, 2010).

1.4.3. Antibacterial Effects of Phenolic Acids

The studies investigating antibacterial effects of phenolic acids commonly focus on the minimum inhibitory concentrations, minimum bactericidal concentrations, and the effect on the membrane structures of different pathogenic strains. In a study conducted by Lou and his colleagues (2011), the action mechanism of chlorogenic acid against *Staphylococcus aureus* 6538, *Streptococcus pneumonie* ATCC49619, *Bacillus subtilis* 9372, *Escherichia coli* ATCC25922, *Shigella dysanteriae* 51302 and *Salmonella* Typhimurium 50013 was investigated. MIC of chlorogenic acid was found via pour plate method by preparation of different concentrations. The lowest concentration that inhibited the bacterial growth (MIC) was determined against all these pathogenic bacteria and found to be between 20-80 μg/ml. Mode of chlorogenic acid action was tested by measuring outer membrane permeability in Gram-negative bacteria. They observed increase in permeability of the membrane and leakage of potassium following chlorogenic acid treatment. Use of transmission electron microscopy confirmed the effect of chlorogenic acid on *S. dysanteriae* and *S. pneumonia* after 90 minutes of treatment (Lou et al., 2011). This study referred to the effect of phenolic acids on the bacterial membranes.

Presence of phenolic acids in daily consumed foods in high amounts increases their value to be used as therapeutic agents. Nohnyek and his colleagues (2006) tested the antimicrobial effects of berries that contain derivatives of hydroxycinnamic and hydroxybenzoic acids on pathogenic bacteria including *S. aureus*. They examined the viability of the cells treated with berry extracts with nucleic acid probes SYTO9 and propidium iodide (PI). Treated cells stained with PI gave red fluorescence indicating damaged bacterial membrane. When *S. aureus* cells were treated with cloudberry and strawberry, they observed red fluorescence. Moreover, plate count results proved 1 to 3 logarithmic decrease in the number of the cells treated with strawberry and 4 to 5 logarithmic unit decrease in the cells treated with cloudberry extracts. Their conclusion was that berry phenolics can inhibit the bacterial growth and these phenolics are promising for therapeutic usage (Nohynek et al., 2006). Nohnyek's study not only indicated that the phenolic acid effected disruption of membranes in Gram-positive bacteria but also proved the idea of dose dependent daily consume of phenolic acids for the development of alternative antimicrobial compounds.

Although the cell membrane is the first target for antibacterial action of phenolic acids, it is not the only one. Targeting of genetic materials is also shown in several studies. In a study conducted by Lou et al. (2012), dual effect of *p*-coumaric acid on bacteria including *Staphylococcus aureus* 6538, *Shigella dysanteriae* 51302 and *Salmonella typhimurium* 50013 was shown in case of membrane permeability and interaction with bacterial DNA. Increased amount of K+ ion leakage from the cells when they were treated with *p*-coumaric acid was observed DNA binding assays proved the binding of *p*-coumaric acid to bacterial DNA which then may inhibit the cellular functions (Lou et al., 2012).

Utility of phenolic acids as potential antimicrobials, especially for drug-resistant microorganisms was also hypothesized by Alves and his colleagues in 2013. In this study, the antibacterial effects of 2,4-dihydroxybenzoic, vanillic, syringic and *p*-coumaric acids were tested against MRSA and MSSA. It was observed that these compounds had more inhibitory effect on MRSA than MSSA when they were used at the same concentrations. Moreover, by using structure-activity relationship (SAR) analysis and docking studies against PBP2a of MRSA, they displayed possible action mechanism of these compounds on this protein. The information provided by this study showed the importance of substituents of phenolic acids for anti-MRSA activity (Alves et al., 2013). This study was important with respect to its findings on (i) susceptibility of two antibiotic-resistant strains of *S. aureus* differing only in methicillin resistance and (ii) effect of different substituents of phenolic acids on the same protein.

The antimicrobial effects of phenolic acids on cellular structures of some bacteria were studied; however, their mode of action with respect to global effect is not defined in detail yet and needs to be clearly investigated for further therapeutic purposes. For characterization of biological systems in more detail molecular-based studies are required.

1.5. Proteomic Analyses for Determination of Phenolic Acid Action Mechanisms

As a term, proteomics refers to the qualitative and quantitative analysis of all proteins present in an organism or in a cell under certain conditions (Ferguson and Smith, 2003). While genes remain usually constant in each cell, physiological or

environmental conditions change the mRNA, proteins and metabolites within a cell (Baggerman et al., 2005). For understanding relative expression levels of proteins, transcriptome analyses can be performed; however, mRNA levels within the cell do not correlate with the activity of the proteins. Moreover, protein functions may change by the presence of several posttranslational modifications (Ferguson and Smith, 2003). While mRNAs are responsible for transmitting genetic information, proteins are the real reflectors of function (Baggerman et al., 2005). Consequently, data obtained from proteomic studies would be accepted as more relevant for characterization of biological systems (Aslam et al., 2017).

Apart from the global pattern of gene expression, proteomics covers the regulation of gene expression. This regulation ensures the coding of the proteins required for the growth and the survival of the cell. Since production of these proteins in the necessary amount and the transportation of them to corresponding locations are tightly regulated, specific parts of the genome are activated under different conditions. For investigation of changes in the protein profiles of the cells, production of a reference map is fundamental. By comparison of a reference map obtained from the cells grown under standard conditions with the protein map obtained from the cells subjected to different stimuli, changes in the protein expression can be determined. This comparison provides information about the proteins which are repressed under standard conditions and which are activated under different stimuli (Hecker et al., 2003).

Mass spectrometry-based analysis of proteins is commonly used in proteomic researches. Mass spectrometric analyses involve either gel-based or gel-free approaches for separation of proteins prior to identification of individual proteins or protein profiles.

1.5.1. Mass Spectrometry for Protein Identification

Mass spectrometry (MS) is a technique used for identification of proteins which can be targets for many drugs. Beyond sensitivity and speed of this technique, its capability to be combined with separation techniques increases its role in identification of new targets for therapeutic agents. MS is used for determination of the mass-to-charge (m/z) ratio of the analyte and consists of an ionization source, a mass analyzer and a detector.

In mass spectrometry, different ionization techniques can be used such as electron impact, chemical ionization, fields desorption, electrospray ionization (ESI) and laser desorption. In proteomic studies, the most commonly used ionization methods are ESI and matrix-assisted laser desorption ionization (MALDI) (Karpievitch et al., 2010). MALDI is an ionization technique in which ion desorption is done from solid phase. This technique requires the co-crystallization of the analyte with the appropriate matrix on a MALDI target plate. Following transition into the gas phase under the laser beam, desorbed and ionized analytes are directed towards the mass analyzer (Glish and Vachet, 2003). On the other hand, ESI is known as a soft ionization technique in which chemical bonds of the molecules are not broken and allows further fragmentation of the peptides. In the ESI, electrostatic repulsion ensures the leaving of drops of the solvent from a spray. While the solvent evaporates from a fine spray, the disappearing of droplets as highly charged molecules is achieved. Since ESI allows accommodation of peptides in a liquid solution, it is highly efficient for being an interface in LC-MS/MS studies (Karpievitch et al., 2010).

The ion source of the mass spectrometer is the place for production of charged species and provides a passage to the analyzer and detector. In the analyzer, separation of ions is performed according to their mass to charge (m/z) ratio (El-Aneed et al., 2009). Time of flight (TOF) mass spectrometer is a commonly used mass analyzer which separates ions according to their velocity. The logic behind TOF is that the higher velocities can be reached by ions with lower m/z. Therefore, following formation of the ion, the measurement of the time that is taken by the ion until it reaches the detector determines the m/z ratio (Glish and Vachet, 2003).

1.5.1.1. Gel-Based Proteomics: Two Dimensional-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) is a commonly used technique for protein separation. Presence of sodium dodecyl sulfate in the gel is required for denaturation of the proteins to separate them based on their molecular weight. Otherwise, migration of proteins according to their charge and mass cannot be prevented. Therefore, all proteins would be capable of migrating to the same direction on the electrical field (Aslam et al., 2017).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is one of the approaches used for the characterization of protein mixtures. In this technique, proteins are firstly separated according to their isoelectric point and then to their molecular weight (Ferguson and Smith, 2003; Baggerman et al., 2005; Aslam et al., 2017). Prior to second separation, protein solubility and denaturation should be carried out by applying detergents such as SDS and CHAPS and chaotropic agents such as urea and thiourea (Zhang et al., 2013). Since these two kinds of separation parameters are not related, proteins are uniformly distributed on a gel providing a map of protein spots that reflects the protein fingerprint of the sample of interest. Visualization of the proteins on the gel is provided by staining. Coomassie blue staining is one of the most preferred staining methods due to its properties such as being inexpensive, user-friendly and allowing easy relative quantification (Baggerman et al., 2005). Following excision of the protein spots, digestion with a protease provides removal of peptides from the gel. Analysis is done by matrix-assisted laser desorption ionization coupled to mass spectrometry by observation of the peptide peaks in the spectra. Comparison of obtained peak pattern with the predicted fragments of proteins in a database provides protein identification (Ferguson and Smith, 2003). In this technique, hydrophobicity of the proteins, the isoelectric point and the molecular weights of the proteins may limit the comprehensive proteome analysis. Inadequate separation of proteins on the immobilized pH gradient during isoelectric focusing can lead to the formation of overlapping spots on the gel. Besides, due to presence of posttranslational modifications, the same gene products may locate to the different positions on the gel and the observation of low-abundance proteins may be difficult (Ferguson and Smith, 2003; Dung et al., 2013; Baggerman et al., 2005).

Despite the fact that 2D-PAGE technique has some difficulties, it is commonly used in comparative proteomic studies. 2D-PAGE analyses of cellular or extracellular proteins of the bacterial strains in different growth conditions provide reference maps for identification of the cell membrane or the cytoplasmic proteomes. These findings are essential for understanding the significance of the bacterial response to the stress conditions and to provide insights into antibacterial mechanisms of potential antibacterial drugs (Sianglum et al., 2011). Pasqua and his colleagues (2010) used this technique to investigate the antibacterial action mechanism of thymol, a phenolic compound, against *Salmonella enterica* serovar Thompson. For documentation of targets of thymol in the bacterial cell, 2D-PAGE and MALDI- TOF/MS analyses were combined. Comparison of thymol-treated cells with control group revealed that different

chaperone proteins and thermal stress proteins were upregulated upon thymol treatment; while proteins with roles in some metabolic pathways were significantly downregulated. This work emphasized the adaptation ability of the bacteria to the antimicrobial stress conditions in the presence of sublethal concentrations (Pasqua et al., 2010).

In 2011, the same combinational techniques were used for determination of the antibacterial effect of rhodomyrtone, an acylphloroglucinol derivative of *Rhodomyrtus tomentosa*, on MRSA and *S. aureus* ATCC 29213. Rhodomyrtone showed inhibitory effect and displayed MIC and MBC values as 0.5 μg/ml and 2 μg/ml, respectively. To understand the action mechanism of this compound, proteome analyses was conducted with subinhibitory concentrations against MRSA. Proteomic profiling of MRSA with 2D-PAGE and MALDI-TOF/MS analyses revealed the proteins whose expression changed upon treatment. The proteins that were upregulated or downregulated corresponded to the proteins with roles in cell wall biosynthesis, cell division, protein degradation, stress responses, virulence factor and some metabolic pathways (Sianglum et al., 2011). The findings of this study revealed the primary targets and/or the effects of thymol action within the cell which was important for showing global response of bacteria against the tested antimicrobial compound.

Although commonly used in proteomic studies, 2D-PAGE, a top-down proteomic approach, has some limitations in the identifications of proteins found in low abundance, proteins located on the membrane or proteins having extreme physicochemical properties (Yang et al., 2012). These limitations prompt the use of first-dimension liquid chromatography separation that provides better compatibility with downstream analysis such as mass spectrometer (Yang et al., 2012; Resing and Ahn, 2005). Liquid phase separations of proteins coupled to a mass spectrometer provide more comprehensive and less biased proteome analysis when compared with 2D-PAGE separation technique (Ferguson and Smith, 2003).

1.5.1.2. Gel-Free Proteomics: Bottom-up Shotgun Proteomics

The main difference between top-down proteomics and bottom-up proteomics method is while intact proteins are analyzed in the first approach, the second deals with the analysis of peptides (Resing and Ahn, 2005; Karpievitch et al., 2010). Performing bottom-up protein analysis on a protein mixture is called shotgun proteomics (Zhang et

al., 2013). Bottom-up shotgun proteomics is also known as multidimensional liquid chromatography tandem mass spectrometry (LC-MS/MS) or multidimensional protein identification technology (MudPIT) (Resing and Ahn, 2005). In shotgun proteomics, high performance liquid chromatography (HPLC) provides separation and is comprised of a stationary phase that contains a non-polar (hydrophobic) column, a pump to form pressure and to mobilize the mobile phase that contains polar eluents, and a detector for capturing the retention time. With the separation achieved by HPLC, only the peptides eluted from the column at a time is introduced into the mass spectrometer. Since the peptides having unlike hydrophobicity elute from the column at different times, they are analyzed in mass spectrometer at different times and so, their overlap is prevented (Karpievitch et al., 2010).

In shotgun proteomics, the solution containing a mixture of complex proteins is separated to its peptides via chromatography before performing MS/MS analysis. Peptide sequencing is done by automatic selection of ions and subsequent fragmentation by MS/MS which then enables obtaining thousands of spectra from a single analysis (Resing and Ahn, 2005; Karpievitch et al., 2010; Carrera et al., 2017; Meissner and Mann, 2014). However, after digestion of proteins with proteases, an intermediate step may be required to clean, desalt and concentrate the peptide mixture. This is mostly achieved by pipette-based devices. Ionization of eluted peptides was achieved with an electrospray located at the tip of the column and then transferred into mass spectrometer. The mass, ion intensity and the list of peptide fragments are collected for each peptide within the mass spectrometer. The first record of the peptide mass referred as MS or MS1. After fragmentation of individual peptides, obtained second mass spectra is recorded as MS/MS, MS2 or tandem mass spectrum. While the information about the mass of the peptide and masses of the fragments is used for peptide identification, ion intensity is used for quantification of the peptide (Meissner and Mann, 2014). For identification of peptides based on MS/MS spectra, search programs such as MASCOT or Sequest are used for comparison of observed fragment ions against a protein database (Resing and Ahn, 2005; Carrera et al., 2017).

It has been known that single liquid chromatography followed by tandem mass spectrometry is limited when compared with fractionation of protein samples before LC-MS/MS analysis. Like 2D-PAGE, multidimensional chromatic separation is crucial for decreasing the complexity of the sample. Therefore, before MS/MS analyses two-dimensional liquid chromatography (2D-LC) separation is commonly performed (Yang

et al., 2012). 2D-LC is a technique that provides improved analytical range and proteomic coverage. This technique is one of the most common approaches used in MS/MS proteomic studies due to its effective LC separation. Each separation provides different chromatographic resolving power. High-pH reverse phased liquid chromatography (RPLC) as a first dimensional separation provides a significant difference in charge distribution of peptides within the mobile phase. Concatenation of the fractions of RPLC obtained during early, middle and late separation in the first-dimension separation of RPLC provides improved analysis coverage (Wang et al., 2011), decreased number of peptide fractions and more efficient elution in the second dimensional separation (Yang et al., 2012). Low-pH RPLC is mostly used as a second dimensional separation due to its convenience in coupling to mass spectrometry via electrospray ionization. These types of LC separations performed in different pHs are prevalently used in proteomic studies and it has been proven to have more orthogonality when compared with other approaches (Wang et al., 2011; Yang et al., 2012).

Walters and Mobley (2009) claimed the use of two dimensional liquid chromatography coupled to a mass spectrometer may be useful for identification of membrane proteome of bacteria grown under different conditions that may help development of vaccines against pathogenic microorganisms. In their study, investigation of the surface proteome of uropathogenic *Escherichia coli* (UPEC) while they grow in human urine was achieved. Since UPEC are responsible for the majority of urinary tract infections, the researchers aimed to identify candidate antigens for vaccine production. One of the methods they used for this purpose was labeling surface-exposed peptides with biotin tags and applying two-dimensional liquid chromatography-tandem mass spectrometry (2-DLC-MS/MS). Following isolation of outer membrane proteins of *E. coli,* strong cation exchange chromatography was used to fractionate the peptides. By using MALDI-TOF/TOF analyzer mass spectra was obtained and by using MASCOT search engine identifications of peptides were achieved. With this technique, 25 predicted outer membrane proteins which produced during growing in urine were found (Walters and Mobley, 2009).

Shotgun proteomics can also be used for characterization of bacteria for assignment of species-specific biomarkers. Carrera et al. (2017) used shotgun proteomics for characterization of different strains of *Staphylococcus aureus*. The results obtained from LC-MS/MS were searched against the preset database of *S. aureus* in UniProt/TrEMBL database. The researchers identified a total of 644 proteins and

predicted network of protein-protein interaction. Some of the identified proteins belong to pathways related with energy, lipid metabolism and virulence of the bacteria. Species-specific peptide biomarkers were also screened and 21 of them were assigned to identify species of *Staphylococcus aureus* (Carrera et al., 2017).

These studies reflect the practical usage of proteomics for investigation of differentially expressed proteins of bacteria grown under different environmental conditions. The information obtained from such experiments is essential for clearly defined targets of antimicrobial compounds on pathogenic bacteria which then may help us to combat with antibiotic-resistant bacteria.

CHAPTER 2

ANTIMICROBIAL EFFECTS OF PHENOLIC ACIDS ON *Staphylococcus aureus* AND DETERMINATION OF THEIR RESISTANCE DEVELOPMENT

2.1. Introduction

The revolutionary discovery of antibiotics not only helped saving many lives, but also alternatively used in agriculture, animal-care, cleaning products and in research. This overuse resulted in release of excessive amounts of antibiotics into the biosphere and provided constant selection to resistant strains in the environment. The excessive use of antibiotics for the treatment of a disease caused by a human pathogen generates multidrug resistant (MDR) strain. Then, many of these bacterial pathogens evolve into superbugs and it leads to overall increased morbidity and mortality rates. The resistance to antibiotics remains its importance and results in financial and clinical problems all over the world (Davies and Davies, 2010).

Staphylococcus aureus is accepted as one of the most serious superbugs that are notorious for nosocomial infections. However, it is also a community-acquired pathogen and the diseases caused by this bacterium are difficult to cure due to its multidrug resistance (Davies and Davies, 2010). Therefore, the search for novel antimicrobial agents is essential to fight against pathogens resistant to multiple antibiotics. Use of plants and plant extracts for treatment of many diseases is very common in traditional medicine (Howes et al., 2003) and in phytotherapy. Plants have these therapeutical properties due to presence of compounds such as alkaloids, lignans, flavonoids, essential oils, and phenolic compounds (Chandra et al., 2017). Potential of plant-derived compounds as antimicrobial agents that would be used against pathogens resistant to multiple antibiotics is curiously investigated in many studies (Akiyama et al., 2001; Daglia, 2012; Vaquero et al., 2007; Nostro et al., 2007). Investigation of phenolic acids might provide a foundation of alternative antimicrobials due to presence in the plants in the high amounts (Borges et al., 2013) and their antimicrobial properties

(Oliveira et al., 2010; Cueva et al., 2010) as well as aforementioned health beneficial properties. Other advantages of using plant-derived compounds as antimicrobial agents are their abundance in the environment, their eco-friendly and safe nature, and broad-spectrum activity against microorganisms. Moreover, plant metabolites can be used as enhancers of bioactivity of the therapeutically used antibiotics. Exhibition of such synergistic activity help inhibition of antibiotic-resistant bacteria (Shin et al., 2018).

All aforementioned properties of plant-based metabolites are included in the phenolic acids used in this study, namely, vanillic acid and 2-hydroxycinnamic acid. Apart from their antimicrobial and health-beneficial properties, there is no known resistance mechanism of *S. aureus* to any of the phenolic acids, although literature lists many studies showing gain of resistance to an antibiotic following exposure of bacteria to subinhibitory or inhibitory concentrations (Kohanski et al., 2010; Pillai et al., 2009; Nair et al., 2013). Investigation of resistance to antimicrobial compounds other than antibiotics is rarely studied. Blair et al. (2009) used *Leptospermum* honey to determine its antibacterial effect in terms of its spectrum, resistance development and transcriptome analysis on several pathogens. The induction of resistance in bacteria to antibiotics and honey was achieved by exposure to sub-lethal concentrations of them. This exposure resulted in increased MIC value for tested antibiotics; however, any resistance to *Leptospermum* honey was not developed (Blair et al., 2009). This study showed how quickly antibiotic resistance was gained by pathogens when they are treated with subinhibitory concentrations in contrast to plant-based compound. The development of resistance to presence of phenolic acids was tested for a well-known food-borne pathogen *Listeria monocytogenes* by Takahashi and his colleagues in 2015. They compared gain of resistance to a bacteriocin (nisin) obtained from another microorganism, and to ferulic acid which is a derivative of hydroxycinnamic acids. While exposure of bacteria to nisin resulted in increased MIC, there was no change for MIC of ferulic acid at the end of the exposure process. Their conclusion was lower risk of development of resistance against ferulic acid when used in foods as food preservatives (Takahashi et al, 2015).

Studies showed that the presence of subinhibitory concentrations of antibiotics in the environment creates a selective pressure on bacteria for more resistant strains. On the other hand, presence of phenolic acids in their environment does not result in development of a natural resistance against them unless the pathogens are not phenolic compound degrading bacteria. Therefore, the aims of this study were to investigate the

antibacterial effects of vanillic acid and 2-hydroxycinnamic acid (*o*-coumaric acid) on MRSA and MSSA by determination of minimum inhibitory concentrations of each phenolic acid and to determine the resistance development ability of these bacteria against them by inducing bacteria with subinhibitory phenolic acid concentrations.

In this part of the study, two hypotheses were tested:

I. If vanillic acid and 2-hydroxycinnamic acid (*o*-coumaric acid) have antibacterial effects, then the treatment of MRSA and MSSA with a specific amount of any of these phenolic acids would inhibit the total growth of bacteria.

II. If antibiotic-resistant MRSA and MSSA develop resistance against these phenolic acids, then continuous exposure to subinhibitory phenolic acid concentrations would generate phenolic acid resistant bacterial population.

2.2. Materials and Methods

In this study, methicillin-resistant *Staphylococcus aureus* (MRSA) (39) N315 type II SCC*mecA* and methicillin-susceptible *Staphylococcus aureus* (MSSA) (27) ATCC 29213 strains were used.

2.2.1. Bacterial Culture Conditions

Bacteria were grown in tryptic soy broth (TSB) (Sigma-Aldrich 22092) and agar (TSA). Stock cultures of MRSA and MSSA were maintained in TSB containing 20% glycerol and stored at –80^{o}C. For short-term storage, cultures were maintained at +4^{o}C by sub-culturing onto TSA plates in every week.

2.2.2. Determination of Bacterial Growth Curve

A single colony of bacteria was inoculated into 4 ml of TSB and placed into 37^{o}C for overnight incubation. Then, it was transferred into fresh TSB as 2% inoculum and this was accepted as initial time point (0th hour). After first optical density (OD_{600nm}) was measured spectrophotometrically, the cultures were incubated at 37^{o}C. Optical densities were measured every three hours during 24-hour (h) incubation period.

Determination of the viable cell number in the cultures was accomplished by spread plating 100 µl of the bacteria onto TSA plates in every three hours during the incubation period and, incubated overnight at 37°C. The colonies on these plates were enumerated and bacterial count was noted as colony forming unit/ml (cfu/ml).

2.2.3. Determination of Antimicrobial Activities of Phenolic Acids

Preparation of bacterial culture was performed in a same way for each antimicrobial experiment. For this purpose, a single colony of bacteria (MRSA or MSSA) was inoculated in 4 ml TSB media and incubated in 37°C for overnight incubation. Following optical density measurement, MRSA and MSSA cultures were refreshed into TSB media as 2% inoculums.

For determination of initial bacterial number, viable cell count method was used in every experiment. Following the overnight incubation at 37°C, the colonies were enumerated and the count results were noted as cfu/ml.

2.2.3.1. Determination of Effects of Ethanol and Dimethyl Sulfoxide on Bacteria

Since vanillic acid and 2-hydroxycinnamic acid were not soluble in the water, different concentrations of ethanol (Sigma-Aldrich 32221) and dimethyl sulfoxide (DMSO) (Sigma-Aldrich D5879) were tested against MRSA and MSSA to be used for the preparation of phenolic acids.

Ethanol and DMSO concentrations were prepared by mixing certain amount of ethanol and DMSO with TSB media. The tested concentrations of ethanol and DMSO ranged between 0.1% and 25%. The prepared bacterial culture (See section 2.2.3.1) was added into DMSO or ethanol containing tubes which had 2-fold of intended final concentrations. Blanks were prepared with the same concentrations of DMSO and ethanol without the addition of bacteria. The bacteria inoculated into TSB were used as positive control.

The OD_{600nm} measurements were taken at 0^{th}, 2^{nd}, 4^{th}, 8^{th}, 9^{th} and 24^{th} hours of incubation at 37°C.

2.2.3.2. Preparation of Phenolic Acid Solutions

Vanillic acid (VA) (Sigma-Aldrich 94770) and 2-hydroxycinnamic acid (2-HCA) (Sigma-Aldrich H22809) were purchased commercially to keep the standard. Phenolic acids were prepared freshly before each experiment. Different concentrations of these phenolic acids were prepared by dissolving them in DMSO in TSB media with 2-fold of the intended final concentration of the phenolic acids.

2.2.3.3. Determination of Antimicrobial Activities of Phenolic Acids

Antimicrobial activities of phenolic acids were determined both spectrophotometrically and by performing viable cell count. The phenolic acids were used at the final concentrations from 0.8 mg/ml to 2 mg/ml for 2-HCA and to 2.5 mg/ml for VA. MRSA and MSSA strains were prepared as explained in section 2.2.3.1. The bacteria grown in TSB were used as control. Blanks were the same concentrations of phenolic acids without addition of bacteria. By measuring OD_{600nm} at 0^{th}, 8^{th} and 24^{th} hours of incubation, antimicrobial activities of phenolic acids were determined. At the 24^{th} hour, the concentrations that inhibit the total bacterial growth were assigned as minimum inhibitory concentration (MIC). From the tubes that gave no visible growth at the 24^{th} hour, 100 µl of bacterial cultures was spread plated and incubated for overnight at $37^{o}C$. Cell count results were noted as cfu/ml. These experiments were performed for at least two times for each phenolic acid on MRSA and MSSA.

2.2.4. Morphological Study of Bacteria in the Presence of Phenolic Acids

Morphological examination of bacterial cells grown in the presence and absence of phenolic acids was carried out with scanning electron microscopy (SEM). For surface characterization, effect of vanillic acid and 2-hydroxycinnamic acid were investigated both for MIC values and about MIC_{50} values and compared with control cells. MRSA and MSSA cultures were prepared as explained in section 2.2.3.1. They were inoculated (2%) into 4 ml TSB containing 1.3 mg/ml and 2.5 mg/ml VA or 1.2 mg/ml and 1.6

mg/ml 2-HCA. The 4 ml TSB was inoculated with bacteria as 2% inoculation for the control. Tubes were incubated for 24 hours at 37°C. The OD_{600nm} measurements from all tubes were taken for confirmation of antimicrobial effect. Then the SEM samples were prepared as following: One ml from each culture was centrifuged at 14,000x*g* for 10 minutes. The pellet was washed with 1 ml of 0.1% peptone water times. Control MRSA and MSSA cells were resuspended in 100 µl of 0.1% peptone. The cells treated with 1.3 mg/ml VA and 1.2 mg/ml 2-HCA were resuspended in 50 µl of 0.1% peptone. The cells treated with MIC values of phenolic acids were resuspended in 25 µl of 0.1% peptone due to less amount of obtained pellet. Then, 10 µl of prepared samples was spreaded onto aluminum foil with an inoculation loop. After cells were air dried on the aluminum foil in the BSL2 cabinet, the foils were cut into squares approximately 1 cm^2 in size. These were covered with gold for 120 seconds prior to examination for distinct visualization of the bacterial cells. Scanning electron microscopes Phillips XL-30S FEG and FEI Quanta 250 FEG at İYTE-MAM were used to take micrographs.

2.2.5. Induction of Phenolic Acid Resistance Mechanism via Exposure to Subinhibitory Concentrations

Prior to induction of resistance to phenolic acids, different types of antibiotics were tested against MRSA and MSSA to find the effective concentration.

2.2.5.1. Screening of Inhibitory Concentrations of Antibiotics

The antibiotics ampicillin (Roche), kanamycin sulfate (abbreviated as kanamycin throughout the text) (Roche) and vancomycin (Koçak Farma) were tested against MRSA and MSSA. The bacterial cultures were prepared as explained in section 2.2.3.1. The antibiotics were prepared in double distilled water (ddH_2O) as 10 mg/ml stocks concentration. They were filter sterilized and were kept at -20°C.

The tested concentrations of ampicillin and kanamycin were ranged from 10 µg/ml to 200 µg/ml for both bacteria. The tested vancomycin concentrations were ranged between 1 µg/ml and 50 µg/ml for MRSA and were ranged between 2.5 µg/ml and 10 µg/ml for MSSA (Concentrations of the antibiotics were indicated as ‘ug/ml’

within the graphics). All tested concentrations of the antibiotics freshly prepared prior to the experiments in TSB from the stocks. The addition of 2% bacterial inoculum into the antibiotic containing media was accepted as initial time point (0^{th} hour) and first measurement was taken at OD_{600nm}. After incubation at $37^{o}C$ for 24 hours, the OD was measured. The tests were performed at least two times for each antibiotic.

2.2.5.2. Determination of the Effect of Increasing Subinhibitory Concentrations of Phenolic Acids and Vancomycin

The design of the experiments to induce resistance against VA and 2-HCA was based on the study of Blair and his colleagues (Blair et al, 2009) with minor modifications. The VA, 2-HCA and vancomycin solutions were prepared freshly before each experiment at the desired concentrations in TSB. For preparation of phenolic acid concentrations 0.6% DMSO was used to increase the solubility of the compounds. The bacterial cultures were prepared as in section 2.2.3.1. Control groups were MRSA and MSSA grown in TSB without addition of any antimicrobial compound. In this experiment, the initial subinhibitory concentrations were tested as 1.3 mg/ml VA and 1.2 mg/ml 2-HCA for both MRSA and MSSA; 5 μg/ml vancomycin for MRSA and 7.5 μg/ml vancomycin for MSSA by considering the results obtained from the experiments explained in section 2.2.3.4. Also, the predetermined MIC values of all antimicrobials were tested against both bacteria.

Determination of resistance development to VA, 2-HCA and vancomycin was started with the addition of 2% inoculum from overnight culture of bacteria into the media containing both subinhibitory and inhibitory concentrations of all tested antimicrobial agents. Following 24-hour incubation at $37^{o}C$, ODs of cultures were measured. For determination of the viable cell number of the cultures after 24 hours of incubation, 100 μl of bacterial cultures was spread plated and incubated at $37^{o}C$ for 24 hours. Then, the 2% inoculum from the media containing subinhibitory concentration of antimicrobials was transferred into the freshly prepared phenolic acid and vancomycin solutions (4 ml total volume) that had increased concentrations. The increments in the subinhibitory concentrations were 0.1 mg/ml and 0.5 μg/ml for phenolic acids and vancomycin, respectively. The increments in predetermined MICs were 0.2 mg/ml and 5 μg/ml, for phenolic acids and vancomycin, respectively. Namely, 2% inoculums of

bacteria (MRSA or MSSA) from the test tube that contain 1.3 mg/ml VA was transferred into 1.4 mg/ml (increased subinhibitory concentration) VA, 2.5 mg/ml VA (which was predetermined as MIC), 2.7 mg/ml (increased MIC) VA containing test tubes and into 4 ml TSB as control. The other transfers were performed in the same manner as indicated increments. For the positive control 80 µl bacterial culture from the previous control tube was transferred into 4 ml TSB. Subsequently, 0^{th} hour OD measurement was taken and the transferred cultures were incubated at 37^{o}C for 48 hours. Following 48-hour incubation, OD measurements were taken at 600 nm.

To continue to test the effect of subinhibitory concentrations of phenolic acids and vancomycin, second increments in the concentrations of the compounds were carried out. As before, the increments in the tested subinhibitory concentrations were 0.1 mg/ml for phenolic acids and 0.5 µg/ml for vancomycin. And increments in the MICs were 0.2 mg/ml and 5 µg/ml for phenolic acids and vancomycin, respectively. The transfer time was accepted as 0^{th} hour and first OD measurement was taken at 600 nm. All tubes were placed into 37^{o}C for 72-hour incubation.

After 72-hour incubation, the ODs of bacterial cultures were measured at 600 nm by transferring them into the wells of 96-well plate. From all test and control tubes, spread plating was performed by spreading 100 µl bacterial culture following serial dilutions of the samples. Plates were placed into 37^{o}C for 24-hour incubation. Then, the cultures grown in the presence of indicated concentrations of phenolic acids and vancomycin for 72 hours were shifted to TSB media as 2% inoculums and 0^{th} hour OD measurement was taken. These cultures transferred into TSB were incubated for 48 hours at 37^{o}C without the addition of any phenolic acids or vancomycin.

After the incubation period, ODs of the cultures were measured again. Then, 2% inoculations from the cultures grown in TSB were transferred into test tubes containing initially tested MIC and subinhibitory concentrations of phenolic acids and vancomycin (also with increased MIC only for vancomycin). These tubes were incubated for 24 hours at 37^{o}C.

Following the OD measurement at 24^{th} hour, spread plating was done from all tubes to learn the number of viable cells within each culture. After incubating plates for 24 hours, cell count results were noted as cfu/ml. These experiments were performed for at least two times independently for both MRSA and MSSA.

2.3. Results and Discussion

In these types of studies, it was important to understand the growth behavior of bacteria grown under normal conditions. Therefore, to be able to understand their growth, firstly growth curves of bacteria were plotted.

For determination of bacterial growth, OD measurements were taken in every three hours and the growth curves of MRSA and MSSA plotted as $OD_{600\ nm}$ value versus time. The growth curves of MRSA and MSSA during incubation at 37°C for 24 hours were shown in Figure 2.1. Growth curves showed that exponential phase of bacterial growth took place in the first 9 hours of incubation and then the stationary phase began for both MRSA and MSSA.

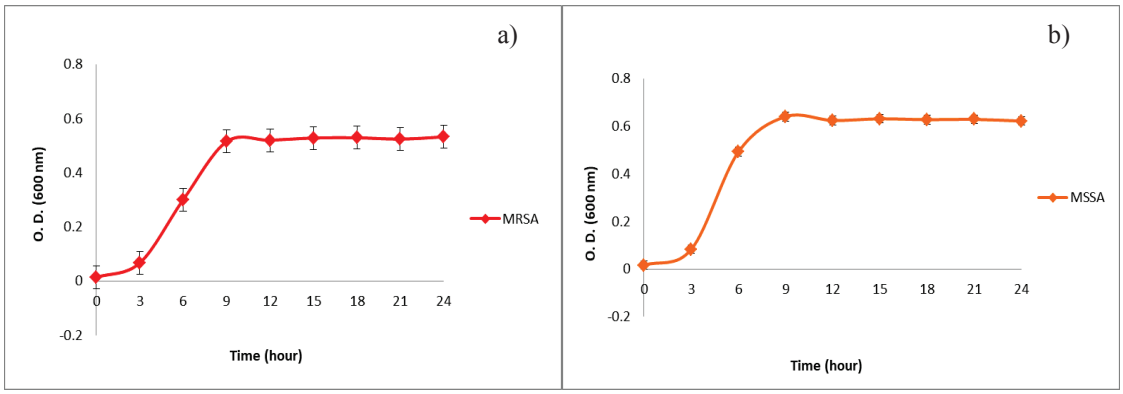

Figure 2.1. Growth curves of (a) methicillin-resistant *Staphylococcus aureus* (MRSA) and (b) methicillin-susceptible *Staphylococcus aureus* (MSSA). Standard deviations were lower than 0.042 and 0.018 for MRSA and MSSA, respectively.

Moreover, parallel with spectrophotometer measurements every three hours, viable cell count was performed by spread plating of bacterial culture taken from the test tubes. According to the cell count results, the initial bacterial number in the beginning of the experiment was 10^6 cfu/ml for both MRSA and MSSA. At 24th hour of incubation, cell number reached to 10^8 cfu/ml for MRSA and to 10^9 cfu/ml for MSSA. The viable cell count results of both bacteria were given in Table 2.1.

Table 2.1. Viable count results of bacteria during 24 hour incubation at 37°C.

Spread Plating Time Points during Incubation (Hour)	MRSA Viable Cell Count (cfu/ml)	MSSA Viable Cell Count (cfu/ml)
0th	4.2×10^6	9.1×10^6
3rd	3.2×10^7	5.5×10^7
6th	1.3×10^8	5.2×10^8
9th	2.5×10^8	7.3×10^8
12th	2.4×10^8	6.3×10^8
15th	2.5×10^8	9.1×10^8
18st	2.9×10^8	8.6×10^8
21th	3.1×10^8	1.0×10^9
24	4.5×10^8	1.0×10^9

2.3.1. Effects of Ethanol and Dimethyl Sulfoxide on Bacterial Growth

Since tested highest concentrations of vanillic and 2-hydroxycinnamic acids were not completely soluble in the water, different concentrations of dimethyl sulfoxide (DMSO) (Sigma-Aldrich D5879) were tested against MRSA and MSSA to be used for the preparation of phenolic acids. For determination of the any antibacterial effect, different concentrations of DMSO ranged between 25% and 0.1% were tested against both bacteria. For the first 9 hours of incubation, OD was measured every two hours to understand their effect on exponential phase of the bacterial growth. According to the spectrophotometer measurements at 24th hour, percent inhibitions of each concentration were calculated both for ethanol and DMSO (See Appendix A for percent inhibitions of ethanol).

The percent inhibition values of tested concentrations of DMSO were given in Table 2.2. The concentrations of DMSO ranged between 25% and 1% showed inhibitory effects on both bacteria and inhibited the growth. Therefore, 0.6% DMSO concentration was determined as the highest concentration which had no or negligible effect on bacterial growth (Figure 2.2). Percent inhibitions were calculated according to the formula 100*(1-(sample OD/control OD)).

Table 2.2. Percent inhibitions of DMSO concentrations on MRSA and MSSA.

Concentration of DMSO (vol/vol) (%)	MRSA Percent Inhibition (%)	MSSA Percent Inhibition (%)
25	96	96
20	95	94
15	82	84
10	43	35
5	10	0
1	6	8
0.6	0	2
0.5	-9	3
0.4	-3	0
0.3	-10	0
0.2	-5	0
0.1	-4	0

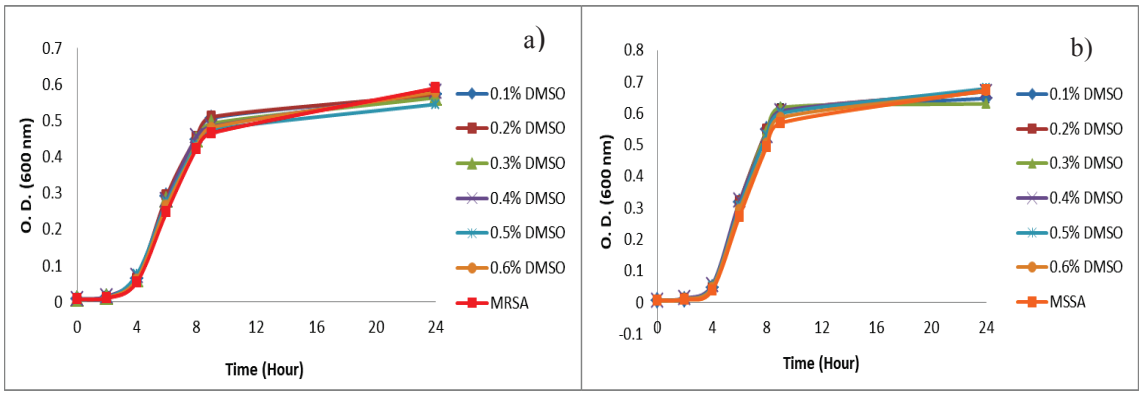

Figure 2.2. Growth curves of (a) MRSA and (b) MSSA in the presence of different dimethyl sulfoxide (DMSO) concentrations. Standard deviations for were lower than 0.015 and 0.013 for MRSA and MSSA, respectively.

Different ethanol concentrations were also tested against both MRSA and MSSA (Appendix A). Since solubility of phenolic acids in DMSO was better, 0.6% DMSO concentration was chosen to be used in preparation of phenolic acids in this study.

2.3.2. Antimicrobial Effects of Phenolic Acids on Bacteria

Antibacterial effects of vanillic acid and 2-hydroxycinnamic acid on MRSA and MSSA were determined according to the absorbance measurement values obtained during 24 h incubation at 37°C. The growth curves in the presence of each phenolic acid were plotted as OD versus time. Growth curves of MRSA and MSSA in the presence of 2-hydroxycinnamic acid (2-HCA) and vanillic acid (VA) were given in Figure 2.3 and Figure 2.4, respectively.

While some concentrations (<1.2 mg/ml for 2-HCA) of these phenolic acids retarded the growth of MRSA and MSSA, others (>1.2 mg/ml for 2-HCA) inhibited the growth totally. When the Figure 2.3 was closely examined, it was seen that 2-HCA delayed the beginning of the exponential phase for 8 hours both in MRSA and MSSA at the concentration of 1.2 mg/ml. On the other hand, the concentrations of 1.6 mg/ml and 2 mg/ml 2-HCA showed inhibitory effect on MRSA and MSSA.

Minimum inhibitory concentration (MIC) of each phenolic acid was determined as the lowest concentration that inhibited the growth of bacteria after 24 h incubation period. According to the results, MIC value of 2-HCA was determined as 1.6 mg/ml for both for MRSA and MSSA (Figure 2.3).

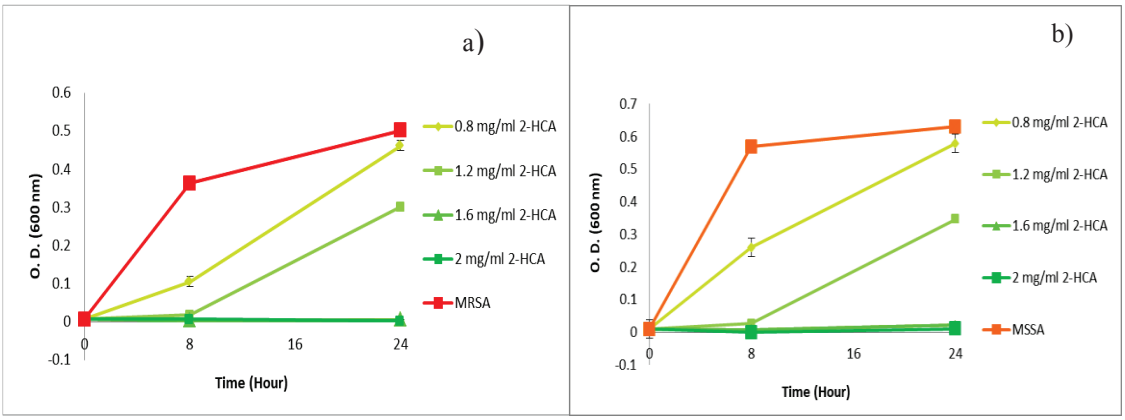

Figure 2.3. Antibacterial effects of 2-hydroxycinnamic acid (2-HCA) on MRSA and MSSA. ODs were measured at 0th, 8th and 24th hours of incubation at 600nm. Standard deviations for 2-HCA concentrations were lower than 0.015 and 0.028 for MRSA and MSSA, respectively.

The viable cell count results and the percent inhibition values of tested 2-HCA concentrations on MRSA and MSSA at the 24th hour of incubation and were presented in Table 2.3. The MRSA and MSSA control (Indicated as 0) reached to 10^8 cfu/ml bacterial count at the end of the incubation period. While 0.8 mg/ml and 1.2 mg/ml 2-HCA concentrations did not result in log decrease in the bacterial count, 1.6 mg/ml 2-HCA resulted in 3 log reduction in the bacterial growth for both MRSA and MSSA. Viable count results from the cultures treated with 2 mg/ml 2-HCA showed difference: While it caused 4 log reductions in the MRSA growth, it caused 3 log reductions in the growth of MSSA.

Percent inhibitions of the tested vanillic acid concentrations and viable cell count results of MRSA and MSSA in the presence of vanillic acid were given in Table 2.4. While the MRSA and MSSA controls reached to 10^8 cfu/ml bacterial counts at the end of the incubation period, 1.7 mg/ml and 2.5 mg/ml VA concentrations resulted in only some log decreases in the bacterial counts.

Table 2.3. Percent inhibition values of 2-hydroxycinnamic acid on bacteria and viable cell count results. The bacteria indicated as 0 corresponds to the control.

	2-Hydroxycinnamic Acid Concentration (mg/ml)	**Viable Count After 24 h of incubation (cfu/ml)**	**Percent Inhibition (%) at 24th h of Incubation**
MRSA	0	4.3×10^8	0
	0.8	4.1×10^8	8
	1.2	2.2×10^8	40
	1.6	2.5×10^5	99
	2.0	4.2×10^4	100
MSSA	0	6.3×10^8	0
	0.8	7.3×10^8	8
	1.2	2.4×10^8	45
	1.6	4.9×10^5	97
	2.0	1.9×10^5	98

Initial MRSA load: 2.0×10^6

Initial MSSA load: 1.5×10^7

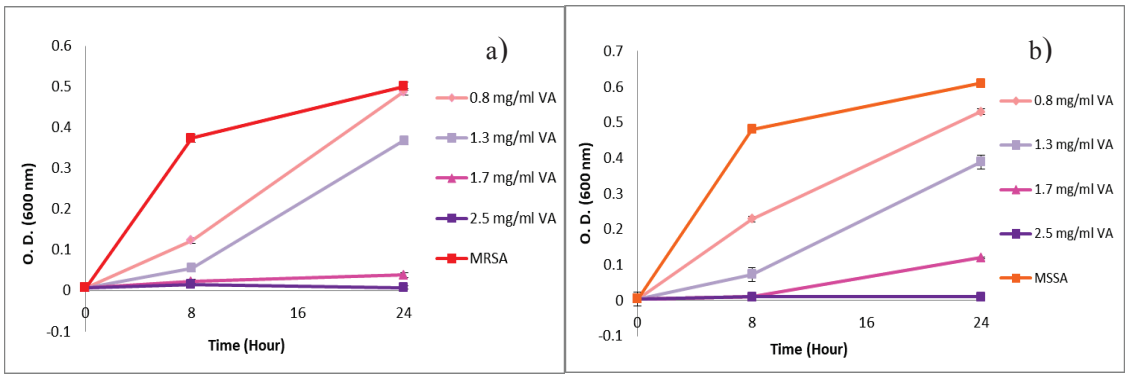

Figure 2.4. Antibacterial effects of vanillic acid (VA) on MRSA (a) and MSSA (b). ODs were measured at 0^{th}, 8^{th} and 24^{th} hours of incubation at 600 nm. Standard deviations for VA concentrations were lower than 0.010 and 0.019 for MRSA and MSSA, respectively.

Table 2.4. Percent inhibition values of vanillic acid on bacteria and viable cell count results. The bacteria indicated as 0 corresponds to the control.

	Vanillic Acid Concentration (mg/ml)	**Viable Count After 24 h of incubation (cfu/ml)**	**Percent Inhibition (%) at 24^{th} h of Incubation**
MRSA	0	2.0×10^8	0
	0.8	2.0×10^8	3
	1.2	8.0×10^8	27
	1.7	4.4×10^6	92
	2.5	5.9×10^4	99
MSSA	0	5.0×10^8	0
	0.8	3.4×10^8	13
	1.2	8.0×10^7	36
	1.7	8.0×10^6	80
	2.5	6.3×10^5	98

Initial MRSA load: 2.4×10^6

Initial MSSA load: 1.2×10^7

It can be concluded that, MRSA was affected more than that of MSSA when treated with the same concentrations of VA and 2-HCA. In a study carried out by Alves and his colleagues (2013), phenolic acids including vanillic acid and *p*-coumaric acid was tested on MRSA and MSSA. Similarly, they determined the MICs of these phenolic acids as 0.5 mg/ml and 1 mg/ml for VA and *p*-coumaric acid on MRSA. On the other hand, MICs of both phenolic acids were higher than 1 mg/ml for MSSA (Alves et al., 2013). Obtaining different MIC for the same phenolic acid (in case of vanillic acid) might be due to (i) the use of different bacterial load for initial inoculum, (ii) using a different approach to determine MIC or (iii) using different strains of MRSA and MSSA. Nevertheless, these findings are promising for the usage selected phenolic acids as alternative antimicrobials to overcome the problems caused by MRSA and MSSA.

2.3.3. Morphological Investigation of MRSA and MSSA in the Presence of Phenolic Acids

The possible changes in the cell surface and morphology of MRSA and MSSA upon treatment with VA and 2-HCA were examined by scanning electron microscopy (SEM). Both bacteria were grown in the presence of the subinhibitory concentrations and in the MIC levels of the phenolic acids. The micrographs of MRSA grown in the presence of 1.3 mg/ml VA and 1.2 mg/ml 2-HCA were shown in Figure 2.5. According to the images, MRSA protected the intactness of the cells and displayed regular cocci shape after treatment with subinhibitory concentrations of both phenolic acids. The presence of the impurities in the image of MRSA treated with 2-HCA in Figure 2.5 (E) was due to peptone water in which the bacteria was resuspended. However, the shape of the bacteria could be seen in the micrographs. The staphylococcal shape of *S. aureus* was evidently observed in Figure 2.6 (A) which MSSA was seen as grape-like clusters. Figure 2.6 (C-D) showed the morphologies of MSSA treated with VA. As in VA treated MRSA, VA treated MSSA cells remained intact and displayed regular cocci shape. Similarly, treatment of bacteria with subinhibitory concentration of 2-HCA did not affect the shape of the cell as presented in Figure 2.6 (E-F). For determination of whether the concentration of phenolic acids had different effect on cell morphology, bacteria treated MIC values of phenolic acids. However, since these concentrations inhibited the growth of bacteria, no bacterial cell could be observed under SEM.

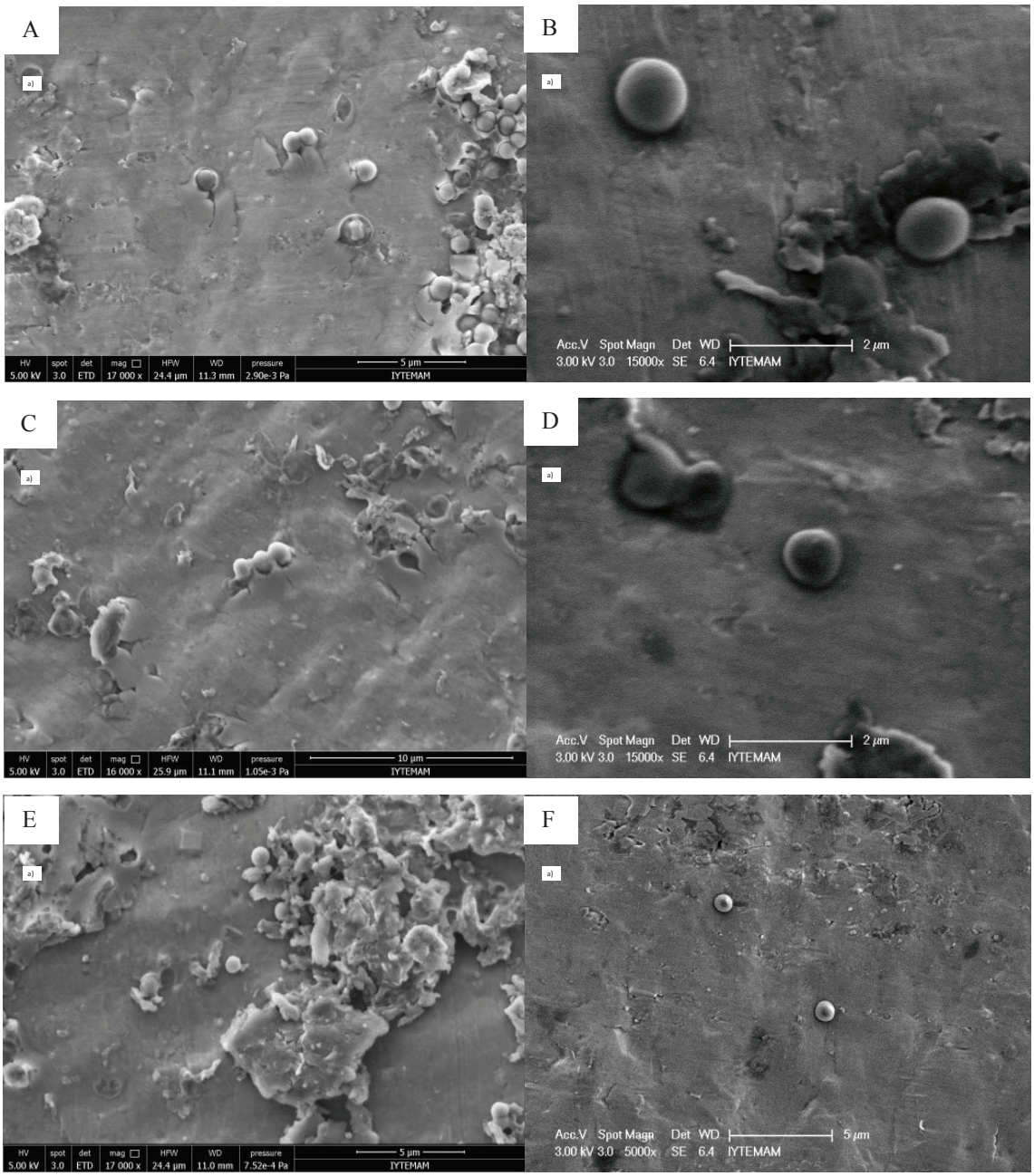

Figure 2.5. SEM micrographs of MRSA. Staphylococci shape (A) and cocci shape (B) of MRSA control. Staphylococci shape (C) and cocci shape (D) of MRSA treated with 1.3 mg/ml VA. Staphylococci shape (E) and cocci shape (F) of MRSA treated with 1.2 mg/ml 2-HCA. On the left part staphylococci shape and on the right part cocci shape of *S. aureus* could be seen. The images shown on the left and on the right parts were taken on different days by using different microscopes. The magnifications were indicated at the bottom of each micrograph.

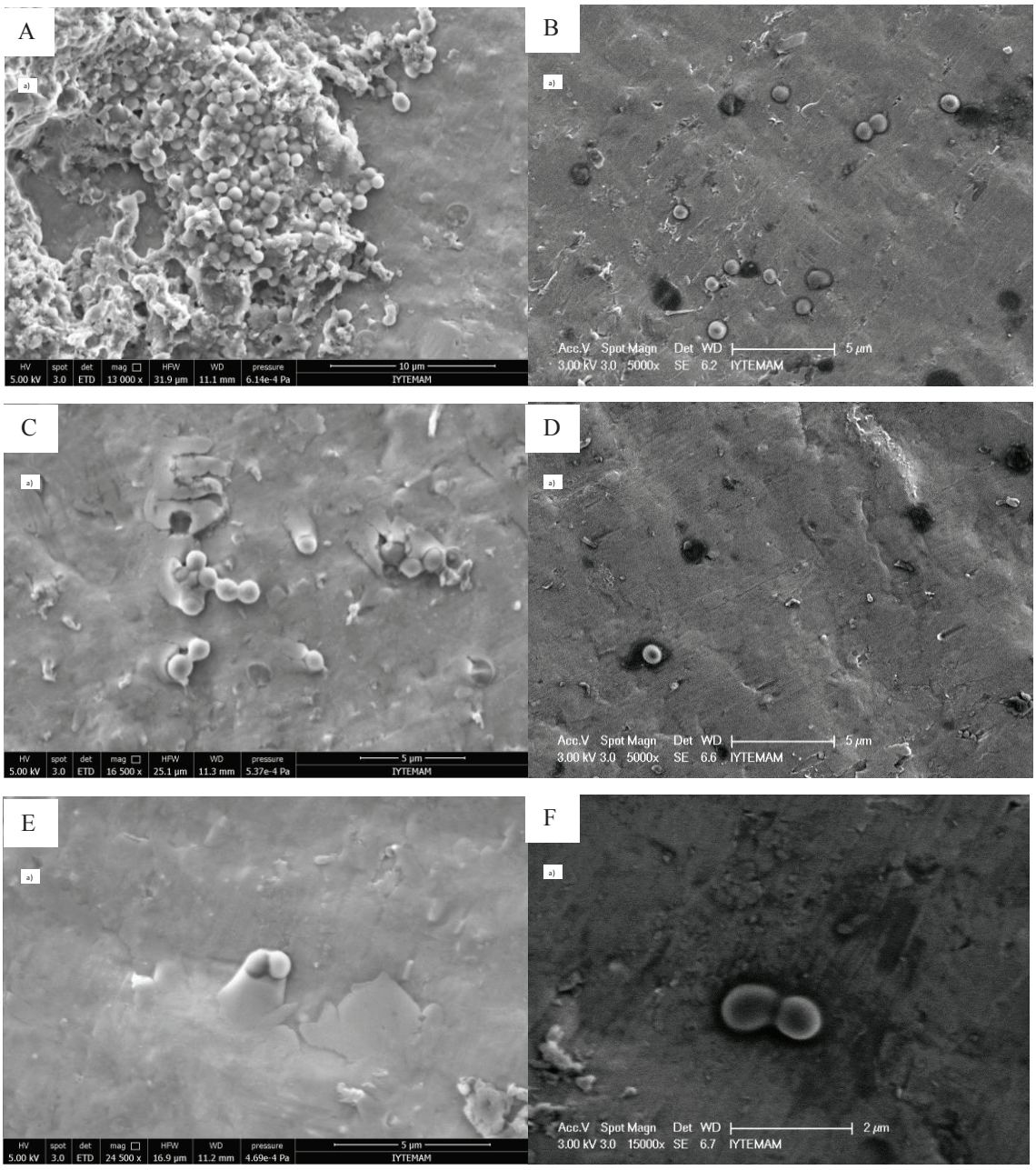

Figure 2.6. SEM micrographs of MSSA. Staphylococci shape (A) and cocci shape (B) of MSSA control. Staphylococci shape (C) and cocci shape (D) of MSSA treated with 1.3 mg/ml VA. Staphylococci shape (E) and cocci shape (F) of MSSA treated with 1.2 mg/ml 2-HCA. On the left part staphylococci shape and on the right part cocci shape of *S. aureus* could be seen. The images shown on the left and on the right parts were taken on different days by using different microscopes. The magnifications were indicated at the bottom of each micrograph.

When the images taken from control groups and phenolic acid treated bacteria were compared, it could be seen that the surface characteristics and the morphology of the bacteria were not changed upon treatment. By taking this into consideration, it could be concluded that these phenolic acids affected the bacteria through other inhibitory ways than interruption of intactness of the cells.

2.3.4. Effects of Antibiotics on Bacteria

Three antibiotics, namely ampicillin, kanamycin and vancomycin were tested against MRSA and MSSA. The reason for screening antibiotics was to use one of them as a control in the experiments aimed to investigate the ability of bacteria to acquire resistance against VA and 2-HCA. The antibiotic concentrations that showed inhibitory effect on MRSA and MSSA were different for same antibiotics (μg/ml values were indicated as ug/ml in the graphics). For the selection of one antibiotic as a control, firstly the antibacterial effect of ampicillin was tested. The highest concentration of the ampicillin used against bacteria was 200 μg/ml. Even at the highest concentration, MICs of ampicillin for MRSA and MSSA could not be determined (Figure 2.7). Although ampicillin showed inhibitory effect on MSSA when compared with MRSA, still 200 μg/ml was not enough to inhibit the total growth of bacteria.

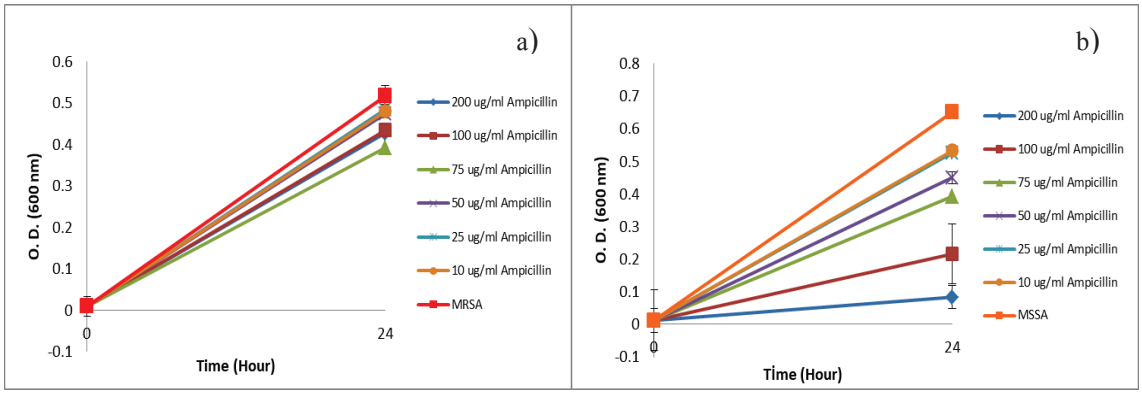

Figure 2.7. Antibacterial effect of ampicillin on (a) MRSA and (b) MSSA. ODs were taken at 0^{th} and 24^{th} hour of incubation. Standard deviations for ampicillin concentrations were lower than 0.016 and 0.092 for MRSA and MSSA, respectively.

Table 2.5. Percent inhibitions of ampicillin concentrations on MRSA and MSSA.

Ampicillin Concentration (µg/ml)	MRSA Percent Inhibition (%)	MSSA Percent Inhibition (%)
200	17	87
100	16	67
50	8	31
25	8	19
10	7	18

While 200 µg/ml ampicillin inhibited the 87% of the MSSA growth (Figure 2.7), its presence caused 17% inhibition in the growth of MRSA. Percent inhibition values for each tested concentration of ampicillin were given in Table 2.5. MRSA and MSSA had resistance against even at the high concentrations of ampicillin.

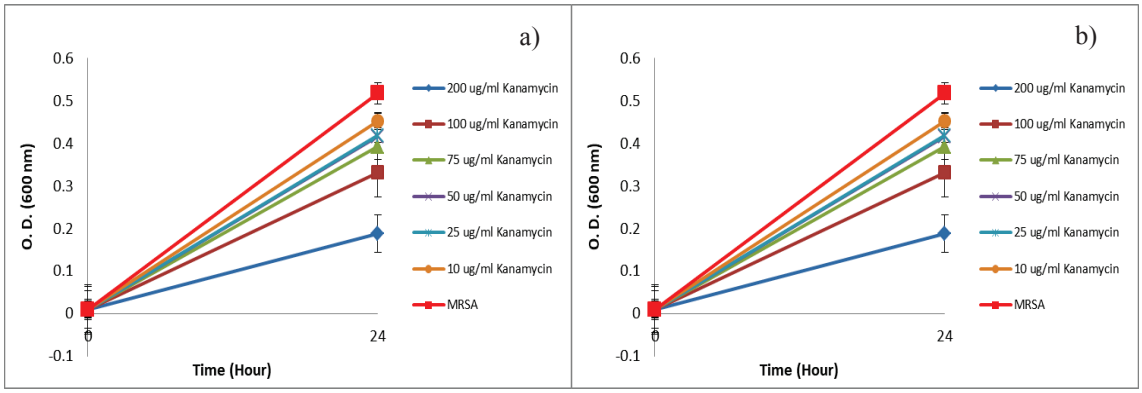

Figure 2.8. Antibacterial effects of kanamycin on (a) MRSA and (b) MSSA. ODs were taken at 0^{th} and 24^{th} hour of incubation. Standard deviations for kanamycin concentrations were lower than 0.057 and 0.081 for MRSA and MSSA, respectively.

Kanamycin at the concentration of 200 μg/ml had more inhibitory effect on MRSA growth when compared with ampicillin as shown in Figure 2.8. However, it did not display total growth inhibition. When the antibacterial effect of kanamycin on MSSA was studied, it was observed that same tested concentrations were more effective on inhibition of MSSA than of MRSA as the results obtained for ampicillin.

As seen in Table 2.6, from the concentration of 25 μg/ml, kanamycin displayed more than MIC_{90} (The concentration inhibits the 90% of total bacteria) values for MSSA. However, the inhibition of MRSA was much lower when compared with MSSA. While 10 μg/ml kanamycin inhibited the 65% of MSSA growth, 200 μg/ml kanamycin inhibited the 64% of MRSA growth barely.

Table 2.6. Percent inhibitions of kanamycin concentrations on MRSA and MSSA.

Kanamycin Concentration (μg/ml)	**MRSA Percent Inhibition (%)**	**MSSA Percent Inhibition (%)**
200	64	95
100	36	95
50	20	94
25	19	92
10	12	65

The total inhibition of MRSA could not be obtained with the highest tested concentration of kanamycin; therefore, the antibacterial effect of vancomycin was tested on MRSA and MSSA to find the antibiotic showed inhibitory effect on both bacteria. Since the comparison of MRSA and MSSA in terms of their resistance profiles was one of the aims of this work, it was important to use same antibiotic for MRSA and MSSA.

The inhibitory concentrations of vancomycin on MRSA and MSSA were shown in Figure 2.9. When compared with ampicillin and kanamycin, lower concentrations of vancomycin had more inhibitory effect on both bacteria. Concentrations higher than 5 μg/ml vancomycin resulted in more than MIC_{90} values for MRSA (Table 2.7).

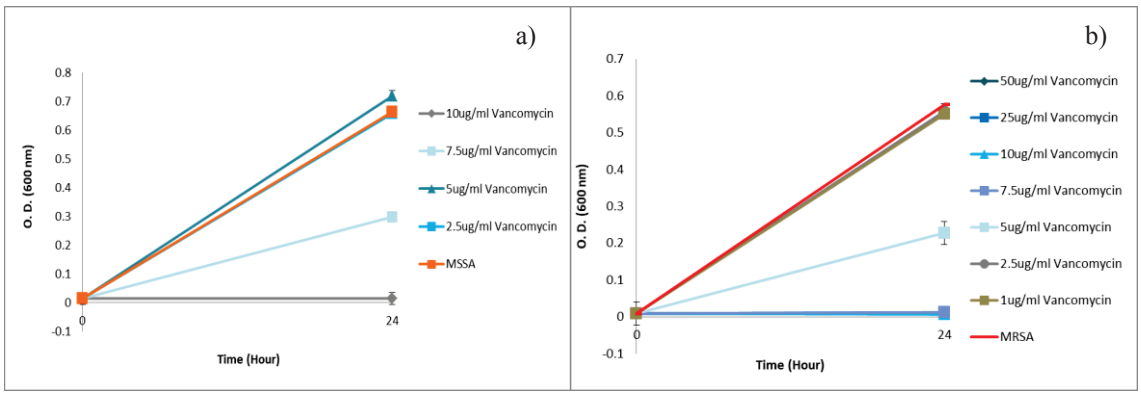

Figure 2.9. Antibacterial effect of vancomycin on (a) MRSA and (b) MSSA. ODs were taken at 0^{th} and 24^{th} hour of incubation. Standard deviations for vancomycin concentrations were lower than 0.030 and 0.020 for MRSA and MSSA, respectively.

Different from the resistance profiles against ampicillin and kanamycin, MSSA displayed more resistance against vancomycin than MRSA did. As indicated in the Table 2.7, vancomycin at the concentration of 5 μg/ml had inhibition values near MIC_{50} (The concentration inhibited the growth of 50% of the bacteria) for MRSA; however, the similar inhibition level for MSSA was observed with 7.5 μg/ml vancomycin. Vancomycin at the concentration of 5 μg/ml did not result in growth inhibition in MSSA. MIC was determined as 10 μg/ml vancomycin for both MRSA and MSSA. Concentrations higher than 10 μg/ml and lower than 2.5 μg/ml vancomycin were only tested for MRSA and were not tested for MSSA.

Therefore, to be used as controls in the experiments trying to promote phenolic acid resistance, subinhibitory concentrations of vancomycin were chosen as 5 μg/ml and 7.5 μg/ml for MRSA and MSSA, respectively.

Table 2.7. Percent inhibitions of vancomycin concentrations on MRSA and MSSA.

Vancomycin Concentration (μg/ml)	MRSA Percent Inhibition (%)	MSSA Percent Inhibition (%)
50	98	Not determined
25	98	Not determined
10	99	99
7.5	97	55
5	60	-8
2.5	3	1
1	4	Not determined

2.3.5. Determination of Acquisition of Resistance against Phenolic Acids and Antibiotic

Bacteria are known for their ability to gain resistance against antibiotics. Overuse and misuse of the antibiotics result in drug-resistant bacteria. Moreover, exposure of bacteria to sublethal concentrations of antibiotics induces resistance and results in formation of drug-resistant bacteria. Thus, in this part of the study, it was aimed to test the ability of MRSA and MSSA to gain resistance against vanillic and *2*-hydroxycinnamic acids following treatment with increasing subinhibitory concentrations. For this purpose, the MIC levels of phenolic acids and vancomycin (as control drug) were determined at the beginning of exposure and after each transfer of the sample to an increasing concentration. The obtained MIC values following exposure to phenolic acids and vancomycin were tested at the end of the experiment to demonstrate if the increased or unchanged MICs were stable.

In the previous section, subinhibitory and minimum inhibitory concentrations of VA and 2-HCA against bacteria were given. Therefore, in the experiments to test ability of cells to gain resistance against phenolic acids, 1.3 mg/ml VA and 1.2 mg/ml 2-HCA were chosen. Vanillic acid at the concentration of 2.5 mg/ml, and 2-HCA at the concentrations of 1.6 mg/ml and 2 mg/ml were used to test predetermined MIC levels

for MRSA and MSSA. For confirmation of the effect of subinhibitory concentrations, the antibacterial effects of VA, 2-HCA and vancomycin were assessed against MRSA and MSSA before continuous exposure to these compounds (Figure 2.10).

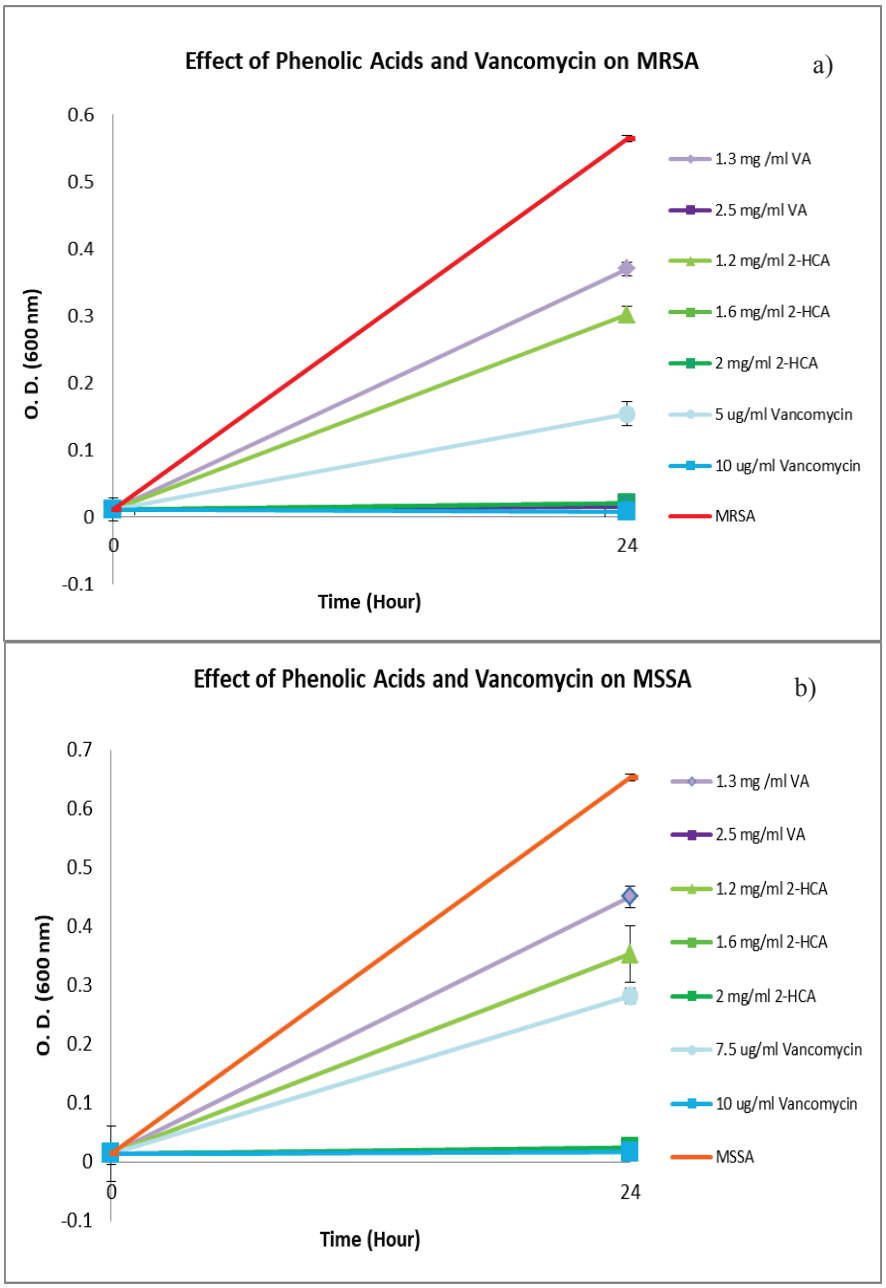

Figure 2.10. Growth of (a) MRSA and (b) MSSA in the presence of different concentrations of vanillic acid (VA), 2-hydroxycinnamic acid (2-HCA) and vancomycin. Standard deviations were lower than 0.017 and 0.047 for MRSA and MSSA, respectively.

As expected, 1.3 mg/ml VA and 1.2 mg/ml 2-HCA displayed subinhibitory levels around MIC_{50}, and higher tested concentrations inhibited the growth of both bacteria after 24 hour incubation. Moreover, 5 μg/ml and 7.5 μg/ml vancomycin concentrations showed subinhibitory effects on MRSA and MSSA, respectively. As before, vancomycin at the concentration of 10 μg/ml inhibited the growth of both bacteria similar to the results obtained in the screening of antibiotics experiments (See section 2.3.5). As shown in Table 2.8, the percent inhibitions obtained from subinhibitory concentrations were similar to those obtained in the previous sections. While 1.3 mg/ml VA inhibited the bacterial growth about 30%, 1.2 mg/ml 2-HCA inhibited the growth 46% in both MRSA and MSSA. Percent inhibitions of vancomycin concentrations were also similar with the previously obtained values.

Table 2.8. Percent inhibition values and viable cell count results of normally grown MRSA and MSSA.

Sample	Percent Inhibition of MRSA (%)	Viable Count of MRSA after 24 h (cfu/ml)	Percent Inhibition of MSSA (%)	Viable Count of MSSA after 24 h (cfu/ml)
1.3 mg/ml VA	34	1.5×10^8	31	1.6×10^8
2.5 mg/ml VA	98	1.7×10^5	97	5.7×10^5
1.2 mg/ml 2-HCA	46	1.6×10^8	46	1.7×10^8
1.6 mg/ml 2-HCA	97	1.2×10^5	97	6.7×10^5
2.0 mg/ml 2-HCA	97	9.7×10^4	96	2.0×10^5
5 μg/ml vancomycin	72	1.1×10^8	Not determined	Not determined
7.5 μg/ml vancomycin	Not determined	Not determined	57	2.8×10^8
10 μg/ml vancomycin	99	7.0×10^4	98	4.0×10^6
Initial MRSA 0 hour: 4.3×10^6 cfu/ml; MSSA 0 hour: 1.4×10^7 cfu/ml				
Control MRSA 24 hour: 2.9×10^8 cfu/ml; MSSA 24 hour: 7.4×10^8 cfu/ml				

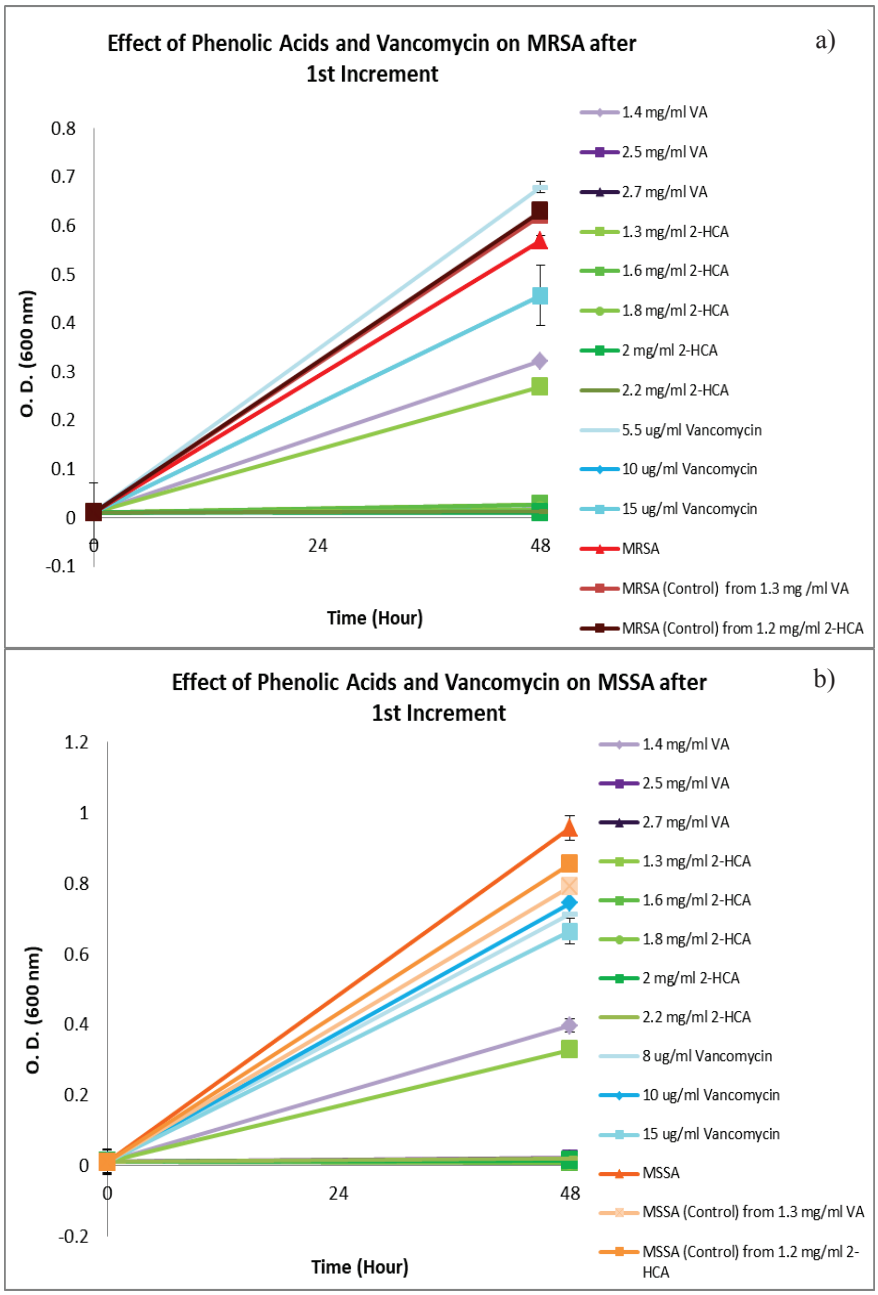

Figure 2.11. Growth of (a) MRSA and (b) MSSA after the first increment in the concentrations of VA, 2-HCA and vancomycin. Standard deviations were lower than 0.061 and 0.035 for MRSA and MSSA, respectively.

According to Figure 2.11, increasing subinhibitory concentrations of vanillic acid and 2-hydroxycinnamic acid did not affect the growth of MRSA and MSSA during 48-hour incubation. Although inhibitory effects of 1.4 mg/ml VA and 1.3 mg/ml 2-HCA were observed to be increased slightly, their subinhibitory effects remained and

previously determined MIC values unchanged for both phenolic acids and for both bacteria. However, 10 μg/ml vancomycin which was assessed as MIC before the exposure to subinhibitory vancomycin concentration lost its MIC degree. Even 15 μg/ml vancomycin was not enough to be MIC neither for MRSA nor for MSSA (Table 2.9).

Table 2.9. Percent inhibition of MRSA and MSSA treated with phenolic acids and vancomycin for 48 h. The percent inhibitions indicated the inhibition values after the first increment in the subinhibitory concentrations of phenolic acids and vancomycin.

Sample	**Percent Inhibition of MRSA after 48 hour (%)**	**Percent Inhibition of MRSA after 48 hour (%)**
1.4 mg/ml VA	43	59
2.5 mg/ml VA	97	98
2.7 mg/ml VA	98	98
1.3 mg/ml 2-HCA	53	66
1.6 mg/ml 2-HCA	95	99
1.8 mg/ml 2-HCA	97	99
2 mg/ml 2-HCA	98	99
2.2 mg/ml2-HCA	97	98
5.5 μg/ml vancomycin	-19	Not determined
6 μg/ml vancomycin	Not determined	25
10 μg/ml vancomycin	-9	22
15 μg/ml vancomycin	46	31
Control from 1.3 mg/ml VA	-5	11
Control from 1.2 mg/ml 2-HCA	-7	11

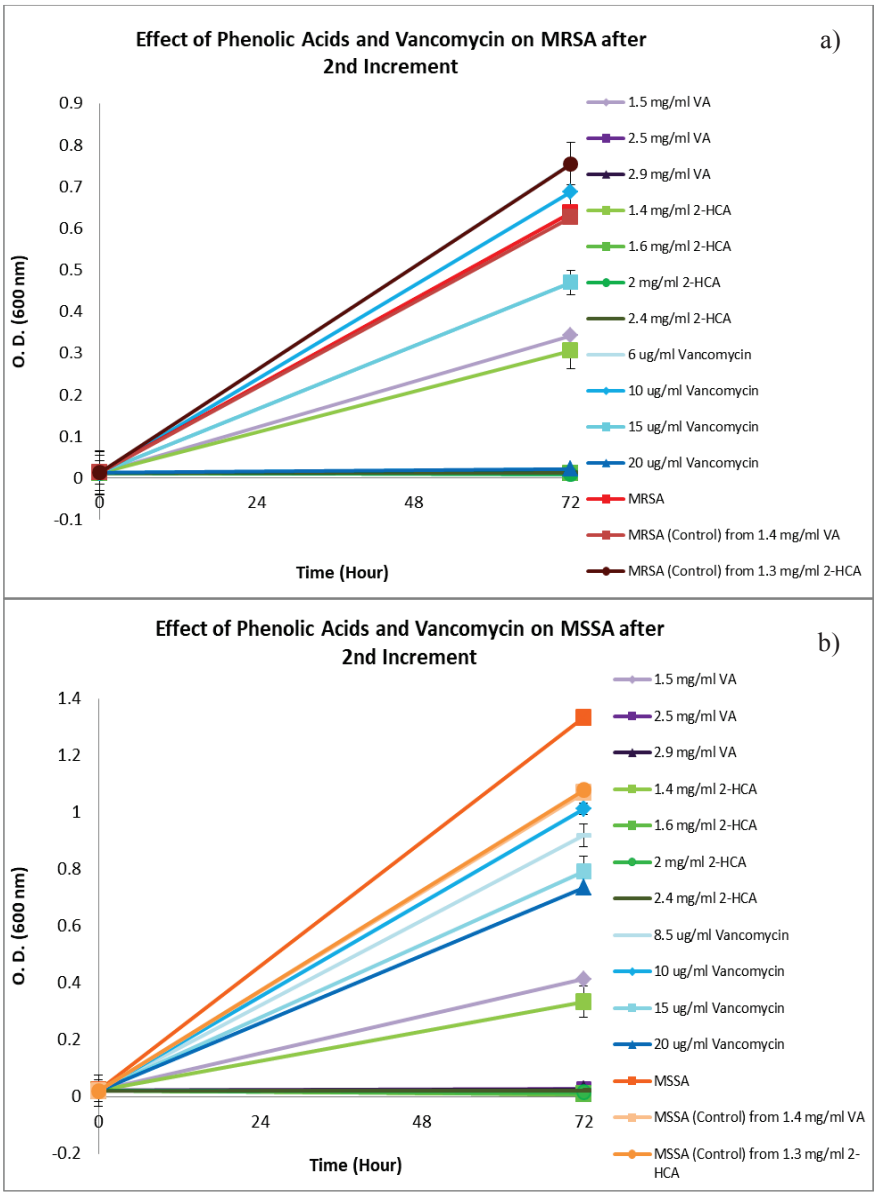

Figure 2.12. Growth of (a) MRSA and (b) MSSA after second increment in the concentrations of VA, 2-HCA and vancomycin. Standard deviations were lower than 0.053 and 0.054 for MRSA and MSSA, respectively.

The highest percent inhibition obtained from vancomycin treatment was 46% for MRSA and 31% for MSSA. Control from 1.3 mg/ml VA and control from 1.2 mg/ml 2-HCA showed that the transfer of bacteria treated with subinhibitory phenolic acid concentrations to TSB showed similar OD values to control groups which were always subcultured in TSB. After the first increment in the subinhibitory concentrations of antimicrobial agents, treatment of bacteria with subinhibitory vancomycin

concentrations immediately increased the MIC levels and induced resistance. On the other hand, MRSA and MSSA did not show resistance against increased subinhibitory phenolic acids concentrations. Therefore, another transfer was performed to incubate bacteria for a longer period within the presence of increased concentrations of antimicrobial agents. Following the 72-hour incubation after the second increment in the subinhibitory concentrations, MIC levels of phenolic acids against MRSA and MSSA remained unchanged (Figure 2.12). However, increase in the MIC value for vancomycin was observed (Table 2.10).

Table 2.10. Percent inhibition values and viable cell count results of MRSA and MSSA treated with phenolic acids and vancomycin for 72 h.

Sample	**Percent Inhibition of MRSA after 72 hour (%)**	**Viable Count of MRSA after 72 hour (cfu/ml)**	**Percent Inhibition of MSSA after 72 hour (%)**	**Viable Count of MSSA after 72 hour (cfu/ml)**
1.5 mg/ml VA	46	5.5×10^6	69	6.2×10^6
2.5 mg/ml VA	98	7.0×10^2	98	4.2×10^2
2.9 mg/ml VA	98	6.0×10^2	98	5.0×10^2
1.4 mg/ml 2-HCA	52	1.7×10^7	75	2.0×10^7
1.6 mg/ml 2-HCA	98	Not determined	99	1.7×10^2
2 mg/ml 2-HCA	99	4.0×10^3	99	4.0×10^3
2.4 mg/ml 2-HCA	98	Not determined	98	3.1×10^3
6 μg/ml vancomycin	-18	3.3×10^8	Not determined	Not determined
8.5 μg/ml vancomycin	Not determined	Not determined	31	5.5×10^8
10 μg/ml vancomycin	-8	3.8×10^8	24	3.0×10^8
15 μg/ml vancomycin	26	1.2×10^8	40	1.0×10^8
20 μg/ml vancomycin	96	3.8×10^6	45	$\sim 10^8$
Control from 1.3 mg/ml VA	4	1.2×10^8	19	1.0×10^9
Control from 1.2 mg/ml 2-HCA	-4	1.2×10^8	20	1.1×10^9

Control MRSA 72 hour: 1.3×10^8 cfu/ml. Control MSSA 72 hour: 9.0×10^8 cfu/ml

It could be seen that MRSA developed about 2-fold increase in the MIC for vancomycin. For MSSA, even 2-fold increase in the initial MIC of the vancomycin was not enough for total growth inhibition. A 20 µg/ml vancomycin had 45% inhibition in the MSSA growth following continuous exposure to subinhibitory concentrations of vancomycin. Viable cell count results indicated that increased incubation period within the phenolic acid concentrations resulted in more log reduction in the number of bacteria (Table 2.10) when compared with the untreated 24-hour incubated cultures (Table 2.8). However, the number of the cells incubated in vancomycin increased during longer period of incubation.

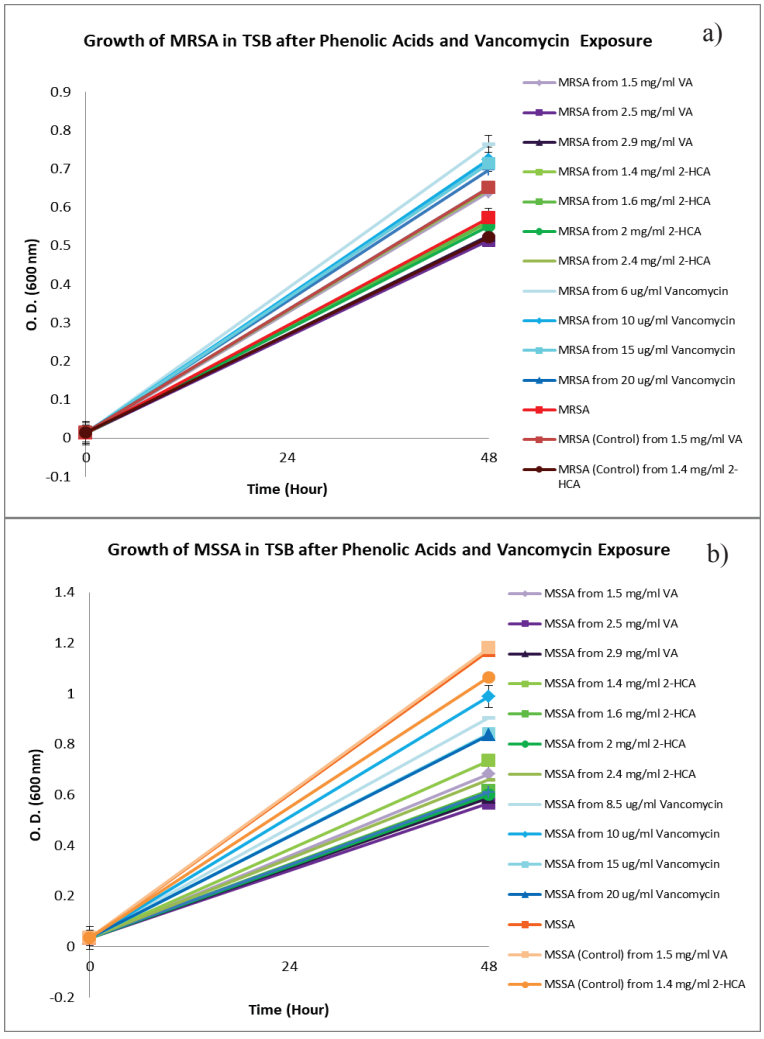

Figure 2.13. Growth of (a) MRSA and (b) MSSA cultures transferred from phenolic acid and vancomycin containing tubes to TSB. Standard deviations were lower than 0.031 and 0.044 for MRSA and MSSA, respectively.

Since the treatment with subinhibitory levels of vancomycin resulted in increased MIC, the cultures that were grown in the presence of phenolic acids and vancomycin were transferred and grown in the phenolic acid/vancomycin-free environment for 48 h (Figure 2.13). The reason of shifting cultures into TSB was to understand whether the increased MICs for vancomycin were transient or stable. Although their growth patterns were the same, quantitatively their growth was unalike due to the difference in the bacterial load in the same amount of inoculum. This was because of the reason that the inoculums were transferred from the tubes containing varying concentrations of the antimicrobial compounds. After the cultures were grown in phenolic acid/vancomycin-free environment for 48 hours, transfer of 2% inoculums into freshly prepared phenolic acid and vancomycin solutions allowed the final MIC test. The effect of phenolic acids and vancomycin on the growth of MRSA and MSSA -treated with subinhibitory concentrations- were shown in Figure 2.14. The exposure to the subinhibitory concentrations of vancomycin induced development of resistance in MRSA and MSSA and resulted in almost 2-fold increase in the MIC (Table 2.11). On the other hand, treatment of MRSA and MSSA with increasing subinhibitory concentrations of VA and 2-HCA could not raise the predetermined MICs. Viable count results obtained from phenolic acid treated MRSA and MSSA were similar to initially obtained results (Table 2.8); while, increased number of bacteria was observed in case of vancomycin treatment.

Considering the information given about the antibiotics in the literature review, development of resistance to vancomycin was not surprising. Although vancomycin is one of the antibiotics used for the treatment of MRSA infections, development of resistance and drug-dependent problems result in unsuccessful treatment processes (DeLeo et al., 2010). When MRSA and MSSA were compared in terms of their resistance profile against vancomycin in this study, it could be concluded that both bacteria developed resistance to vancomycin following exposure of subinhibitory concentrations. However, their resistance degree showed some difference. While treatment of MRSA with 20 μg/ml vancomycin resulted in 96% of growth inhibition, the same concentration inhibited only 45% of MSSA growth after 72-hour incubation period. Final MIC tests for MRSA and MSSA cultures exposed to subinhibitory concentrations (Table 2.11) showed that 15 μg/ml vancomycin inhibited almost the total MRSA growth, while for MSSA 25 μg/ml vancomycin displayed the same effect.

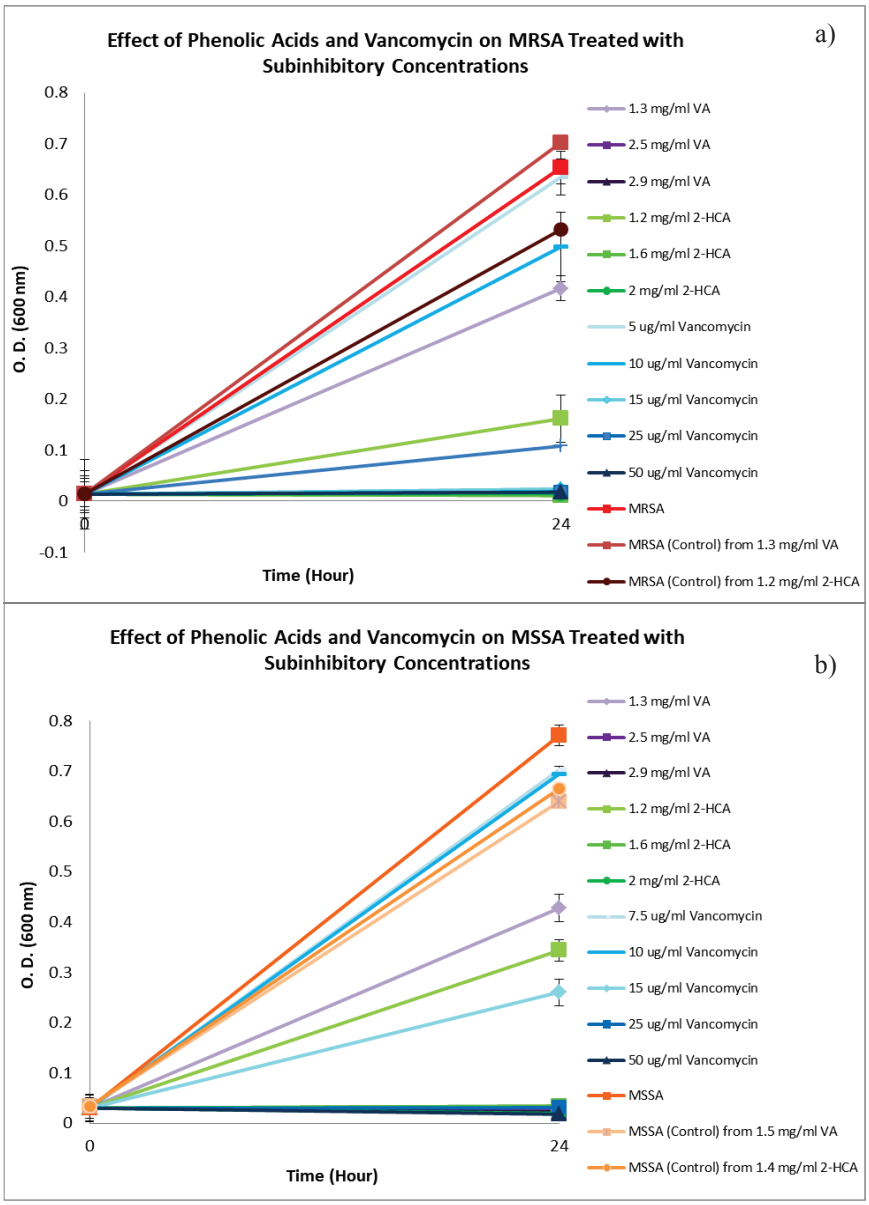

Figure 2.14. Growth of (a) MRSA and (b) MSSA -treated with subinhibitory antimicrobial concentrations- in the presence of VA, 2-HCA and vancomycin. Standard deviations were lower than 0.067 and 0.026 for MRSA and MSSA, respectively.

According to this information, it could be said that the resistance ability against methicillin did not lead to difference for development of vancomycin resistance. On the other hand, shifting bacteria from antimicrobial-containing media in which they were continuously exposure to antimicrobials to antimicrobial-free media resulted in a negligible shift in the final minimum inhibitory concentration for vancomycin.

Therefore, it can be speculated that longer incubation time in the presence of vancomycin resulted in emergence of more resistant strains.

The significance of this study was being one of the first studies showing that bacteria do not gain resistance to the tested antimicrobial agents. The literature mentioning the similar phenomenon is limited to a few microorganisms and antimicrobial agents as mentioned in section 2.1 (Blair et al., 2009; Takahashi et al., 2015). Difference of our study was showing the maintenance of antimicrobial activity during continuous exposure and after shifting the cells to antimicrobial-free environment for the first time for MRSA and MSSA against tested phenolic acids.

Table 2.11. Percent inhibition values and viable cell count results of MRSA and MSSA treated with subinhibitory concentrations of phenolic acids and vancomycin.

Sample	**Percent Inhibition of MRSA**	**Viable Count of MRSA after 24 hour (cfu/ml)**	**Percent Inhibition of MSSA**	**Viable Count of MSSA after 24 hour (cfu/ml)**
1.3 mg/ml VA	36	3.5×10^8	45	1.5×10^8
2.5 mg/ml VA	97	6.0×10^5	97	1.3×10^6
2.9 mg/ml VA	97	1.6×10^5	97	2.1×10^5
1.2 mg/ml 2-HCA	75	2.5×10^8	55	1.5×10^8
1.6 mg/ml 2-HCA	98	3.1×10^5	96	2.5×10^6
2 mg/ml 2-HCA	98	1.1×10^5	98	5.0×10^4
5 μg/ml vancomycin	3	4.5×10^8	Not determined	Not determined
7.5 μg/ml vancomycin	Not determined	Not determined	9	1.3×10^9
10 μg/ml vancomycin	24	3.2×10^8	10	1.1×10^9
15 μg/ml vancomycin	96	Not determined	66	1.1×10^6
25 μg/ml vancomycin	98	1.2×10^5	96	7.8×10^6
50 μg/ml vancomycin	98	1.6×10^4	98	5.1×10^4

Control MRSA 24 hour: 6.6×10^8 cfu/ml. Control MSSA 24 hour: 1.1×10^9 cfu/ml

2.4. Conclusion

This study emphasized the use of VA and 2-HCA as potent antimicrobials against antibiotic resistant bacteria. The first outcome of this study was consistent with the general knowledge about the antibiotic resistance ability of bacteria. The second and the exciting outcome of this study was the maintenance of susceptibility of bacteria to phenolic acids. The difference between resistance ability for antibiotics and phenolic acids might be due to distinct origins of these compounds or due to different inhibition mechanisms provided by phenolic acids. Differently from the antibiotics which commonly targets for one specific cellular structure within the cell, phenolic acids display their inhibitory effects via several mechanisms. Among these, disruption of the permeability of the cell wall and membrane, change in the levels of potassium efflux, leakage of nucleotides from the cell (Lou et al., 2011), altering the surface charge of the cell membrane (Borges et al., 2013), interaction with DNA (Lou et al., 2012), and interaction with the proteins (Alves et al., 2013), can be considered. Therefore, it seems unexpected for MRSA and MSSA to develop resistance against phenolic acids in the near future. Nevertheless, investigation of molecular action mechanisms of phenolic acids is required to provide information about the potential specific targets in bacteria.

CHAPTER 3

DETERMINATION OF PROTEOMIC RESPONSES OF *Staphylococcus aureus* TO THE PRESENCE OF PHENOLIC ACIDS VIA GEL-BASED AND GEL-FREE APPROACHES

3.1. Introduction

Clinical importance of *S. aureus* infections and the increased resistance to almost all commercial antibiotics require the search for alternative antimicrobial compounds. Besides their antimicrobial activities, antioxidant, anticarcinogen, antiinflamatuar, antimutagenic and cardioprotective properties (Andjelkovic et al., 2006) make phenolic acids promising candidates for combatting with antibiotic-resistant bacteria. Since phenolic acids are found in vegetables and the fruits in high amounts, they are important for human diet and their dietary consumption provides plenty of health beneficial properties (Heleno et al., 2015).

Proteomic approach is commonly used in the studies aiming to understand the action mechanisms of antibiotics or several antimicrobial compounds on bacteria (Bandow et al., 2003; Fernandez-Reyes et al., 2009; Visutthi et al., 2011; Hesketh et al., 2015). These kinds of studies indicate the importance of the proteomic methods in terms of providing novel insights to the responses of the bacteria against antimicrobial compounds. Exploration of new antimicrobials involves two steps: evaluation of the action mechanism of the potential drug and its origin point to understand whether it has intrinsic antibacterial activity or it is structurally modified compound (Bandow et al., 2003). When the process of discovery of novel antimicrobial agents is considered, proteomic approaches can be useful to enlighten the modes of action of the agents and to remark the potential targets in antibiotic-resistant bacteria.

In proteomic approaches use of two different protein separation techniques and two different ionization techniques provide comparative information due to the differences between the techniques.

The differences between MALDI and ESI can be listed as: (i) The introduction of the samples into the ion source is different in MALDI and ESI: While ESI uses samples in solution; MALDI uses solid samples co-crystalized with a matrix. (ii) The position of the laser beam and the experience of the operator affect the quality of the spectra obtained from MALDI. (iii) Presence of salt and detergent in small quantities does not influence the results of MALDI; while in ESI presence of such compounds may compute with the analytes and may affect the results. (iv) Finally, MALDI produces singly charged ions in contrast to ESI produces multiply charged ions help to broaden the mass range (El-Aneed et al., 2009).

The success of bacteria to develop resistance against antibiotics that commonly target for a single specific structure or mechanism within the cell is defined clearly so far. However, elucidation of action mechanisms of alternative antimicrobial compounds such as phenolic acids is not fully defined. Since they display more complicated actions on the microbes with multiple targets they have great importance. Power of proteomic approach can be used for investigation of biological differences in bacteria treated with phenolic acids to understand the action mechanism on the bacteria at a molecular level.

The aim of this study was to investigate the differences in proteomes of MRSA and MSSA upon treatment with effective phenolic acid concentrations. To achieve the aim of the study, gel-based (2D-PAGE & MALDI-TOF-MS/MS) proteomics was used for identification of protein profiles of both MRSA and MSSA in the presence of each phenolic acid in comparison with their control groups. Moreover, gel-free (LC-ESI-MS/MS) shotgun proteomics was used to identify the protein profile of MRSA in the presence of each phenolic acid.

In this part of the study, three separate hypotheses were tested:

I. If the treatment of bacteria with each phenolic acid affect the growth as observed in the antimicrobial studies, then the growth of bacteria in the presence of subinhibitory phenolic acid concentrations would generate an observable response in the protein profiles.

II. If different subclasses of phenolic acids have different effects on protein expression of bacteria, then protein profiles of the same bacteria treated with vanillic acid and 2-hydroxycinnamic acid would be dissimilar.

III. If shotgun proteomics allow more comprehensive data, then the results of shotgun proteomic analyses would be more informative to reveal the action mechanisms of phenolic acids and to provide candidate proteins on MRSA.

3.2. Materials and Methods

For gel-based and gel-free proteomic approaches, the same protein isolation protocol was carried out. SDS-PAGE and 2D-PAGE techniques in gel based proteomic approach; LC-ESI-MS/MS technique in gel-free approach were used.

3.2.1. Total Protein Isolation from Bacteria

For total protein isolation from MRSA and MSSA cells, firstly single colonies of these bacteria were inoculated into 4 ml TSB media and incubated at 37^{o}C. Then, bacteria were inoculated into flasks containing either TSB (control group) or subinhibitory concentrations of phenolic acids: 1.3 mg/ml for VA; 1.2 mg/ml for 2-HCA. MRSA and MSSA cultures were inoculated into media and phenolic acids as 2% inoculums and 0th hour OD_{600} measurement was taken. Control of initial number of bacteria was provided with spread plating from the 2% inoculated control groups of MRSA and MSSA. Flasks containing control and test groups were incubated at 37^{o}C for 18 hours. Cells were harvested by centrifugation at 10,000xg at 4^{o}C for 20 minutes.

The protein isolation was done according to the steps in the study of Sianglum and his colleagues (Sianglum et al., 2011) with minor modifications. After removal of supernatant, pellets were dissolved in 20 ml of 0.85% (w/v) sodium chloride (NaCl) solution. Resuspended cells were washed twice by centrifuging at 20,000x*g* at 4^{o}C for 20 minutes and kept at -80^{o}C until resuspension. After resuspension of cells in 2 ml of phosphate buffered saline (PBS) solution (pH 7.4); cells were subjected to sonication process on ice for 15 minutes with 9 seconds on and off intervals. To remove the cell debris, the lysed cells were centrifuged at 20,000x*g* at 4^{o}C for 20 minutes. The supernatant containing proteins were stored at -80^{o}C until usage. Quantification of protein concentrations was done by Bradford protein assay (See Appendix B).

3.2.2. SDS-PAGE Analyses

SDS-PAGE analysis was carried out in Mini-PROTEAN Tetra Cell (Bio-Rad). Amounts of proteins were adjusted to desired concentration by diluting proteins with phosphate buffered saline (pH 7.4), or concentrating them by evaporating via SpeedVac

concentrator. Before loading samples into the wells, protein samples were mixed with sample buffer (1.4 ml of 0.5 M Tris-HCl pH 6.8, 1.6 ml glycerol, 1.6 ml of 10% SDS, 3 ml ddH_2O, 0.4 ml β-mercaptoethanol and 0.5% w/v bromophenol blue) and heated at 95^oC for five minutes. Pre-stained protein marker (Applichem Protein Marker VI) was used for determination of the molecular weights of the proteins.

- Resolving gel (12%) (5 ml): Two ml of 30% acrylamide-bisacrylamide solution (19T:1) was mixed with 1.675 ml of ddH_2O. Following addition of 1.25 ml of 1.5 M tris (pH 8.8) and 50 µl of 10% SDS into the acrylamide solution, 25 µl of 10% freshly prepared ammonium per sulfate (APS) and 2.5 µl tetramethylethylenediamine (TEMED) were added. Immediately after adding TEMED, the gel was poured in the cast and layered with 200 µl ethanol for faster polymerization. When the gel was completely solidified, the alcohol was discarded and the top of gel was washed with distilled water. Stacking gel was prepared and added onto the separating gel.
- Stacking gel (4%) (2.5 ml): 0.325 ml of acrylamide-bisacrylamide solution was mixed with 1.525 ml of ddH_2O. Following addition of 0.625 ml of 0.5 M tris (pH 6.8) and 25 µl of 10% SDS into solution, 12.5 µl of 10% APS and 2.5 µl of TEMED were added. The prepared gel was poured onto the resolving gel and the comb was placed.

When the polymerization was completed, the comb was removed and 1X run buffer was added. The prepared protein samples were loaded into the wells as 40 µg protein per well. Three microliter of protein marker was also loaded into a well.

- 5X Run Buffer (1X: 25 mM Tris, 192 mM glycine, 0.1% SDS): 15 g of tris and 72 g of glycine were dissolved in 600 ml of ddH_2O. Then, 5 g of SDS was added and the volume was completed to 1 liter. Run buffer was kept at +4^oC and warmed at room temperature prior to use.

Electrophoresis was carried out at 100 V for 120 minutes, after the addition of 1X run buffer. For visualization of protein bands, gel was placed into staining solution.

- Staining Solution 'Coomassie Blue G-250' (500 ml): 40 g of ammonium sulfate was mixed with 50 ml of water. Also, 0.5 g of Coomassie Blue G-250 was dissolved in 50 ml of distilled water. When ammonium sulfate had dissolved, the dye solution was added. Following addition of 100 ml of methanol and 8 ml of *ortho*-phosphoric acid, the solution was completed to 500 ml with distilled water.

The images of the stained gels were taken with VersaDoc Imaging System at IYTE-BIOMER. SDS-PAGE analyses were performed twice for confirmation of obtained differences.

3.2.3. 2D-PAGE Analyses

Before analysis of proteins with 2D-PAGE system, the impurities in isolated proteins were clarified using a 2D-clean up kit (GE Health Care) by following the steps provided by the manufacturer.

For concentrating the proteins prior to rehydration, the excessive of PBS buffer in protein solution was evaporated by centrifuging in SpeedVac (Thermo Electron Corporation). Protein concentration was adjusted to 1 μg/μl (350 μg protein in 350 μl rehydration buffer) within a rehydration buffer that contains urea (Sigma), CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (Sigma), dithiothreitol (DTT) (Sigma) and ampholytes (pH 3-10, Bio-Rad).

- Rehydration Buffer: 4.8 g urea and 0.2 g CHAPS were dissolved in double distilled water (ddH_2O) and then the volume was adjusted to 10 ml. This solution was kept at +4^oC. Prior to experiment, 0.0035 g DTT and 8.75 μl of pH 3-10 ampholytes were added into 350 μl sample.

Protein samples were dispensed into the wells of isoelectric focusing (IEF) tray and immunomobilized pH gradient (IPG) strips (pH 3-10, 17 cm, Bio-Rad) were placed onto samples as their gel side down. The tray was incubated at room temperature for 2 hours for passive rehydration. Then, 2 ml of mineral oil (Bio-Rad) was added on the IPG strips to reduce the evaporation and to prevent the precipitation of urea. The tray was covered and active rehydration was started at 20^oC for 16 hours.

The first separation of proteins by 2D-PAGE technique was carried out by using a Protean IEF Cell (Bio-Rad). After the rehydration had completed, paper wicks were wetted with ultrapure water and placed onto the electrodes under the IPG strips.

Isoelectric focusing of proteins was carried out at 20^oC by applying the six different parameters given below:

- Linear gradient, 200 V, 300 Vhours
- Linear gradient, 500 V, 500 Vhours
- Linear gradient, 1000 V, 1000 Vhours

- Linear gradient, 4000 V, 4000 Vhours
- Rapid gradient, 8000 V, 24000 Vhours
- Rapid gradient, 8000 V, 30000 Vhours

After the isoelectric focusing of proteins, the strips were taken from the focusing tray carefully, covered with stretch wrap and stored at -80^{o}C for further steps.

Prior to second separation, focused proteins were equilibrated with two equilibration buffers. Equilibration step was required to solubilize the proteins and to provide binding of SDS to the proteins.

- Equilibration Buffer I: 7.2 g urea was dissolved in 5 ml of ultrapure water. Subsequently, 5 ml of 1.5 M tris-HCl (pH 8.8), 4 ml of glycerol and 0.4 g of SDS were added. After completion of dissolving process, the volume was adjusted to 20 ml by ultrapure water. The solution was stored at +4^{o}C. 400 mg/20 ml DTT was added into equilibration buffer just before the experiment to reduce sulfhydryl groups.
- Equilibration Buffer II: Contains the same amounts of urea, 1.5 M tris-HCl (pH 8.8), glycerol and SDS. However, instead of DTT, 500 mg/20 ml iodoacetamide was added into buffer prior to usage to alkylate sulfhydryl groups.

For equilibration process, strips were placed into disposable tray as their gel side up. The strips were treated with 6 ml of equilibration buffer I for 10 minutes with gentle shaking. Then, first buffer was discarded and 6 ml of equilibration buffer II was added. Strips were incubated in this buffer for 10 minutes with gentle shaking.

Second separation of proteins by SDS-PAGE was performed in PROTEAN II Cell (Bio-Rad). 12% polyacrylamide gel containing 1.5 M tris-HCl (pH 8.8), 10% SDS, 10% APS and TEMED was prepared for separation of proteins according to their molecular weight.

- 12% Polyacrylamide Gel (for 2 gels): 32 ml of 30% acrylamide-bisacrylamide solution (19T:1) was mixed with 26.8 ml of ultrapure water. Following addition of 20 ml of 1.5 M tris (pH 8.8) and 0.8 ml of 10% SDS, 400 µl of 10% APS and 40 µl of TEMED were added and the gel was immediately poured. Faster polymerization was provided with addition of 2 ml ethanol on the top of the gels.

After the equilibration, the IPG strips were rinsed by dipping into 1X run buffer and were placed onto the polyacrylamide gels. By addition of about 2 ml of melted overlay agarose (0.5% agarose) the strips were sealed to the gels.

- Overlay agarose (25 mM tris, 192 mM glycine, 0.1% SDS and Bromophenol Blue): For 50 ml total volume; 0.25 g agarose, 0.15 g of tris, 0.72 g glycine, 0.05 g SDS, and 8 ml bromophenol blue was mixed in 50 ml ddH_2O. The solution was homogenized by boiling at microwave oven.

After the overlay agarose had solidified, the gel stands were placed into the electrophoresis cell. Following addition of the required amount of 1X run buffer, electrophoresis was started with the current at 16 mA/gel for the first 30 minutes, and then adjusted to 24 mA/gel for about 5 hours.

After the electrophoresis had completed, the gels were rinsed into distilled water for 5 minutes. The gels were stained with colloidal coomassie blue G-250 for about 18 hours at room temperature with gentle shaking and they were washed with deionized water. For distinct spot visualization, the gels were subjected to destaining solution (25% methanol, 5% Acetic Acid) for about an hour.

The images of the gels were taken with VersaDoc imaging system. The gels were kept in 5% acetic acid solution at +4^{o}C for further analyses.

3.2.4. Protein Identification

For the identification of proteins, in-gel digestion of separated proteins was carried out with trypsin enzyme. This was followed by Zip-Tip assay and MALDI-TOF-mass spectrometry.

3.2.4.1. In-Gel Digestion

For the cleavage of proteins within the excised protein spots, tryptic in-gel digestion process was performed (Kinter and Sherman, 2000). Excision of spots from the gel, and all chemical applications were performed in BSL2 cabinet to prevent any contamination.

Preparations of required chemicals were done as follows:

- Wash solution [50% (v/v) methanol and 5% (v/v) acetic acid]: 25 ml of methanol was added into 12.5 ml of ddH_2O. Following addition of 2.5 ml of acetic acid, the volume was adjusted to 50 ml with ddH_2O.

- 100 mM Ammonium Bicarbonate (ABC): 0.2 g ammonium bicarbonate (Applichem) was dissolved in 20 ml of ddH_2O.
- 50 mM Ammonium Bicarbonate: 2 ml of 100 mM ABC was mixed with 2 ml of ddH_2O.
- 10 mM DTT: 1.5 mg of dithiothreitol was added into 1 ml of 100 mM ABC.
- 100 mM Iodoacetamide: 18 mg of iodoacetamide was weighed and dissolved by addition of 1 ml of ABC.
- Trypsin solution (20 μg/ml) (Sigma): 1 ml of ice-cold 50 mM ABC was added into 20 μg of trypsin and dissolved by pipetting.
- Extraction Buffer (50% acetonitrile and 5% formic acid): 10 ml of acetonitrile was added into 5 ml of ddH_2O. After addition of 1 ml of formic acid, the solution was adjusted to 20 ml with ddH_2O.

In the first day of this protocol, 5% acetic acid solution was removed and the spots were cut into small pieces with a micro pipette tip in microcentrifuge tubes. Then, they were treated with 200 μl of wash solution that was changed three times during treatment at room temperature with gentle shaking.

The second day protocol of in-gel digestion procedure was carried out in room temperature. The wash solution was removed and 200 μl of acetonitrile was added for 5-minute dehydration. Following removal of acetonitrile, the gels in microcentrifuge tubes were completely dried in a vacuum centrifuge for 5 minutes. With addition of 30 μl of 10 mM DTT solution proteins were reduced during 30-minute incubation. Then, DTT solution was removed and 30 μl of 100 mM iodoacetamide was added into tubes which were kept in dark for 30-minute incubation for alkylation. After removal of iodoacetamide solution, 200 μl of acetonitrile was added and incubated for 5 minutes to provide dehydration. Then, acetonitrile was pipetted out and rehydration of gel pieces was provided by addition of 200 μl of 100 mM ammonium bicarbonate during 10-minute incubation. The ammonium bicarbonate was removed and 200 μl of acetonitrile was added onto the gel pieces for another round of dehydration for 5 minutes. And, acetonitrile was removed and the gel pieces were completely dried in a vacuum centrifuge for 5 minutes. As a final step, 30 μl of trypsin solution with the concentration of 20 ng/μl was added and gels were kept on ice for 10 minutes for rehydration. Then, the microcentrifuge tubes were placed in 37^{o}C heater block and gel pieces were covered with 50 mM ammonium bicarbonate (ABC) solution to prevent the gels to dry.

In the third day procedure, firstly, 30 µl of 50 mM ABC solution was added and samples were incubated for 10 minutes at room temperature with occasional vortex. After 30 seconds of centrifugation at maximum speed, the supernatant was transferred into 1.5 ml protein Lo-bind Eppendorf tubes. Then 30 µl of extraction buffer was added and incubated for 10 minutes with occasional vortex. Following centrifugation of samples at maximum speed for 30 seconds, the supernatant was collected and combined with previous supernatant. Extraction step was repeated one more time and obtained supernatant was mixed with previously obtained supernatant.

After collection of all supernatant in the same tube, the tubes were placed into -20^{o}C until usage.

3.2.4.2. Zip-Tip Assay

Prior to Zip-Tip assay, the excess of supernatant was evaporated under vacuum centrifuge to concentrate peptides until the total volume of the samples were 20 µl or less. Millipore Zip-Tip C18 tips were used for purification, desalting and concentration of the samples.

The procedure contains wetting, equilibration, washing and elution steps. All solutions were prepared prior to usage with fresh ddH_2O.

- Wetting solution: 100% acetonitrile (ACN) (Sigma-Aldrich).
- Equilibration solution: 0.1% trifluoroacetic acid (TFA) in ddH_2O was prepared by mixing 10 µl of TFA with 9.990 ml of ddH_2O.
- Wash solution: 0.1% TFA in ddH_2O.
- Elution solution: 0.1% TFA/50% ACN was prepared by mixing prepared TFA solution with ACN in equal amounts.

Firstly, 10 µl of wetting solution was aspirated into Zip-Tip pipette tip and was dispensed slowly for three times. For equilibration of tips, 10 µl of equilibration solution was aspirated and dispensed for three times. To bind the samples to the resin in the tip, 10 µl of peptide sample was pipetted up and down at least 10 times without dispensing the solution. After dispensing the wash solution, about 2-3 µl of elution solution was used to elute peptides and the sample was collected in 0.1 ml microcentrifuge tubes.

3.2.4.3. MALDI-TOF Mass Spectrometry

For preparation of two-layer matrix system for MALDI-TOF mass spectrometry, alpha-cyano-4-hydroxycinnamic acid (CHCA) (Sigma) matrix was used.

- First layer of matrix: 5 mg of CHCA was added into 100 µl of methanol. Then, 400 µl of acetone was added and the mixture was dissolved by vortex.
- Second layer of matrix: 5 mg of CHCA was added into 200 µl of methanol. After dissolving all matrices in methanol, 300 µl of 0.1% TFA in ddH_2O was added. The mixture was centrifuged at maximum speed for 8 minutes.

For preparation of samples to MALDI-TOF mass spectrometry, the samples were mixed with second layer of matrix in the ratio of 1:3 within 0.1 ml microcentrifuge tubes prior to spotting process. Apart from the samples, peptide mixture containing standards angiotensin I, angiotensin II, bradykinin and adrenocorticotropic hormone (ACTH) mixture was prepared for MS calibration. Also, ACTH was mixed with second layer of matrix in the ratio of 1:1 and used for MS/MS calibration. Initially, first layer of matrix was spotted as 1 µl on gold coated target plate (MTP384 target plate ground steel: Target plate with transponder technology, Bruker, Daltonics). Then, prepared samples were spotted on target as 1 µl onto the first layer. All samples were spotted on three different areas in case of inadequate first layer or sample. After 15-20 minutes, the spots on target were dried and the target was placed into the MALDI-TOF/MS equipment (Bruker, Autoflex III Smartbeam).

For MALDI-TOF/MS analysis, Bruker autoflex III smartbeam equipment was used with the program flexControl version 3.0. Analyses of peaks were done by using flexAnalysis version 3.0 and connection to databases was provided by Biotools version 3.1. MASCOT search engine was used as ion search tool for obtained mass spectra. For identification of proteins, optimized parameters were used which were as follows: Taxonomy: Bacteria; enzyme: trypsin; modifications: carbamidomethyl (C), oxidation (M); mass values: monoisotopic; peptide mass tolerance +/- 200 ppm; fragment mass tolerance: +/- 0.5 Da (or 1 Da).

Obtained peptide peaks from MS analysis were combined prior to database search in both NCBIprot and SwissProt databases. Molecular weights of proteins, pH values and ion scores were considered for protein identification.

By using the protein accession numbers (Protein IDs), the functions of proteins were determined via searching on UniProt database.

3.2.5. Gel-Free Approach (LC-ESI-MS/MS)

The acetone precipitation was carried out for elimination of the impurities from the protein samples. Four times of the protein sample volume of cold acetone (Cooled at -20^{o}C overnight) was added onto the supernatant containing proteins. Then, the tube was vortexed and incubated overnight at -20^{o}C. Following centrifugation at 15,000xg at +4^{o}C for 20 minutes, the supernatant was removed. After acetone was evaporated, the protein pellet was dissolved in resuspension buffer containing 7 M urea, 2 M thiourea and 0.1 M Tris-HCl (pH: 7.8) for downstream applications. The concentration of protein samples were adjusted to 400 μg. Experiments were performed with two technical runs and two biological repeats.

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3.2.5.1. In-Solution Digestion

For the cleavage of proteins, tryptic in-solution digestion process was performed. All solutions were prepared freshly with fresh ddH_2O and all applications were performed in room temperature in the flow cabinet to prevent any contamination.

- 0.2 M Dithiothreitol (DTT) in 50 mM Tris-HCl (pH: 7.8) was added into the protein sample as final concentration of 10 mM and incubated for 50 minutes.
- 0.2 M Iodoacetamide in 50 mM Tris-HCl (pH: 7.8) was added into the protein sample as final concentration of 20 mM and incubated at dark for 50 minutes.
- 0.2 M DTT in 50 mM Tris-HCl (pH: 7.8) was added into the protein sample as final concentration of 20 mM and incubated for 50 minutes.
- To decrease the concentration of urea, samples were transferred into 10K cut-off filters and centrifuged for 30 minutes with 10 minute-breaks.
- Then, protein samples were washed with 200 μl of 50 mM Tris-HCl (pH: 7.8) and centrifuged for 20 minutes at 14.000 rpm.
- 200 μl of Trypsin solution containing 8 μg trypsin (stock concentration: 1μg/μl) in 50 mM Tris-HCl (pH: 7.8) was added onto the samples as final concentration 0.04 μg/μl and samples were kept at 37^{o}C for overnight incubation.

After overnight incubation, trypsin-digested samples were transferred into lo-bind protein Eppendorf tubes and the tubes were kept in -20^{o}C.

3.2.5.2. Mass Spectrometry Analyses

Mass spectrometry analyses for gel-free proteomic approach included the following steps: fractionation of peptides, Zip-Tip assay, liquid chromatography coupled to mass spectrometry and protein identification via Mascot search engine.

3.2.5.2.1. Fractionation of Peptides

First fractionation of samples was performed with reverse phase liquid chromatography by using HPLC instrument (Shimadzu, LC20AD) that has a column durable to high pH. Prior to first fractionation, samples taken from -20^{o}C were vacuum-concentrated by using SpeedVac.

Since the injection volume of the HPLC column was 100 µl, the volume of the concentrated sample was completed to 100 µl with phase A.

The mobile phases used during fractionation were as follows:

- Phase A: ddH2O pH adjusted to 10 with ammonium hydroxide.
- Phase B: 90 % Acetonitrile prepared with pH 10 adjusted-upH2O.

By performing fractionation of samples (LCsolution program), chromatographic separation of peptides was achieved. The samples eluted from high pH HPLC column were collected in 96-well plate via MALDI-Spotter instrument (SunCollect program) with the flow rate of 5 µl/min. The obtained eluents were concatenated into 12 fractions in 2 ml Eppendorf tubes by combining samples from random wells of the 96-well plate.

After 12 fractions for each sample (Control groups of MRSA and MSSA; 1.3 mg/ml VA treated MRSA and MSSA; 1.2 mg/ml 2-HCA treated MRSA and MSSA) were collected, the volume of the samples within the centrifuge tubes was concentrated with SpeedVac until all liquid was evaporated from the tubes. The tubes were stored at -20^{o}C until ESI-MS/MS analysis.

3.2.5.2.2. Zip-Tip Assay

For purification, desalting and concentration of samples prior to ESI-MS/MS analysis, Zip-Tip assay was performed by using Millipore Zip-Tip C18. All solutions were prepared freshly prior to usage with fresh ddH_2O.

- Wetting solution: 100% acetonitrile (ACN) (Sigma-Aldrich).
- Conditioning solution: 1% formic acid (FA) in ddH_2O was prepared by mixing 10 µl 990 µl of ddH_2O.
- Wash solution: 1% FA in ddH_2O.
- Elution solution: 50% ACN and 75% ACN were prepared with ddH_2O.

Prior to Zip-Tip process, firstly sample preparation was done by adding 20 µl of 2% ACN-0.1% FA in ddH_2O onto samples. Three cycles of 15 second-sonic bath and 5 second-vortex processes were performed, and then the samples were centrifuged at 14.000 rpm for 10 minutes.

For wetting the Zip-Tip, 10 µl of wetting solution was aspirated into Zip-Tip pipette tip and dispensed slowly for five times. For conditioning of tips, 10 µl of equilibration solution was aspirated and dispensed for five times. Then, 10 µl of peptide sample was aspirated and dispensed by preventing drop of the sample from the tip about 10-15 times. The tip was washed by aspirating and dispensing 10 µl of 1% FA. After dispensing the wash solution, about 2 µl of each elution solution (50% ACN and 75% ACN, respectively) was used to elute peptides and the obtained sample was collected in 0.5 ml microcentrifuge tubes. Prior to addition of the sample into glass vial inserts for mass analyses, the samples were mixed with 25 µl of 0.1 %FA and transferred into glass vials.

3.2.5.2.3. Liquid Chromatography Coupled to Mass Spectrometry

Liquid chromatography-Electrospray ionization mass spectrometry (LC-ESI-MS/MS) analyses were performed with Ultimate 3000 (Dionex) HPLC instrument coupled to LTQ XL mass spectrometer (Thermo Scientific) equipped with electrospray ion source.

Chromatographic separation of peptides was performed by using Chromeleon software with 15 cm length HPLC column with the flow rate of 5 µl/min. The mobile phases used for separation of peptides were as follows:

- Phase A: 0.1% formic acid (FA) in ddH_2O.
- Phase B: 0.1% FA in acetonitrile.

Injection volume was adjusted to 10 µl and two technical runs were performed for each fraction. The elution program was as follows: 98% phase A and 2% phase B at the start; the percentage of phase B was increased up to 90%. Total elution time was 46 minutes for each injection of the sample. Helium gas was used as collision gas.

3.2.5.2.4. Identification of Proteins by MASCOT Search Engine

Analysis of the obtained peaks was done with LTQ tune software. MS/MS ion search was achieved by using MASCOT search engine against UniProt database. The obtained ESI-MS/MS spectra were compared with dataset of *Staphylococcus aureus* strain N315 proteome (UniProt proteome ID: UP000000751) for identification of MRSA proteins.

Parameters for protein identification were as follows:

Database: *Staphylococcus aureus* strain N315 proteome, enzyme: Trypsin; fixed modifications: carbamidomethylation (C); variable modifications: oxidation (M); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: +/- 1.2 Da; fragment mass tolerance: +/- 0.6 Da; maximum missed cleavages: 1; instrument type: ESI-trap. False discovery rate (FDR) was kept below 1% for validation of peptide assignments.

Molecular functions and the biological processes of the proteins were found by searching each protein ID against UniProt database.

3.3. Results and Discussion

For determination of proteomic changes of MRSA and MSSA in the presence of phenolic acids, isolated proteins were firstly separated by SDS-PAGE. This method provided the general aspects of the protein bands in the phenolic acid stress.

3.3.1. SDS-PAGE Analyses

Following protein isolation from MRSA and MSSA cells treated with 1.3 mg/ml VA, 1.2 mg/ml 2-HCA and their control groups, the proteins were separated by SDS-PAGE. Protein concentrations were adjusted to 40 μg and separated on a 12% acrylamide gel. Gel images were shown in Figure 3.1 for (A) MRSA and (B) MSSA.

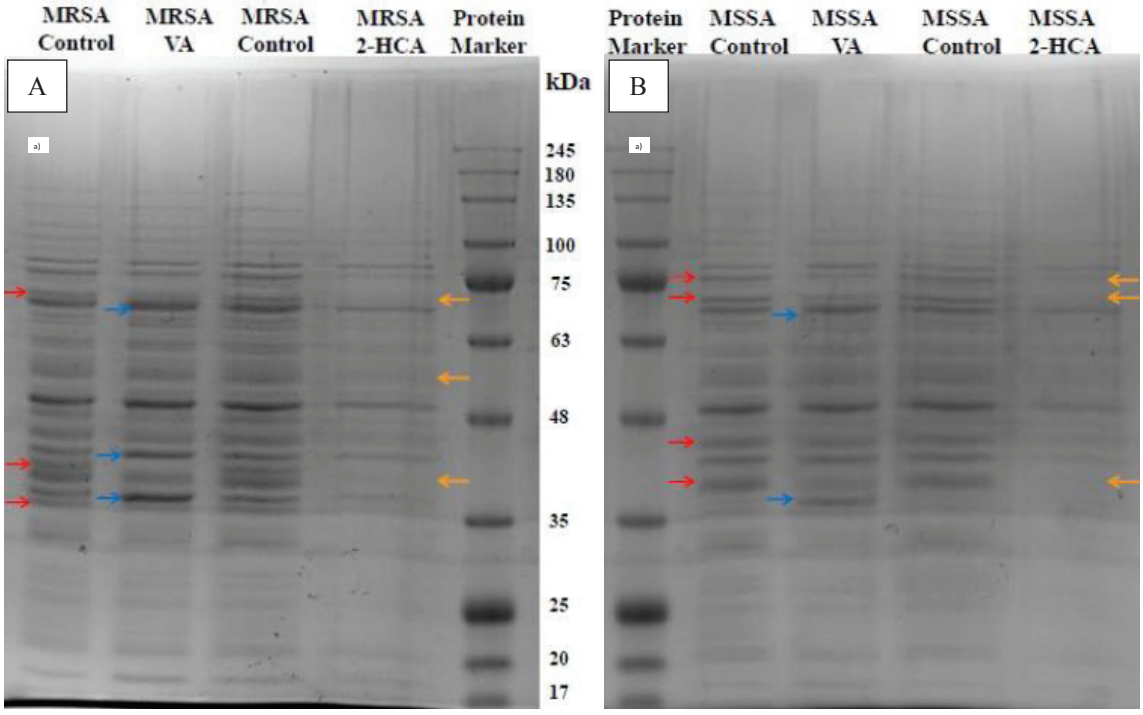

Figure 3.1. SDS-PAGE images of (A) MRSA and (B) MSSA treated with VA and 2-HCA. Red arrows indicated the proteins whose expressions were higher in control. Blue arrows indicated the proteins whose expression had increased following VA treatment. Orange arrows corresponded to 2-HCA treated proteins whose expressions were decreased.

According to the Figure 3.1, expression of the some of the VA treated MRSA proteins having molecular weights of around 75 kDa, 40 kDa and 35 kDa had decreased, whereas; upon treatment expression of proteins having molecular weights of around 70 kDa, 42 kDa and 37 kDa had increased. When the bands that containing 2-

HCA treated proteins were examined, decrease in the density of three bands was seen (Indicated by orange arrows). Differences in the expression of VA treated MSSA proteins having molecular weights around 75 kDa and between 48 and 35 kDa. Decrease of 2-HCA treated MSSA proteins was also seen when the proteins having molecular weights around 75 kDa and 40 kDa were examined.

3.3.2. 2D-PAGE and MALDI-TOF/MS/MS Analyses of Proteins

For determination of the effects of phenolic acid stress on MRSA and MSSA, gel based- proteomic approach was used. The proteins isolated from MRSA and MSSA treated with phenolic acids were separated according to their isoelectric points and then to their molecular weights for more detailed separation. The protein amounts of all samples were adjusted to 350 μg prior to first separation.

Following separation of proteins with 2D-PAGE technique, MALDI-TOF mass spectrometry allowed identification of proteins whose expressions had changed as response to phenolic acid stress. NCBIProt and SwissProt databases were used as protein sequence databases. High quality protein sequence information can be obtained from these databases by means of previously loaded scientific literature and analysis. The functions of proteins were determined based on the information provided on UniProt database. UniProt database provides manually annotated protein sequences of high quality by uniting scientific information obtained from the literature and computationally evaluated analysis results (Dung, et al., 2013).

According to the mass spectrometry results, treatment of MSSA with 1.2 mg/ml 2-HCA resulted in identification of 4 upregulated proteins and 2 downregulated proteins; while treatment with 1.3 mg/ml VA resulted in identification of 8 upregulated proteins. The proteomic analysis of MRSA treated with 2-HCA showed that 4 proteins were upregulated and 6 proteins were downregulated. Moreover, identification of proteins in the presence of vanillic acid showed 11 upregulated proteins and 2 downregulated proteins. As shown in the tables, some of the proteins were mutual for both bacteria and both phenolic acid stresses.

Detailed list of proteins of MRSA and MSSA were given in the tables as upregulated or downregulated proteins and also for the proteins identified with low score number during MASCOT search. While the Table 3.1 the proteins of VA treated

MRSA, Tables 3.2 to 3.4 listed the 2-HCA treated MRSA proteins. MSSA proteins treated with VA were listed in Tables 3.5 and 3.6; and 2-HCA treated MSSA proteins were listed in Tables 3.7 and 3.8. As seen in the Figures 3.2 to 3.5, most of the *S. aureus* proteins were acidic according to their localization on the IPG strip (pH 3-10) and consequently on the polyacrylamide gel.

According to Figure 3.2, MRSA cells treated with subinhibitory concentrations of VA (Figure 3.2B) displayed different protein expression pattern when compared with control and allowed the observation of different sizes and intensities of spots besides (Figure 3.2A).

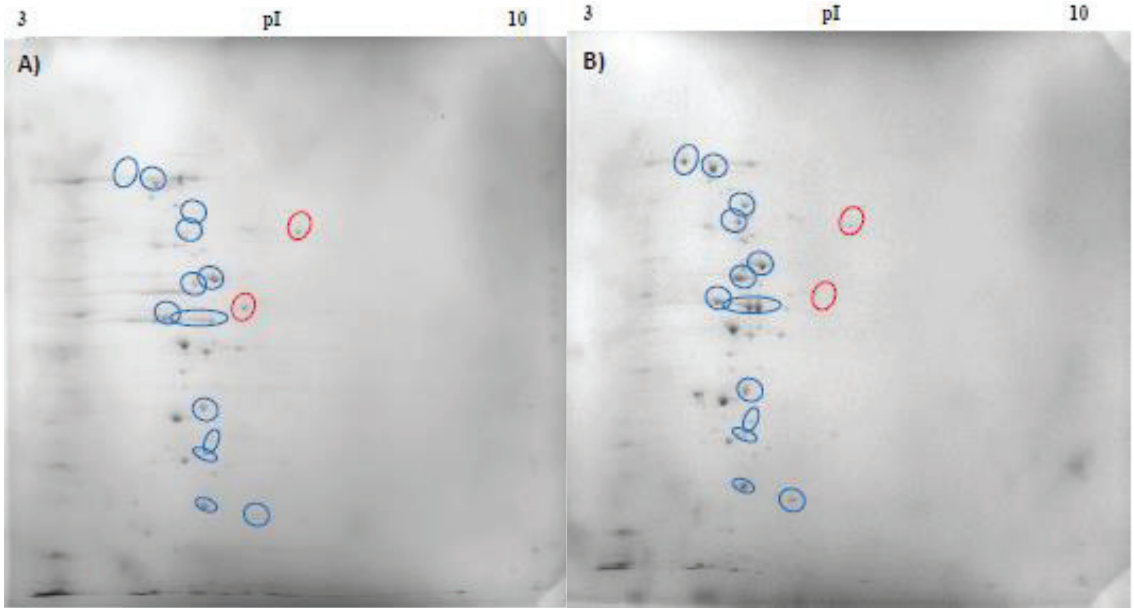

Figure 3.2. 2D-PAGE images of (A) control and (B) VA treated MRSA proteins. 350 µg protein containing gels were stained with Coomassie blue staining. The protein spots of MRSA control marked in red circles indicated the proteins whose expression were decreased or completely disappeared in VA treated proteins. On the other hand, blue circles indicated the proteins spots whose expression had increased as a result of VA treatment.

Identified upregulated proteins of MRSA upon treatment with 1.3 mg/ml vanillic acid were listed in Table 3.1. Upregulation of chaperone protein DnaK might prove the

struggle of cells to prevent newly synthesized polypeptide aggregation and protein misfolding due to phenolic acid stress (Lv et al., 2016). This was consistent with the increase in the expression of putative universal stress protein SA1532. VA treatment increased the expression of proteins which induce the formation of NADH.

The increased production of NADH by dihydrolipoyl dehydrogenase, D-lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase within the cell indicated the need for energy (Reddy et al., 2015). Detection of upregulation in probable transglycosylase IsaA (or called as Immunodominant Staphylococcal Antigen A Precursor) protein indicated possible disruptions on the cell membrane. This was correlated with our findings for MSSA and also with the literature showing the effect of phenolic acids on the cell membrane (Rivas-Sendra et al., 2011; Borges et al., 2013). On the other hand, phosphate acetyltransferase upregulation which was also observed in 2-HCA treated MSSA (Table 3.7) indicated increased acetyl-CoA formation from acetate. Increased acetyl-CoA biosynthesis was related with the production of fatty acids that might be used for membrane modifications as a result of phenolic acid action (Rivas-Sendra et al., 2011) and also for increased production of metabolic intermediates for energy.

Upregulation in the expression of trigger factor was correlated with the observation of increase in the chaperone proteins. By functioning as a chaperone, trigger factor maintains open conformation of proteins, as well as taking roles in protein export and cell division. This upregulation might indicate cells' attempt to block misfolding of newly synthesized proteins and correctly transport them to their locations. The other upregulated protein was cell division protein; FtsZ. FtsZ takes role in cell division by forming contractile ring structure with the help of other recruited proteins. Therefore, increased amount of FtsZ might indicate the requirement of protein production for formation of new cell wall between dividing cells.

When other glycolysis related proteins were considered (Enolase and glyceraldehyde 3-P dehydrogenase), it was reasonable to observe increase in the expression of phosphoglycerate kinase. Since this protein functions in carbohydrate degradation metabolism, energy production via substrate level phosphorylation might have been increased.

Upregulation in diacetyl reductase protein might indicate the increase in the catabolic reactions, as well as increase in the amount of NADH within the cell due to reaction catalyzed by the protein.

Table 3.1. Identified upregulated MRSA proteins upon treatment with VA.

Protein ID	Protein Name	Function	Mass (Da)	Isoelectric Point
DNAK_STAAB	Molecular chaperone DnaK	Stress response	66338	4.66
DLDH_STAAC	Dihydrolipoyl dehydrogenase	Dihydrolipoyl dehydrogenase activity	49592	4.95
TIG_STAAC	Trigger factor	Protein export	48579	4.34
PGK_STAAB	Phosphoglycerate kinase	Phosphoglycerate kinase activity	42632	5.17
FTSZ_STAAC	Cell division protein, FtsZ	Contractile ring structure formation	41012	4.87
LDHD_STAAC	D-lactate dehydrogenase	D-lactate dehydrogenase activity	36716	5.10
G3P1_STAAC	Glyceraldehyde 3-phosphate dehydrogenase	Glyceraldehyde 3-phosphate activity	36372	4.89
PTAS_STAAC	Phosphate acetyltransferase	Phosphate acetyl transferase activity	35044	4.72
ISAA_STAAB	Probable transglycosylase IsaA	Cleavage of peptidoglycan, hydrolase activity	24281	5.91
Y1532_STAAN	Putative universal stress protein 1532	Response to stress	18521	5.60

Besides aforementioned upregulated proteins, there were two downregulated proteins: probable malate: quinone oxidoreductase (Protein ID: MQO2_STAAC) and alcohol dehydrogenase (Protein ID: ADH_STAAB). Decreased expression of probable malate: quinone oxidoreductase results in reduced conversion of malate to oxaloacetate in TCA which might indirectly affect the amino acid metabolism in bacteria.

One last protein for vanillic acid treated MRSA -determined with low score number during MASCOT search- was upregulated superoxide dismutase (Protein ID: SODM1_STAAB). Consistently, the same protein upregulation was observed for 2-HCA treated MSSA (Table 3.7) In line with the literature (Reddy et al., 2015),

inducement of this protein under the stress conditions was required to protect cells from reactive oxygen species.

The separation of the 2-HCA treated MRSA proteins were as seen in Figure 3.3. Protein spots on the 2D-PAGE image indicated the upregulation and downregulation of several proteins as response to 2-HCA.

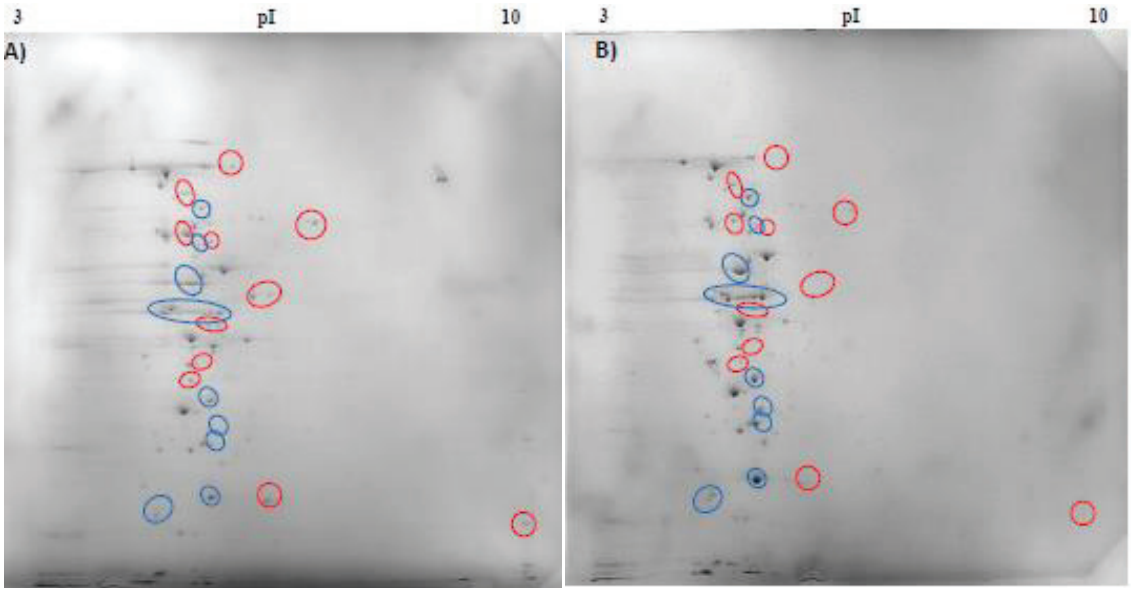

Figure 3.3. 2D-PAGE images of (A) control and (B) 2-HCA treated MRSA proteins. 350 μg protein containing gels were stained with Coomassie blue staining. The protein spots of MRSA control marked in red circles indicated the proteins whose expression were decreased or completely disappeared in 2-HCA treated proteins. On the other hand, blue circles indicated the proteins spots whose expression had increased as a result of treatment with 2-HCA.

The identified upregulated proteins of MRSA treated with 2-HCA were shown in Table 3.2. When MRSA was treated with 2-HCA, the expression of dihydrolipoyl dehydrogenase was upregulated. Bacteria tried to increase the amount of NADH with dihydrolipoyl dehydrogenase to provide energy to the cell through metabolic pathways. Upregulation of D-lactate dehydrogenase in MRSA was mutually observed in phenolic acid treated MSSA. Inducement of this protein might have been required to produce more reducing equivalents to be used in metabolism. The biological processes involving

phosphopentomutase protein are 5-phosphoribose 1-diphosphate biosynthetic pathway, salvage of cellular metabolic compounds and deoxyribonucleotide catabolism. When all these metabolic processes were considered, it could be concluded that the bacteria increased the amount of phosphopentomutase to provide conversion between nucleosides and carbon metabolism. The catabolized nucleotides might be used by other metabolic pathways to provide carbon and energy to the cells. Upregulation of glyceraldehyde 3-phosphate dehydrogenase supported this idea. This enzyme is involved in glycolysis and is responsible of formation of 1,3-bisphosphoglycerate (Condell et al., 2012). The reaction also results in NADH formation that might be used for producing energy.

Table 3.2. Identified upregulated proteins of MRSA upon treatment with 2-HCA.

Protein ID	Protein Name	Function	Mass (Da)	Isoelectric Point
DLDH_STAAC	Dihydrolipoyl dehydrogenase	Dihydrolipoyl dehydrogenase activity	49592	4.95
A0A077ULJ8	Phosphopentomutase	Phosphotransfer between the C1 and C5 carbon atoms of pentose	42824	4.94
LDHD_STAAC	D-lactate dehydrogenase	D-lactate dehydrogenase activity	36746	5.10
G3P1_STAAC	Glyceraldehyde 3-phosphate dehydrogenase	Glyceraldehyde 3-P dehydrogenase activity	36372	4.89

There were two downregulated proteins related with protein synthesis (Table 3.3). Downregulation of translation elongation factor could be seen as a mark of cells to reduce consume of energy for more protein biosynthesis, but instead expending the obtained energy for metabolic activities. Downregulation of threonine t-RNA ligase was consistent with other downregulated protein biosynthesis-related proteins. This protein

is essential for the attachment of threonine to tRNA which is an ATP requiring reaction. The decrease in the expression might have allowed the bacteria to reduce the energy expense. When the metabolism related proteins were considered in Table 3.3, it could be assumed that bacteria reduced the alcohol dehydrogenase levels to decrease metal ions due to its requirement of zinc as a cofactor. Probable malate: quinone oxidoreductase catalyzes the oxidation of malate to oxaloacetate (Condell et al., 2012), while NAD molecule is reduced to NADH. Although the general stress response for bacteria was prone to increase NADH formation, the expression of probable malate: quinone oxidoreductase was decreased upon treatment. This could be due to relation of this enzyme with general amino acid metabolism or direct effect of phenolic acid on the enzyme.

Table 3.3. Identified downregulated proteins of MRSA upon treatment with 2-HCA.

Protein ID	Protein Name	Function	Mass (Da)	Isoelectric Point
SYT_STAAW	Threonine t-RNA ligase	tRNA aminoacylation in protein biosynthesis	74557	4.97
MQO2_STAAC	Probable malate:quinone oxidoreductase	TCA. Malate dehydrogenase activity	56135	6.12
AOH67797.1	Translation elongation Factor, Tu	Translation elongation factor activity in protein biosynthesis	43134	4.74
ADH_STAAB	Alcohol dehydrogenase	Alcohol dehydrogenase activity	36438	5.34
PDXK_STAAC	Putative Pyridoxine Kinase	Phosphomethyl pyrimidine kinase activity	29952	4.85
WP_000634173.1	Universal Stress Protein UspA	Stress response	18494	5.60

Downregulation of the protein putative pyridoxine kinase was consistent with cells' general behavior to reduce the activity to preserve the energy. This protein takes role in the thiamine biosynthetic process with a kinase activity for the formation of vitamin B1. Therefore, it was reasonable for bacteria to suppress the expression of enzymes utilizing ATP. Interestingly, universal stress protein UspA was downregulated following 2-HCA treatment in MRSA.

Other than the proteins mentioned above, there were more determined proteins with low score numbers during MASCOT database search (Table 3.4). However, some of these proteins were detected in MSSA at the same spot localizations. This provided an increased reliability of obtained low score number proteins.

Table 3.4. Identified proteins of 2-HCA treated MRSA with low MASCOT score number.

Protein ID	Protein Name	Function	Mass (Da)	Isoelectric Point	Response to 2-HCA
HCHA_STAAB	Protein/nucleic acid deglycase	Nucleic acid repair	32284	4.97	Upregulation
FABD_STAAC	Malonoyl CoA-acyl carrier transacylase	Fatty acid biosynthesis	33742	4.85	Upregulation
ISAA_STAAB	Probable transglycosylase IsaA	Cleavage of peptidoglycan	24281	5.91	Upregulation
SODM1_STAAB	Superoxide dismutase	Stress response. Destroys superoxide radicals	22697	5.08	Upregulation
ASP23_STAAB	Alkaline shock protein 23	May play a key role in alkaline pH tolerance	19180	5.13	Upregulation
PDXS_STAAC	Pyridoxal 5' biosynthesis synthase subunit PdxS	Glutamine metabolic process	32086	5.10	Downregulation

Pyridoxal 5-phosphate synthase subunit PdxS downregulation was one of the identified proteins with low score number (Table 3.4). The same protein downregulation was observed in 2-HCA treated MSSA. In this case, cells tried to decrease cofactors biosynthesis. Another consistent protein was probable transglycosylase IsaA protein. Similar to VA treated MSSA, treatment of MRSA with 2-HCA caused increased expression of this protein which might result in increased peptidoglycan cleavage. This might indicate that the both phenolic acids disrupt the peptidoglycan structure. The other mutual observation with 2-HCA treated MSSA was superoxide dismutase upregulation that might be required to destroy reactive oxygen species produced in the cell. Upregulation of protein/nucleic acid deglycase HchA might indicate the possible damages on DNA considering its functions in nucleotide repair system.

2D-PAGE image displaying the response of MSSA to VA was shown in Figure 3.4. Treatment of MSSA with VA resulted in upregulation of most proteins.

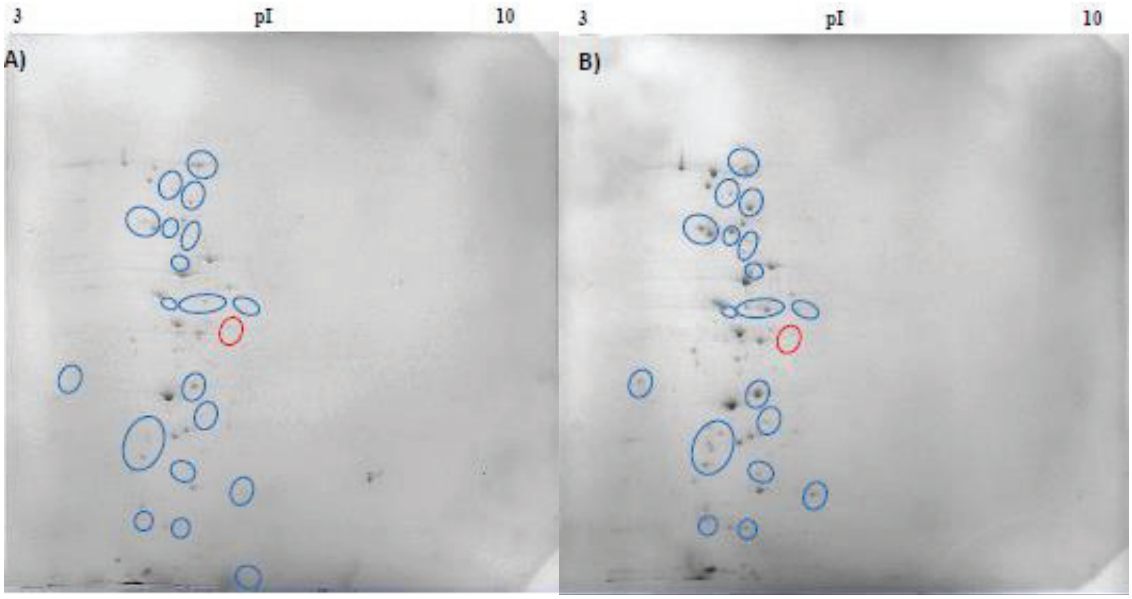

Figure 3.4. 2D-PAGE images of (A) control and (B) VA treated MSSA proteins. 350 μg protein containing gels were stained with Coomassie blue staining. The protein spots of MSSA control marked in red circles indicated the proteins whose expression were decreased or completely disappeared in VA-treated proteins. On the other hand, blue circles indicated the proteins spots whose expression had increased as a result of VA treatment.

When the effect of vanillic acid on MSSA was analyzed (Table 3.5) some mutual proteins that were identified in the presence of 2-HCA stress were observed. These mutual proteins were dihydrolipoyl dehydrogenase and D-lactate dehydrogenase. In that case, it was reasonable to hypothesize that the cells which were subjected to phenolic acid stress increased the expression of proteins to induce production of reducing equivalents for maintenance of cell redox homeostasis.

Transketolase (Table 3.5) was found as an upregulated protein in the presence of VA stress in MSSA. This protein is included in pentose phosphate pathway which is an essential pathway for formation of pentose sugars and reducing equivalents for biosynthesis reactions. Therefore, upregulation of transketolase might indicate the cells' increased demands for sugar phosphates to be used in carbohydrate metabolic pathways.

Another protein with increased expression was enolase OS. This protein is responsible for conversion of 2-phosphoglycerate to phosphoenolpyruvate.

Upregulation of enolase might be seen as another indicator for carbohydrate degradation metabolism whose metabolites probably would be used for providing energy to the cell. Effect of vanillic acid on glycolytic process was also shown with upregulation of pyruvate dehydrogenase E1 component subunit alpha OS. It is responsible for formation of acetyl-CoA and CO_2 from pyruvate and it contains enzymatic components such as dihydrolipoyl dehydrogenase (Condell et al., 2012). Thus, upregulation of this protein was correlated with upregulation of dihydrolipoyl dehydrogenase indicating increased levels of reducing equivalents for metabolic pathways. Moreover, increased acetyl-CoA expression might be related with increased fatty acid synthesis that would be used to alter the lipid composition of the cell membrane due to the effects of phenolic acids (Rivas-Sendra et al., 2011).

Probable transglycosylase IsaA OS was upregulated under vanillic acid stress. Ability of this protein to cleave the peptidoglycan might suggest the presence of some disruptions on the cell membrane.

Another effect of vanillic acid stress on bacteria was related with biological processes as DNA recombination, replication and DNA repair. Upregulation of single-stranded DNA binding protein 1 might indicate that the bacteria increased DNA metabolism to recruit partner proteins for repair of damaged DNA. As expected for stress conditions, a stress protein called putative universal stress protein SA1532 was detected as upregulated protein.

Table 3.5. Identified upregulated proteins of MSSA upon treatment with VA.

Protein ID	Protein Name	Function	Mass (Da)	Isoelectric Point
TKT_STAAC	Transketolase	Transketolase activity	72206	4.97
DLDH_STAAC	Dihydrolipoyl dehydrogenase	Dihydrolipoyl dehydrogenase activity	49592	4.95
ENO_STAAB	Enolase OS	Phosphopyruvate hydratase activity	47145	4.55
ODPA_STAAC	Pyruvate dehydrogenase E1 component subunit alpha	Catalysis of conversion of pyruvate to Acetyl-CoA and CO_2	41357	4.90
LDHD_STAAC	D-lactate dehydrogenase	D-lactate dehydrogenase activity	36716	5.10
ISAA_STAAB	Probable transglycosylase IsaA	Peptidoglycan cleavage	24281	5.91
SSB1_STAAC	Single-stranded DNA binding protein 1	ss-DNA binding in replication, repair and recombination	18642	4.98
Y1532_STAAN	Putative universal stress protein SA1532	Response to stress	18521	5.60

For MSSA under VA stress, there were other detected proteins that were included in Table 3.6 with low score numbers during MASCOT search. 30S ribosomal protein S1 was among them. When considering its localization on the gel and compared with MSSA control group of 2-HCA treated cells, upregulation of 30S ribosomal protein S1 could be justified. This protein was required for providing translational accuracy for the newly synthesized proteins. When requirement of this protein for recognition of translation initiation points were considered, its increase might indicate

the need for proteins to maintain cell stability. Another probable protein with increased expression under vanillic acid stress was pyrimidine-nucleoside phosphorylase OS. This protein is responsible for catalysis of phosphorylation of pyrimidine bases and ribose-1-phosphate. When its roles in pyrimidine nucleoside and nucleobase metabolic processes were considered, upregulation of this protein might support the increase in DNA metabolism. Among probable proteins, an oxidoreductase upregulation was also observed. It was reasonable for cells to upregulate such protein in the presence of phenolic acids with high antioxidant activity due to their ability to scavenge free radicals. This kind of upregulation might be included in oxidative stress response.

Table 3.6. Identified proteins of VA treated MSSA with low MASCOT score number.

Protein ID	Protein Name	Function	Mass (Da)	Isoelectric Point	Response to VA
PDP_STAAB	Pyrimidine nucleoside phosphorylase	Phosphorylate the uridine, thymidine and 2'-deoxyuridine	46338	4.88	Upregulation
RS1_STAAC	30S ribosomal protein S1	RNA binding	43261	4.51	Upregulation
PPI1_STAAB	Putative peptidyl-prolyl cis-trans isomerase	Accelerates the folding of proteins	21605	4.57	Upregulation
TPX_STAAC	Probable thiol peroxidase	Protection against oxidative stress	18005	4.56	Upregulation
WP_050963729.1	Oxidoreductase	Transfer of electrons	31885	4.78	Upregulation
TARI1_STAAB	Ribitol-5-phosphate cytidyltransferase 1	Cell wall biogenesis	26692	5.42	Downregulation
A0A077UMY1	UPF0342 protein ERS140147_02017	Belongs to the UPF0342 family	13348	4.31	Downregulation

Consistent with this information, upregulation in probable thiol peroxidase was observed (Table 3.16). Antioxidant activity of peroxidases might help bacteria to remove H_2O_2 and maintain cell redox homeostasis. Vanillic acid presence might have increased the expression of a putative peptidyl prolyl cis-trans isomerase protein. The function of this protein was to accelerate protein folding. This was in correlation with the increase of chaperone protein expression under stress conditions. Besides these probable proteins with increased expression upon treatment, downregulation in the proteins such as UPF0342 protein SH1117 and ribitol-5-phosphate cytidylyl transferase 1 were determined. The latter protein is involved in the biogenesis of the cell wall and teichoic acid synthesis. This might signal the modifications in the cell wall organization due to inhibition of transfer of functional groups.

Differences in the expression of MSSA proteins treated with 2-hydroxycinnamic acid were seen on Figure 3.5.

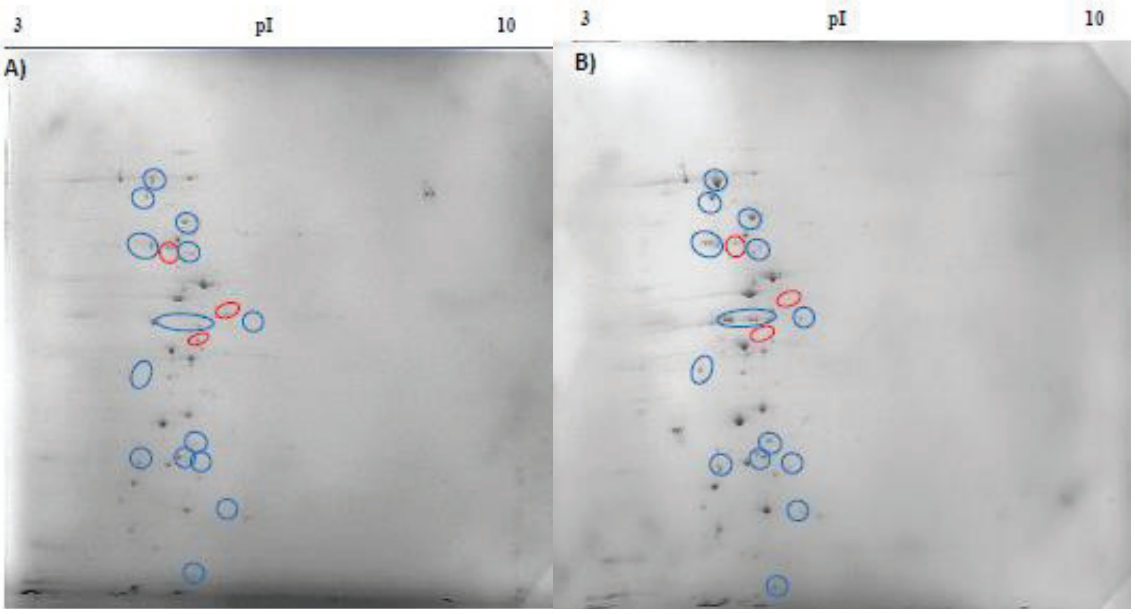

Figure 3.5. 2D-PAGE images of (A) control and (B) 2-HCA treated MSSA proteins. 350 μg protein containing gels were stained with Coomassie blue staining. The protein spots of MSSA control marked in red circles indicated the proteins whose expression were decreased or completely disappeared in 2-HCA-treated proteins. On the other hand, blue circles indicated the proteins spots whose expression had increased as a result of treatment with 2-HCA.

When the effect of 2-hydroxycinnamic acid on MSSA was examined individually considering upregulated (Table 3.7) and downregulated (Table 3.8) proteins, effects on protein folding and protein synthesis were observed. Upregulation of chaperone protein DnaK might prove induce of stress response in bacteria in the presence of 2-HCA. Upregulation of chaperone proteins prevents aggregation of newly synthesized polypeptide chains, as well as prevention of protein misfolding due to stress (Lv et al., 2016).

Upregulation of 30S ribosomal protein S1 indicated struggling for translational accuracy. 30S ribosomal protein is required for mRNA translation and plays role in the recognition of translation initiation point. Increase in the expression of such ribosome associated protein is critical for newly synthesized proteins (Lv et al., 2016). Another protein biosynthesis related protein was found as translation elongation factor Tu. Differently from other two proteins, expression of elongation factor Tu was downregulated as response to 2-HCA stress. During protein biosynthesis, this protein is responsible for promoting the binding of aminoacyl tRNA to the ribosomes in a GTP-dependent manner (Condell et al., 2012). It also has a GTPase activity whose decreased expression may indicate the reducing the energy of cell for producing new proteins. Instead, it tends to increase cells' metabolic activities.

Upregulation of dihydrolipoyl dehydrogenase proves this idea. Dihydrolipoyl dehydrogenase functions in glycolytic process (Sianglum et al., 2011), and is the component of the pyruvate dehydrogenase complex (Condell et al., 2012). Its catalytic activity as dihydrolipoyl dehydrogenase results in formation of NADH. This might indicate the upregulation of the protein is required for generation of reducing equivalents. Energy production in cells is provided by several metabolic pathways such as glycolysis, tricarboxylic acid cycle (TCA) and electron transport chain. Reducing equivalents such as NADH are produced via glycolysis and TCA and are used in generation of proton motive force which then will result in ATP formation (Reddy et al., 2015).

Short chain dehydrogenase was another protein with increased expression that was listed in Table 3.7. This protein is also responsible for formation of NADH due to its cyclopentanol dehydrogenase activity. Thus, increase in expression of these two proteins may indicate the cells' demand for energy under 2-hydroxycinnamic acid stress.

Table 3.7. Identified upregulated proteins of MSSA upon treatment with 2-HCA.

Protein ID	Protein Name	Function	Mass (Da)	Isoelectric Point
DNAK_STAAB	Chaperone protein DnaK	Stress response	66338	4.63
DLDH_STAAC	Dihydrolipoyl dehydrogenase	Dihydrolipoyl dehydrogenase activity	49592	4.95
RS1_STAAC	30S Ribosomal Protein S1	RNA binding in protein Biosynthesis	43261	4.51
EVY05333.1	Short chain dehydrogenase	Cyclopentanol dehydrogenase activity, oxidoreductase activity	23016	4.59

Following 2-HCA treatment, expressions of translation elongation factor Tu (Protein ID: EFTU_STAAN) and alcohol dehydrogenase (Protein ID: ADH_STAAB) were found to be downregulated. Decreased expression of translation elongation factor might indicate the decreased protein synthesis. Downregulation of alcohol dehydrogenase protein might be related with its zinc ion binding activity. Since zinc ion is a cofactor for this protein, detoxification processes to decrease toxicity caused by phenolic acid might be resulted in efflux of metal ions from the cell. This efflux might result in decreased expression of alcohol dehydrogenase.

Apart from the proteins mentioned above, there were proteins identified with low score numbers during MASCOT search (Table 3.8). However, some of these proteins were detected in the other gels at the same places and were identified reliably with higher scores. Therefore, they can be under the assumption of identified proteins. Phosphate acetyltransferase, D-lactate dehydrogenase, Co-chaperone GRPE, superoxide dismutase were detected in 2-HCA treated MSSA with increased expression levels. On the other hand, proteins such as pyridoxal 5' biosynthesis synthase subunit PdxS and ribosome recycling factor were found as downregulated proteins.

Increase in the expression of superoxide dismutase (Table 3.8) was consistent with the information in the literature (Reddy et al., 2015), because this protein was

generally induced under the stress conditions for protection of cells from reactive oxygen species. Co-chaperone GRPE upregulation was also consistent with our data showing upregulation of chaperone protein DnaK. Together, they might prevent misfolding and aggregation of newly synthesized proteins. Although upregulation of D-lactate dehydrogenase was detected with low MASCOT score, the same protein spots were examined in the other gels. D-lactate dehydrogenase was responsible for generation of NADH, and this supported the upregulation of short chain dehydrogenase and dihydrolipoyl dehydrogenase.

Upregulation of phosphate acetyltransferase protein as response to 2-HCA stress might indicate that the bacteria direct metabolic intermediates through pathways such as TCA cycle to supply the required energy. Downregulation of pyridoxal 5' biosynthesis synthase subunit PdxS was indicator of decreased cofactor biosynthesis.

Table 3.8. Identified proteins of 2-HCA treated MSSA with low MASCOT score number.

Protein ID	Protein Name	Function	Mass (Da)	Isoelectric Point	Response to 2-HCA
LDHD_STAAC	D-lactate dehydrogenase	D-lactate dehydrogenase activity	36716	5.10	Upregulation
PTAS_STAAC	Phosphate acetyl transferase	Acetyl CoA biosynthetic process	35044	4.72	Upregulation
EFW32388.1	Co-chaperone GRPE	Protein folding	21634	4.47	Upregulation
SODM1_STAAB	Superoxide dismutase	Stress response	22697	5.08	Upregulation
PDXS_STAAC	Pyridoxal 5' biosynthesis synthase subunit PdxS	Pyridoxal 5'P biosynthesis. Glutamine metabolism	32086	5.10	Downregulation
RRI_STAAB	Ribosome recycling factor	Release of ribosomes from mRNAs	20341	5.04	Downregulation

According to our results, the targets for used phenolic acids were related with the proteins taking roles in protein synthesis and stability, lipid biosynthesis, energy production, carbohydrate metabolism, nucleotide metabolism, DNA repair mechanisms, membrane integrity and cell division machinery and general stress response. It can be concluded that under the phenolic acid stress conditions, both MRSA and MSSA increased the expression of the genes essential for survival while decreasing the ones that could be seen as extra for the cell.

Although some of the proteins that were affected by the presence of 2-HCA and VA were identified in this study, not many proteins were covered by 2D-PAGE and MALDI-TOF/MSMS approach. For validation of distinct alterations and determination of changes in the protein profiles of these bacteria under the phenolic acid stress, shotgun proteomics experiments would be more effective for providing information about the action mechanism of these phenolic acids.

3.3.3. Determination of Gel-Free Proteomic Changes of MRSA upon Phenolic Acid Treatments

Shotgun proteomics (LC-ESI-MS/MS) was used for detailed examination of proteins of MRSA under VA and 2-HCA stresses. Apart from comparison of control and phenolic acid treated MRSA proteomes, comparison of proteins obtained under two phenolic acid stresses was also performed to understand different action mechanisms.

The proteins expressed under VA and 2-HCA stresses were analyzed by obtaining cellular proteins of phenolic acid treated and untreated MRSA. For separation of peptides, two-dimensional liquid chromatography was used. While high-pH liquid chromatography was used for fractionation of peptides, low-pH chromatography was used for separation of peptides. Therefore, more effective separation was provided. Ionization of the peptides was achieved by electrospray ionization coupled to a mass spectrometer. For identification of differently expressed proteins, MASCOT search engine was used to compare obtained spectra with reference proteome (*Staphylococcus aureus* N315 proteome) priory uploaded to UniProt database. During protein search, false discovery rate (FDR) was kept below 1%.

After identification of proteins, each protein was categorized according to their function. These eight functional categories were (i) DNA related proteins, (ii) RNA

related proteins, (iii) ribosome and protein synthesis related proteins, (iv) cell wall and membrane related proteins, (v) proteins related with metabolic reactions, (vi) proteins related with cell homeostasis and redox, (vii) proteins related with pathogenicity and (viii) other proteins.

Proteomes of control and VA treated MRSA; control and 2-HCA treated MRSA; VA treated and 2-HCA treated MRSA were compared for all categories.

3.3.3.1. Comparison of Control and Vanillic Acid Treated MRSA Proteomes

When proteins obtained from control MRSA were compared with vanillic acid treated MRSA proteins, it was found that 149 of control proteins (about 34% of total control proteins) were only identified in control and could not be identified in VA treated group. On the other hand, 80 proteins (about 21% of total VA treated proteins) were only identified in VA treated MRSA. The Venn diagram shown in Figure 3.6 was drawn by using a web tool named as 'Bioinformatics and Evolutionary Genomics'.

Each protein was categorized according to their function for both control MRSA and VA treated MRSA and listed in a consecutive way.

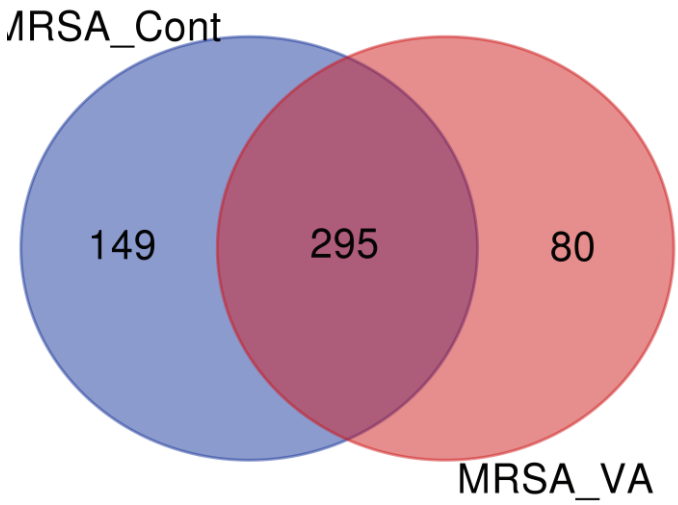

Figure 3.6. Numbers of identified proteins under control conditions (MRSA_Cont) and in the presence of vanillic acid (MRSA_VA).

3.3.3.1.1. Action of Vanillic Acid on DNA

MRSA proteins related with DNA that identified only in control MRSA but not in vanillic acid treated MRSA were shown in Table 3.9.

Table 3.9. DNA related proteins identified in control but not in VA treated MRSA.

Protein ID	Protein Name	Function
A0A0H3JUF7	Uncharacterized protein	DNA binding
A0A0H3JLL1	SA0466 protein	Nucleic acid binding
A0A0H3JML1	Cmp-binding-factor 1	Nucleic acid binding
P65237	Ribose-phosphate pyrophosphokinase	Nucleotide biosynthesis. Ribose phosphate diphosphokinase activity
Q7A5K4	3'-5' exonuclease DinG	DNA replication. Exonuclease activity
Q7A4Q5	DNA ligase (Polydeoxyribonucleotide synthase [NAD(+)])	Essential for DNA replication and repair. Catalyzes the formation of phosphodiester linkages between 5'-phosphoryl and 3'-hydroxyl groups in double-stranded DNA
Q7A5S6	Nuclease SbcCD subunit C	DNA replication, repair, recombination. Cleavage of DNA hairpin structures
P99126	Nucleoid-associated protein SA0437	Binds to DNA and alters its conformation
Q93KF4	DNA topoisomerase 4 subunit A	DNA topological change. Essential for chromosome segregation
P66939	DNA topoisomerase 4 subunit B	Essential for chromosome segregation. Relaxes supercoiled DNA
Q7A6H4	ATP-dependent helicase/nuclease subunit A	Double-strand break repair. Acts as DNA helicase and exonuclease
P99174	Deoxyribose-phosphate aldolase 2 (DERA 2)	Catalyzes an aldol reaction between acetaldehyde and D-glyceraldehyde 3-phosphate

The DNA related proteins identified in control MRSA (Table 3.9) but not in VA treated MRSA pointed the effect of vanillic acid on DNA replication and structure. Identification of proteins such as DNA topoisomerases, 3'-5' exonuclease DinG, DNA ligase and Nuclease SbcCD subunit C indicated the possible differences in the replication mechanism of DNA under the VA stress. Most significant changes among others were probable losses of topoisomerase A and topoisomerase B that take roles in segregation of the daughter chromosomes during replication (Hawkey, 2003). Identification of these proteins in control but not in VA treated MRSA might also explain the delayed growth of bacteria observed in the presence of subinhibitory concentrations of VA (See Chapter 2). Targeting of DNA topoisomerases by VA might be an important outcome because targeting topoisomerases is one of the known action mechanisms of quinolone antibiotics (Hawkey, 2003). Prevention of decatenation of DNA and chromosome unlinking might be one of the main action mechanisms of VA.

When Table 3.10 was examined, presence of DNA gyrases that were not identified in control group attracted attention.

Table 3.10. DNA related proteins identified in VA treated MRSA but not in control.

Protein ID	Protein Name	Function
A0A0H3JMZ3	Uncharacterized protein	Nucleic acid binding
P67047	Thymidylate synthase (TS)	Catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate to 2'-deoxythymidine-5'-monophosphate which is an essential precursor for DNA biosynthesis
P66937	DNA gyrase subunit B	DNA topological change. Negatively supercoils closed circular double-stranded (ds) DNA. Favors strand separation, DNA replication, transcription, recombination and repair
Q99XG5	DNA gyrase subunit A	DNA topological change. Negatively supercoils closed circular ds-DNA. Favors strand separation, DNA replication, transcription, recombination and repair
P65496	Endonuclease MutS2	DNA mismatch repair. Required for the suppression of homologous recombination

DNA gyrases mediate the negative supercoiling of DNA which is important for topology (Hawkey, 2003). Increase in the DNA gyrases (Table 3.10) in the presence of VA might indicate the need for more facilitated progress of replication and also might be required to compensate the loss of DNA topoisomerase 4 A and B that identified in control (Table 3.9).

Identification of endonuclease MutS2 might indicate the requirement of DNA mismatch repair due to possible damages on DNA structure. However, due to homologous recombination repressive property of endonuclease MutS2, this mismatch repair would result in the error-prone DNA synthesis. Considering the unidentifiability of DNA topoisomerase 4 subunits in VA treated MRSA, it could be speculated that the increase in error-prone DNA synthesis might be the reason of inhibitory effect of vanillic acid on MRSA.

3.3.3.1.2. Action of Vanillic Acid on RNA

The RNA related proteins listed in Table 3.11 were only identified in control group indicated the possible disappearance or downregulation in the presence of vanillic acid. These proteins were taking roles in transcription process of the bacteria, ensuring assembly of RNA polymerase, binding of RNA polymerase to DNA, efficient initiation, elongation and termination of transcription.

The lack of these RNA processing and transcription related proteins might be the response of cell to conserve energy by decreasing the transcription of non-essential genes. Moreover, this decreased expression might indicate the problems in initiation of transcription of several genes. This assumption could be corroborated with the identification of transcription termination factor Rho and bifunctional protein PyrR in VA treated MRSA (Table 3.12). Transcription termination factor Rho might be required to increase the termination of several genes' transcription which then might affect the protein synthesis.

Identification of bifunctional protein PyrR might help the clarification of the decrease in the pyrimidine metabolism when the bacteria were treated with vanillic acid. The increased function of this protein might lessen the expression of the genes cotranscribed with pyrimidine nucleotide operon as shown in the metabolism related proteins identified only in the control MRSA (Table 3.17).

Table 3.11. RNA related proteins identified in control but not in VA treated MRSA.

Protein ID	Protein Name	Function
P64235	Methylenetetrahydrofolate-tRNA	tRNA processing. Catalysis of formation of 5-methyl-uridine at position 54 in all tRNAs
P66726	DNA-directed RNA polymerase subunit omega	Transcription. Promotes RNA polymerase assembly
Q99TT5	RNA polymerase sigma factor SigA	Transcription initiation. Promotes the attachment of RNA polymerase to specific initiation sites
P65578	Transcription antitermination protein NusB (Antitermination factor NusB)	Necessary for transcription of ribosomal RNA genes. Involved in transcription antitermination by binding to the boxA antiterminator sequence of the rRNA operons
P0A096	Transcription termination/antitermination protein NusG	Transcription. Involved in transcription elongation, termination and antitermination
P99156	Transcription elongation factor GreA	Regulation of elongation of transcription. DNA binding, RNA polymerase binding
P99175	Catabolite control protein A	Global transcriptional regulator of carbon catabolite repression (CCR) and carbon catabolite activation (CCA)
A0A0H3JM44	DEAD-box ATP-dependent RNA helicase CshB	Probable DEAD-box RNA helicase. May function in ensuring proper initiation of transcription
A0A0H3JNV1	SA2421 protein	DNA binding transcription factor activity
A0A0H3JTG9	Cold-shock protein C	Regulation of transcription
Q7A5P3	Cold shock protein CspA	Involved in cold stress response. Regulation of transcription

Table 3.12. RNA related proteins identified in VA treated MRSA but not in control.

Protein ID	Protein Name	Function
A0A0H3JNE2	Transcription termination factor Rho	Facilitates transcription termination by binding to the nascent RNA
P67182	Probable transcriptional regulatory protein SA0624	Regulation of transcription
P65439	Transcriptional regulator MraZ	Transcription regulation. DNA binding transcription factor activity
A0A0H3JTL1	SA0836 protein	Transcription. DNA binding transcription factor activity
A0A0H3JK71	SA0653 protein	Transcription. DNA binding transcription factor activity
A0A0H3JMM9	SA1329 protein	Transcription. DNA binding transcription factor activity
P65944	Bifunctional protein PyrR	Regulates transcriptional attenuation of the pyrimidine nucleotide (pyr) operon and results in reduced expression of downstream genes

3.3.3.1.3. Action of Vanillic Acid on Ribosome and Protein Synthesis

When the proteins related with the ribosome structure and proteins synthesis were examined, identification of differently expressed 22 proteins in control (Table 3.13) and identification of only 4 differently expressed proteins in response to VA stress (Table 3.14) could be seen. The proteins listed in Table 3.13 mattered in terms of the production of all the proteins required for cell viability. Any difference in the processes responsible for protein production would have at least secondary effect for the inhibition or induce of bacterial growth. The identified proteins of control pointed the negative effect of VA on the protein biosynthesis. These proteins might indicate the disappearance or downregulation of many proteins taking roles in ribosome assembly and stability; as well as, initiation, elongation and termination of translation. Moreover, the proteins of control MRSA that responsible for proper protein folding, modification

and the transport of the proteins were not identified when the bacteria were subjected to VA stress. Prevention of the expression of constituents of 30S and 50S ribosomes, RNA processing and tRNA aminoacylation was also observed in 2-HCA treatment (Table 3.29). Differently from 2-HCA treatment, ribosome recycling factor was not identified in VA treated MRSA. This might result in the deceleration of the recycling of ribosomes during protein synthesis. In addition, lack of peptide chain release factor 1 might contribute to slowing the translation. Decelerated protein synthesis might cause the retarded bacterial growth observed in the presence of VA (See Chapter 2).

Table 3.13. Ribosome and protein synthesis related proteins identified in control but not in VA treated MRSA.

Protein ID	Protein Name	Function
P66153	50S ribosomal protein L28	Structural constituent of ribosome
P66299	50S ribosomal protein L36	Structural constituent of ribosome
P66494	30S ribosomal protein S19	Structural constituent of ribosome
P99130	Ribosome-recycling factor	Releases ribosomes from mRNA at the termination of protein biosynthesis
Q7A682	Ribonuclease J 1	rRNA processing. Has 5'-3' exonuclease and endonuclease activities
P67011	Alanine--tRNA ligase	Alanyl-tRNA aminoacylation. Catalyzes the attachment of alanine to tRNA (Ala)
A0A0H3JME4	Phenylalanine--tRNA ligase beta subunit	Phenylalanine-tRNA ligase activity for translation
P67513	Leucine—tRNA ligase	Leucil-tRNA aminoacylation. Catalyzes the attachment of leucine to tRNA (Leu)
Q7A537	Tyrosine--tRNA ligase	Tyrosyl-tRNA aminoacylation. Catalyzes the attachment of tyrosine to tRNA (Tyr)

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Table 3.13. (cont.)

A0A0H3JKS3	Ribosome-binding ATPase YchF	Binds to the 70S ribosome and the 50S ribosomal subunit
P60392	Ribosomal RNA small subunit methyltransferase H	rRNA processing. Methyltransferase activity
P0A0N7	Ribosomal RNA large subunit methyltransferase H	rRNA processing. Methyltransferase activity
P99077	Peptide deformylase	Removes the formyl groups from newly synthesized proteins during translation
P66019	Peptide chain release factor 1	Directs the termination of translation in codons UAG and UAA
P99082	33 kDa chaperonin	Protein folding. Redox regulated molecular chaperone. Prevention of aggregation of unfolding and damaged proteins
P60748	Foldase protein PrsA	Functions in protein secretion by helping the post-translocational extracellular folding of several secreted proteins
Q7A468	Protein translocase subunit SecY	Protein transport. The central subunit of the protein translocation channel SecYEG
Q7A6Q1	Probable protein-export membrane protein SecG	Involved in protein export
A0A0H3JTY9	Signal recognition particle protein	SRP-dependent co-translational protein targeting to membrane
A0A0H3JRD4	Hydroxamate siderophore binding lipoprotein	Lipoprotein
Q7A3L3	Uncharacterized lipoprotein SA2273	Lipoprotein
A0A0H3JL87	Uncharacterized protein	Ubiquitin-like modifier activating enzyme activity

On the other hand, presence of VA resulted in the identification of endoribonuclease YbeY that responsible for quality control of late stage 70S ribosome. Its presence might indicate the structural changes on the ribosome that might affect the protein synthesis (Table 3.14) consistently with the possible loss of ribosome binding ATPase YchF (Table 3.13). Identification of lipoyl synthase might indicate induce of post-translational modifications of synthesized proteins probably to make them functional in several metabolic reactions.

Table 3.14. Ribosome and protein synthesis related proteins identified in VA treated MRSA but not in control.

Protein ID	Protein Name	Function
A0A0H3JKT6	SA1111 protein	RNA binding
P67137	Endoribonuclease YbeY	rRNA processing. Late-stage 70S ribosome quality control
P65286	Lipoyl synthase	Protein lipoylation
A0A0H3JNQ8	SA2156 protein	Lactate transmembrane transporter activity

3.3.3.1.4. Action of Vanillic Acid on Cell Wall

Treatment of MRSA with VA resulted in the differential expression of 14 proteins related with the cell wall and membrane in the control (Table 3.15) and six proteins in VA treated MRSA (Table 3.16). Apart from the proteins such as UDP-N-acetylenolpyruvoglucosamine reductase, ribulose-5-phosphate reductase, bifunctional protein GlmU and UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase that were important for formation of cell wall; two proteins responsible for the production of ATP (Table 3.15).

ATP synthase epsilon chain and ATP synthase gamma chain located on the membrane produced ATP in the presence of a proton gradient within the cells. Unidentifiability of these proteins in MRSA treated with vanillic acid pointed the

disruptions on the cell membrane which was also observed in 2D-PAGE data and in the literature as mentioned previously (Borges et al., 2013).

Table 3.15. Cell wall and membrane related proteins identified in control but not in VA treated MRSA.

Protein ID	Protein Name	Function
P65463	UDP-N-acetylenolpyruvoylglucosamine reductase	Cell wall formation. Peptidoglycan biosynthesis
A0A0H3JNI1	Ribulose-5-phosphate reductase	Cell wall organization. Polyteichoic acid biosynthesis
Q7A7B4	Bifunctional protein GlmU	Cell wall organization. Catalysis of two reactions in UDP-N-acetylglucosamine biosynthesis
A0A0H3JMW3	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	Cell wall formation. Catalysis of the final step in the synthesis of UDP-N-acetylmuramoyl-pentapeptide
A0A0H3JNG7	Conserved hypothetical protein	Integral component of membrane
A0A0H3JM97	Uncharacterized protein	Integral component of membrane
A0A0H3JNS3	Uncharacterized protein	Integral component of membrane
A0A0H3JLS9	Uncharacterized protein	Integral component of membrane
A0A0H3JMJ8	Uncharacterized protein	Integral component of membrane
P67291	UPF0154 protein SA1178	Integral component of membrane
A0A0H3JND7	SA0168 protein	Transmembrane transport
Q7A3Q5	Protein flp (FmtA-like protein)	Membrane component
P63665	ATP synthase epsilon chain	Production of ATP in the presence of a proton gradient across the cell membrane
Q7A4E8	ATP synthase gamma chain	Production of ATP in the presence of a proton gradient

Treatment of MRSA with VA resulted in the identification of different proteins functioning as integral membrane components (Table 3.16). Alternation in the proteins with similar functions might be due to fix the disruptions on the cell membrane in the presence of VA or might be required for the incorporation of newly synthesized molecules into the membrane. Identification of the UTP-glucose-1-phosphate uridylyltransferase might indicate that MRSA exposed to VA tried to increase the biosynthesis of membrane glycolipids probably to fix the disruptions on the membrane.

Table 3.16. Cell wall and membrane related proteins identified in VA treated MRSA but not in control.

Protein ID	Protein Name	Function
Q7A3J9	UTP--glucose-1-phosphate uridylyltransferase	Catalysis of the formation of UDP-glucose. An intermediate step in the biosynthesis of diglucosyl-diacylglycerol, a glycolipid found in the *S. aureus* membrane
A0A0H3JN07	SA1559 protein	Integral component of membrane
A0A0H3JNL8	SA2103 protein	Integral component of membrane
Q7A341	UPF0397 protein SA2477	Integral component of membrane
A0A0H3JLN8	SA1035 protein	Cell cycle, cell division
A0A0H3JJJ9	SA0200 protein	Transmembrane transport

3.3.3.1.5. Action of Vanillic Acid on Metabolism

Treatment of MRSA with vanillic acid resulted in different expression of 97 proteins taking roles in several metabolic pathways. While about 74% (61 proteins) of the metabolism related proteins were only identified in control (Table 3.17), 26% of them (36 proteins) were identified only in vanillic acid treated MRSA (Table 3.18).

When the effect of VA on metabolism was examined, differences in the expression of proteins related with the purine and pyrimidine metabolism, energy

metabolism, carbohydrate metabolism, fatty acid and phospholipid metabolism, amino acid metabolism, and biosynthesis of cofactors could be seen. When proteins identified only in control MRSA (Table 3.17) and proteins identified only in VA treated MRSA (Table 3.18) were compared, lack of some proteins signals the energy depletion, membrane disruptions, and alterations in the preferences of carbohydrate usage or production of metabolic intermediates. The possible decrease or loss of some proteins taking roles in purine and pyrimidine biosynthesis upon treatment of bacteria with VA might inhibit the purine biosynthesis that would result in decreased production of AMP which directly would affect ATP production.

Identification of proteins such as 3-oxoacyl synthase 2, phosphate acetyl transferase, malonyl-CoA acyl carrier protein transacylase and acetyl coenzyme A carboxylase in control MRSA (Table 3.17) but not in VA treated MRSA might indicate the differences in cell wall composition of phenolic acid treated MRSA. This finding was consistent with the obtained protein differences associated with cell wall and membrane structure.

Another difference in the expression of metabolism related proteins was observed for proteins taking roles in the TCA cycle of bacteria. TCA is essential during aerobic respiration and is responsible for the formation of reducing equivalents for the cell and for production of membrane potential (or proton motive force) across the cell membrane. Therefore, proper function of TCA is essential for the biosynthesis of ATP (Reddy et al., 2015). Identification of TCA related proteins such as aconitate hydratase A and succinate CoA ligase in control MRSA (Table 3.17) but not in VA treated MRSA might signal the repression of TCA and subsequently energy production. On the other hand, VA treated MRSA allowed the identification of citrate synthase, isocitrate dehydrogenase and malate dehydrogenase (Table 3.18) proteins taking roles in TCA. Their presence indicated that the treatment of cells with VA did not result in complete blockage of the pathway. This was also proven by our growth inhibition data obtained during MIC studies (Chapter 2). Used concentrations of phenolic acids did not kill or completely inhibit the growth of bacteria but resulted in retarded growth.

Identification of tagatose-6-phosphate kinase in only control MRSA might indicate the differences in energy source usage of *S. aureus* under the VA stress. This protein is involved in the D-tagatose-6-phosphate pathway and provides *S. aureus* usage of galactose and lactose (Kuroda et al., 2001). Unidentifiability of tagatose-6-phosphate kinase protein in VA treated MRSA was also consistent with the changes in other

carbohydrate metabolism related proteins and its possible loss or downregulation might affect the growth ability of MRSA as observed in the antimicrobial studies (Chapter 2).

Identification of folate metabolism related proteins only in control MRSA might indicate the possible target of VA in the inhibition of MRSA growth. Affect on folate metabolism can be effective inhibition mechanism on MRSA considering the usage of antibiotics (such as sulfonaminds) targeting folate metabolism to fight against pathogenic bacteria (Morgan et al., 2018).

Table 3.17. Proteins related with several metabolic processes identified in control but not in VA treated MRSA.

Protein ID	Protein Name	Function
Q7A7I5	Xanthine phosphoribosyltransferase	Xanthosine 5'-monophosphate biosynthesis (Purine metabolism)
P99085	Hypoxanthine-guanine phosphoribosyltransferase	Purine ribonucleoside salvage. Guanine and hypoxanthine phosphoribosyltransferase activities
P99099	Adenylosuccinate synthetase	AMP biosynthesis. Catalysis of the first step in the biosynthesis of AMP
A0A0H3JM64	Purine nucleoside phosphorylase DeoD-type	Purine nucleoside metabolic process.
A0A0H3JKZ3	SA0537 protein	Phosphomethylpyrimidine kinase activity. Thiamine biosynthesis.
A0A0H3JLN3	SA0514 protein	Nucleoside kinase activity
P99068	Nucleoside diphosphate kinase	CTP, GTP, UTP biosynthetic processes. Required for the synthesis of nucleoside triphosphates other than ATP
P65936	Uridylate kinase	Pyrimidine metabolism. Catalysis of the phosphorylation of UMP to UDP
P99176	Guanylate kinase	Essential for recycling GMP and cGMP

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Table 3.17. (cont.)

Q7A6U7	7-cyano-7-deazaguanine synthase	Queuosine biosynthetic process. Catalysis of conversion of 7-carboxy-7-deazaguanine (CDG) to 7-cyano-7-deazaguanine ($preQ_0$)
A0A0H3JNU5	SA2416 protein	ATP binding, ATPase activity
A0A0H3JUU7	SA1708 protein	Biosynthetic process. ATP binding
Q7A6F8	3-oxoacyl-[acyl-carrier-protein] synthase 2	Fatty acid biosynthesis. Catalysis of the condensation reaction
P65739	Phosphate acyltransferase	Fatty acid and phospholipid biosynthetic processes. Transferase activity
Q7A5Z3	Malonyl CoA-acyl carrier protein transacylase	Fatty acid biosynthesis. S-malonyltransferase activity
Q7A557	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	Fatty acid biosynthetic process. Catalyzes the carboxylation of biotin
Q7A807	PTS system glucose-specific EIICBA component	The phosphoenolpyruvate-dependent sugar phosphotransferase system. Phosphorylation of incoming sugar substrates and their translocation
A0A0H3JSP4	SA0255 protein	Phosphoenolpyruvate-dependent sugar phosphotransferase system
A0A0H3JP54	Tagatose-6-phosphate kinase	D-tagatose 6-phosphate catabolic process. Lactose metabolic process
A0A0H3JUS3	Uncharacterized protein	N-acetylglucosamine metabolic process. Glucosamine-6-phosphate deaminase activity
Q7A5Z4	Uncharacterized protein SA1069	Glycerol metabolic process
P66695	Ribose-5-phosphate isomerase A	Pentose phosphate pathway. Catalysis of the conversion of ribose-5-phosphate to ribulose 5-phosphate
A0A0H3JTC1	Probable N-acetylglucosamine-6-phosphate deacetylase	N-acetylglucosamine-6-phosphate deacetylase activity

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Table 3.17. (cont.)

A0A0H3JMQ8	Acetyl-CoA carboxylase accC	ATP binding, metal ion binding, ligase activity
P99092	Phosphate acetyltransferase	Acetyl-CoA biosynthetic process. Phosphate acetyltransferase activity
P99148	Aconitate hydratase A	TCA. Catalysis of the isomerization of citrate to isocitrate
P99070	Succinate--CoA ligase [ADP-forming] subunit alpha	TCA. Couples the hydrolysis of succinyl-CoA to the synthesis of either ATP or GTP
A0A0H3JNH8	Alpha-acetolactate decarboxylase	Acetoin biosynthetic process. Acetolactate decarboxylase activity
Q7A535	Formate--tetrahydrofolate ligase	Plays role in tetrahydrofolate interconversion
P99079	Dihydrofolate reductase	Tetrahydrofolate biosynthesis. Key enzyme in folate metabolism
Q7A428	Urease accessory protein UreF	Nitrogen compound metabolic process.
P67404	Urease subunit alpha	Urea degradation
A0A0H3JVU8	Nitrite reductase	Nitrate assimilation
P65806	Peptidase T (Aminotripeptidase)	Peptide metabolic process. Cleavage of the N-terminal amino acid of tripeptides
Q7A6H8	NAD-specific glutamate dehydrogenase	Amino acid metabolic process. Glutamate dehydrogenase activity
A0A0H3JQQ7	Aminopeptidase ampS	Aminopeptidase activity
P65602	Ornithine carbamoyltransferase	L-Arginine catabolic process to ornithine
Q7A5B0	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	L-methionine salvage from methylthioadenosine

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Table 3.17. (cont.)

P64218	Probable glycine dehydrogenase subunit 1	Glycine catabolic process. Nucleoside metabolic process
Q7A6B1	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase	Catalysis of the thiamine diphosphate-dependent decarboxylation of 2-oxoglutarate
A0A0H3JUQ8	Protoporphyrinogen oxidase	Protoporphyrin-IX biosynthesis. Catalyzes the 6-electron oxidation of protoporphyrinogen-IX
P64334	Delta-aminolevulinic acid dehydratase	Protoporphyrin-IX biosynthesis. Catalyzes an early step in the biosynthesis of tetrapyrroles
A0A0H3JQ36	Heptaprenyl diphosphate syntase component II	Isoprenoid biosynthetic process. Transferase activity
P99172	Isopentenyl-diphosphate delta-isomerase	Isoprenoid biosynthetic process. Catalysis of the arrangement of the homoallylic substrate isopentenyl to dimethylallyl diphosphate
P60120	Putative pyridoxal phosphate-dependent acyltransferase	Biosynthetic process. Pyridoxal phosphate binding, transferase activity
A0A0H3JNS8	Cystathionine gamma-synthase	Pyridoxal phosphate binding
A0A0H3JPU6	Riboflavin biosynthesis protein	FAD and FMN biosynthetic processes. Riboflavin biosynthetic process
Q99X11	FMN-dependent NADH-azoreductase	Catalyzes the cleavage of azo bond in aromatic azo compounds to the amines
P99150	NH (3)-dependent NAD (+) synthetase	Catalysis of the ATP-dependent amidation of deamido-NAD to form NAD
A0A0H3JJQ6	SA0317 protein	Dehydrogenase activity
A0A0H3JNG6	Uncharacterized protein	Siderophore biosynthetic process

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Table 3.17. (cont.)

A0A0H3JP40	Cytokinin riboside 5'-monophosphate phosphoribohydrolase	Cytokinin biosynthetic process. Hydrolase activity
A0A0H3JNB0	Probable ss-1,3-N-acetylglucosaminyltransferase	Glycosyltransferase activity
Q7A3E8	Putative acetyltransferase SA2342	Acetyltransferase activity
A0A0H3JQ25	Uncharacterized protein	Methyltransferase activity
P65659	Pantothenate synthetase (PS)	Pantothenate biosynthetic process. Catalysis of the condensation of pantoate
P65753	Probable manganese-dependent inorganic pyrophosphatase	Inorganic diphosphatase activity
P99149	UPF0173 metal-dependent hydrolase SA1529	Hydrolase activity
A0A0H3JLB1	Uncharacterized protein	Hydrolase activity
A0A0H3JN42	Uncharacterized protein	Hydrolase activity
Q7A5P6	Uncharacterized hydrolase SA1230	Hydrolase activity

When the metabolism related proteins identified in VA treated MRSA were examined in Table 3.18, increased amino acid catabolism and the production of glutamate with expression of proteins ornithine aminotransferase 2 and 1-pyrroline-5-carboxylate dehydrogenase could be seen. Since glutamine and glutamate function as nitrogen donors for formation of biological molecules, maintenance of glutamine and glutamate levels within the cell has a vital importance. Bacteria can assimilate ammonia, degrade amino acids and use TCA intermediates to stabilize the concentration of glutamate within the cell (Somerville and Proctor, 2009). Induced acetyl-CoA biosynthesis might be required for the TCA cycle or fatty acid metabolism.

Table 3.18. Proteins related with several metabolic processes identified in VA treated MRSA but not in control.

Protein ID	Protein Name	Function
Q7A584	GTPase Obg	Functions in control of the cell cycle, stress response, ribosome biogenesis
Q7A777	GTP cyclohydrolase FolE2	Biosynthetic process. Conversion of GTP to 7,8-dihydroneopterin triphosphate
A0A0H3JNF6	SA1958 protein	ATP binding, ATPase activity
A0A0H3JP36	SA2319 protein	Gluconeogenesis. 4 iron, 4 sulfur cluster binding, L-serine ammonia-lyase activity
A0A0H3JLY6	Glycolytic operon regulator	Carbohydrate binding
P67200	Putative pyruvate, phosphate dikinase regulatory protein	Bifunctional serine/threonine kinase and phosphorylase. Functions in the regulation of the pyruvate, phosphate dikinase
A0A0H3JS20	Acetate-CoA ligase	Acetyl-CoA ligase activity
Q7A7L2	Probable acetyl-CoA acyltransferase	Acetyl CoA C-acetyl transferase activity
A0A0H3JMB8	Citrate synthase	TCA. Transferase activity
P99167	Isocitrate dehydrogenase [NADP]	TCA, glyoxylate cycle. NAD binding, isocitrate dehydrogenase activity
A0A0H3JQE7	SA1524 protein	Malate dehydrogenase activity, metal ion binding, NAD binding
P99144	Orotate phosphoribosyltransferase	UMP biosynthesis. Catalyzes the transfer of a ribosyl phosphate group
P67544	Bifunctional purine biosynthesis protein PurH	IMP biosynthetic process. IMP cyclohydrolase activity
A0A0H3JPM6	SA0958 protein	Phosphatidylinositol phosphorylation
Q7A427	Urease accessory protein UreG	Nitrogen compound metabolic process. Required in the incorporation of the urease nickel metallocenter

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Table 3.18. (cont.)

A0A0H3JQH3	SA1566 protein	Aminopeptidase activity. Metal ion binding
A0A0H3JRQ6	SA2244 protein	Aminopeptidase activity. Metal ion binding
A0A0H3JWB3	Zinc metalloproteinase aureolysin	Metalloendopeptidase activity
A0A0H3JJ63	SA0011 protein	Amino acid biosynthetic pathway. Acetyltransferase
P60298	Ornithine aminotransferase 2	L-proline biosynthesis. Catalysis of the interconversion of ornithine to glutamate
A0A0H3JS27	Sulfite reductase [NADPH] flavoprotein alpha-component	Cysteine biosynthetic process. Hydrogen sulfide biosynthetic process. Catalysis of the 6-electron reduction of sulfite to sulfide
P99076	1-pyrroline-5-carboxylate dehydrogenase	Proline catabolic process to glutamate. Oxidoreductase activity, acting on the aldehyde or oxo group of donors
A0A0H3JNT1	Glycine betaine aldehyde dehydrogenase GbsA	Glycine betaine biosynthetic process from choline. Betaine-aldehyde dehydrogenase activity
P63740	Carbamoyl-phosphate synthase large chain	L-arginine biosynthesis, UMP biosynthesis
P99141	6,7-dimethyl-8-ribityllumazine synthase (DMRL synthase)	Riboflavin biosynthetic process. Catalysis of the formation of 6,7-dimethyl-8-ribityllumazine
Q7A3C4	Uncharacterized hydrolase SA2367	Hydrolase activity
A0A0H3JRT1	SA2278 protein	Hydrolase activity
A0A0H3JLV1	Uncharacterized protein	Hydrolase activity
Q99UT4	Uncharacterized N-acetyltransferase SA1019	Transferase activity

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Table 3.18. (cont.)

A0A0H3JNC9	SA1845 protein	Kinase activity, phosphotransferase activity
A0A0H3JL27	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Metabolic process. Transferase activity
A0A0H3JNN5	SA2327 protein	Catalytic activity. Magnesium ion binding. Thiamine pyrophosphate binding
A0A0H3JNE8	SA0182 protein	Carboxy-lyase activity. Magnesium ion binding. Thiamine pyrophosphate binding
A0A0H3JVC9	Iron-sulfur cluster carrier protein	Metal ion binding. Binds and transfers iron-sulfur clusters to target apoproteins
A0A0H3JU75	Ferrodoxin	Iron ion binding, electron transfer activity
A0A0H3JM38	SA0797 protein	Iron-sulfur cluster assembly. Iron ion binding

Identification of proteins such as SA2319, iron-sulfur cluster carrier protein, ferrodoxin and SA0797 represented the differences in usage of iron in VA treated MRSA. The first two proteins were specific to VA response and could not be identified as response to 2-HCA stress. Formation of different metal ion binding proteins upon VA treatment might indicate the oxidative DNA damage (Zheng et al., 2008). With the help of metal ion binding proteins, bacteria might regulate the acidity of the cells caused by vanillic acid. Interaction of VA with iron might provide protection to the cells against iron-induced oxidative DNA damage (Lodovici et al., 2001) which explains the formation of iron ion binding proteins.

Maintenance of stability of the iron metabolism is essential for the viability of the cell because of its usage in cellular respiration and protection against oxygen intermediates (Chang et al., 2006).

3.3.3.1.6. Action of Vanillic Acid on Cell Homeostasis

The proteins related with the redox and cell homeostasis identified in control and VA treated MRSA, were listed in Tables 3.19 and 3.20, respectively. According to the identified proteins, it could be concluded that treatment of MRSA with VA affected the expression of proteins which maintain cell homeostasis and oxidoreductase activities. Changes in the profiles of the proteins related with the oxidative stress were expected because bacteria have tendency to response the presence of inhibitors (Blair et al., 2009). Identification of oxidative stress related proteins such as catalase was also consistent with our findings indicating the increase in the iron metabolism (Table 3.18) due to property of the iron to act as a cofactor for catalase (Chang et al., 2006).

Table 3.19. Proteins related with cell homeostasis and redox identified in control but not in VA treated MRSA.

Protein ID	Protein Name	Function
P99118	Alkyl hydroperoxide reductase subunit F	Cell redox homeostasis
A0A0H3JQQ0	Uncharacterized protein	Oxidoreductase activity, antioxidant activity
P60386	Redox-sensing transcriptional repressor Rex	Response to redox state by directing the transcription in response to changes in cellular NADH/NAD^+
Q7A759	Putative heme-dependent peroxidase SA0544	Peroxidase activity
A0A0H3JNK2	SA2080 protein	Oxidoreductase activity, FAD binding
Q7A417	Putative 2-hydroxyacid dehydrogenase SA2098	Oxidoreductase activity
Q7A6M9	Organic hydroperoxide resistance protein-like	Response to oxidative stress
Q7A4R2	Bacterial non-heme ferritin	Iron storage protein. Cellular iron ion homeostasis. Iron ion transport

Table 3.20. Proteins related with cell homeostasis and redox identified in VA treated MRSA but not in control.

Protein ID	Protein Name	Function
Q7A5T2	Catalase	Protect cells from the toxic effects of hydrogen peroxide by decomposing hydrogen peroxide into water and oxygen
A0A0H3JLG4	Uncharacterized protein	Oxidoreductase activity
Q7A782	FMN-dependent NADPH-azoreductase	Oxidoreductase activity. Cleaves the azo bond in aromatic azo compounds
A0A0H3JMU4	Alkyl hydroperoxide reductase AhpD	Antioxidant protein with alkyl hydroperoxidase activity
A0A0H3JNN3	Uncharacterized protein	Response to stress

3.3.3.1.7. Action of Vanillic Acid on Pathogenicity

One of the functional categories was made for the proteins related with the pathogenicity of MRSA. When the effect of VA on MRSA pathogenicity was examined, 10 differently identified proteins in control MRSA and only three differently identified proteins in VA treated MRSA were found. Treatment of bacteria with subinhibitory vanillic acid concentrations had a significant effect of bacterial virulence. Identification of protein S-ribosylhomocysteine lyase in control (Table 3.21) but not in vanillic acid treated MRSA might indicate the possible decrease in the quorum sensing ability. This signaling system is responsible for controlling several processes such as secretion of the enzymes, production of the virulence factors and development of biofilm (Liu et al., 2013). The unidentifiability of the proteins such as conserved virulence factor B, conserved virulence factor A, HTH-type transcriptional regulator SarS, HTH-type transcriptional regulator rot in vanillic acid treated MRSA was consistent with the possible reduction of quorum sensing proteins. All those virulence related proteins were expressed in control MRSA (Table 3.21) in a cell density manner and presence of VA decreased the production of virulence related proteins. This

collective downregulation of proteins that are controlled by the same transcriptional regulatory system '*agr*' (Boisset et al., 2007) might indicate the effect of VA on the *agr* locus.

Table 3.21. Proteins related with pathogenicity identified in control but not in VA treated MRSA.

Protein ID	Protein Name	Function
P65330	S-ribosylhomocysteine lyase	Quorum sensing. S-ribosylhomocysteine lyase activity
Q7A5Q1	Conserved virulence factor B	Involved in the expression of virulence factors and pathogenicity in a cell density-dependent manner
P67278	Ribonuclease Y (Conserved virulence factor A)	Pathogenesis. mRNA catabolic process. Initiates mRNA decay
Q7A872	HTH-type transcriptional regulator SarS	Regulation of expression of some virulence factors in a cell density-dependent manner
Q7A514	HTH-type transcriptional regulator rot	Global regulator of several genes involved in virulence
Q99SU9	Staphylococcal complement inhibitor	Involved in host defense. Inhibition of phagocytosis and killing of *S. aureus* by human neutrophils
A0A0H3JPQ1	SA1000 protein	Complement binding
Q7A7R8	ESAT-6 secretion system extracellular protein B	Required for the infection in the host
Q7A4R9	Response regulator protein VraR	Response to antibiotic. Promotes expression of beta-lactam and glycopeptide resistance. Activates transcription of beta-lactam-inducible PBP2, UDP-N-acetylglucosamine enolpyruvyl transferase, monofunctional glycosyltransferase, methicillin and teicoplanin resistance-related proteins
A0A0H3JLY5	PBP2	Penicillin binding. Transferase activity

Among the proteins identified only in control but not in VA treated MRSA (Table 3.21), there were proteins responsible for providing host interactions to bacteria and maintenance of the infection in the host. Presence of staphylococcal complement inhibitor and SA1000 protein in MRSA prevents the immune system of the host from destroying the bacteria. SA1000 is a virulence factor conserved among *S. aureus* strains and provides adherence to epithelial cells (Boisset et al., 2007). This finding was significant, because they were among the unidentified proteins in VA treated MRSA with the proteins such as ESAT-6 secretion system extracellular protein B, response regulator protein VraR and PBP2. Their possible downregulation or loss in VA treated cells might indicate the increased susceptibility to the host defense and the antibiotics.

On the other hand, in VA treated MRSA, sensor protein SrrB, clumping factor A and extracellular matrix-binding protein EbhA proteins were identified as differently expressed proteins from control (Table 3.22). The reason of detection of sensor protein SrrB upon vanillic acid treatment might be due to its involvement for microbial oxidative defense system (Chang et al., 2006).

Identification of clumping factor A in VA treated MRSA might indicate the struggle of cells to provide bacterial clumps for biofilm formation to protect themselves from the presence of vanillic acid. Extracellular matrix binding protein EbhA might be important for the process of adhesion to the host.

Table 3.22. Proteins related with pathogenicity identified in VA treated MRSA but not in control.

Protein ID	Protein Name	Function
Q7A5H7	Sensor protein SrrB	Member of two-component regulatory system SrrA/SrrB. This protein regulates virulence factors in response to oxygen levels
Q99VJ4	Clumping factor A (Fibrinogen-binding protein A)	Promotes the attachment of bacteria to the gamma-chain of human fibrinogen. Induces formation of bacterial clumps and decreases phagocytosis by macrophages
Q99U54	Extracellular matrix-binding protein EbhA	Pathogenesis

3.3.3.1.8. Action on Vanillic Acid on Other Proteins

The predicted proteins with uncharacterized functions were also categorized as a functional group because of their probable effects on the MRSA. Both control MRSA and VA treated MRSA allowed the identification of many uncharacterized proteins that might contribute the response of cells to the presence of phenolic acids.

Proteins that were not grouped under a specific functional category were listed in Table 3.23 for control MRSA. These 10 proteins were mainly predicted proteins with uncharacterized functions.

On the other hand, the proteins that were not grouped under a specific functional category for vanillic acid treated MRSA were listed in Table 3.24. For VA treated MRSA, 14 proteins were categorized in this group due to uncharacterized functions. The protein families for five of these proteins were given under the function section of the table.

Table 3.23. Other proteins identified in control but not in VA treated MRSA.

Protein ID	Protein Name	Function
A0A0H3JVC0	Uncharacterized protein	Predicted protein
A0A0H3JL42	Uncharacterized protein	Predicted protein
A0A0H3JLT4	Uncharacterized protein	Predicted protein
A0A0H3JPD8	Uncharacterized protein	Predicted protein
A0A0H3JUM1	Uncharacterized protein	Predicted protein
A0A0H3JNI4	SA0248 protein	Predicted protein
A0A0H3JSJ2	SA0165 protein	Predicted protein
A0A0H3JNW8	SA0522 protein	Predicted protein
A0A0H3JMZ4	SA2317 protein	Predicted protein
A0A0H3JU44	SA1243 protein	Predicted protein

Table 3.24. Other proteins identified in VA treated MRSA but not in control.

Protein ID	Protein Name	Function
A0A0H3JUT6	Uncharacterized protein	Predicted protein
A0A0H3JLT3	Uncharacterized protein	Predicted protein
A0A0H3JLE7	Uncharacterized protein	Predicted protein
A0A0H3JLS0	Uncharacterized protein	Predicted protein
A0A0H3JM08	Uncharacterized protein	Predicted protein
A0A0H3JN43	Uncharacterized protein	Predicted protein
A0A0H3JM34	Uncharacterized protein	Predicted protein
P60108	TelA-like protein SA1238	Predicted protein. Belongs to TelA family
A0A0H3JJX7	Veg protein	Predicted protein
Q7A593	UPF0337 protein SA1452	Belongs to UPF0337 protein family
P67337	UPF0178 protein SA0636	Belongs to the UPF0178 family
Q99UZ6	UPF0637 protein SA0957	Belongs to the UPF0637 family
Q7A4S2	UPF0435 protein SA1696	Belongs to the UPF0435 family
A0A0H3JTZ5	SA1121 protein	Metal ion binding

To summarize the action of vanillic acid on MRSA, the percentages of proteins belonging to each functional category were displayed in Figure 3.7 for the proteins that identified only in control and only in VA treated MRSA. When the percentage of identified proteins were compared in terms of the functional categories that they belong, it could be seen that most of the proteins were related with the metabolism. Metabolism related proteins contained the 41% of all identified control proteins and 45% of all identified VA treated MRSA proteins. The lowest percentages that obtained were cell homeostasis related proteins of control (5%) and pathogenicity related proteins of VA treated MRSA (4%).

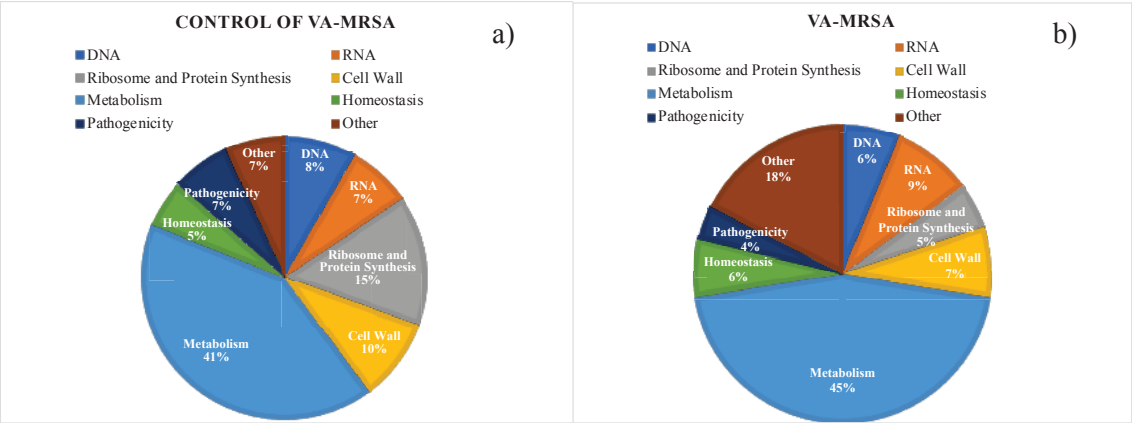

Figure 3.7. The percentages of the identified proteins for each functional category for (a) only in control MRSA and (b) only in VA treated MRSA.

3.3.3.2. Comparison of Control and 2-Hydroxycinnamic Acid Treated MRSA Proteomes

The numerical comparison of control and 2-hydroxycinnamic acid treated MRSA proteins was shown in Figure 3.8. The Venn diagram shown in the figure was drawn by using a web tool named as 'Bioinformatics and Evolutionary Genomics'.

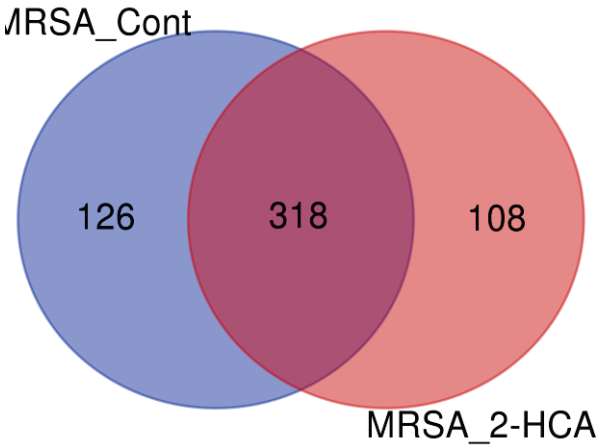

Figure 3.8. Numbers of identified proteins under control conditions (MRSA_Cont) and in the presence of 2-hydroxycinnamic acid (MRSA_2-HCA).

When proteins obtained from control and 2-HCA treated MRSA group were compared, it was observed that 126 proteins (about 28% of total control proteins) were only identified in control indicating probable disappearance or lower expression levels of these proteins upon 2-HCA treatment. On the other hand, 108 of 2-HCA treated MRSA proteins (about 25% of total 2-HCA treated MRSA proteins) were identified as response proteins to stress (When the FDR was kept below 1%).

Following the UniProt search of each protein, they were categorized according to their functions. Functions and biological processes of proteins were determined by searching corresponding protein IDs on the UniProt database.

3.3.3.2.1. Action of 2-Hydroxycinnamic Acid on DNA

The MRSA proteins related with DNA that identified only in control MRSA when compared with 2-hydroxycinnamic acid (2-HCA) treated MRSA were shown in Table 3.25. Treatment of MRSA with subinhibitory 2-HCA concentrations resulted in unidentifiability of several proteins related with nucleotide metabolisms that could be assumed as disappeared or downregulated proteins as response to 2-HCA stress.

Identification of proteins such as 3'-5' exonuclease DinG, ATP dependent helicase/nuclease subunit A, DNA repair protein RecN and nuclease SbcCD subunit C in control MRSA (Table 3.25) but not in 2-HCA treated MRSA might indicate the loss of recombinational repair for damaged DNA. The presence of ATP dependent helicase/nuclease subunit A, DNA repair protein RecN and nuclease SbcCD subunit C proteins provided process of homologous recombination to correctly repair DNA.

According to our findings, presence of 2-HCA hindered or downregulated the expression of these proteins and promoted SOS response for DNA repair (Table 3.26). Identification of UvrABC system protein B and protein RecA suggested the error prone synthesis of DNA which is not depend on homologous recombination. This might result in formation of mismatches and increased mutations (Cirz et al., 2006). Identification of proteins taking roles in nucleotide biosynthesis and replication might be required for the repair of the damaged DNA. Presence of DNA gyrase subunit A that functions as facilitating the movement of the replication forks (Hawkey, 2003) might support the need of DNA synthesis.

Since SOS response indicates the indispensable DNA repair for survival in the presence of 2-hydroxycinnamic acid, it can be speculated that tendency in the error-prone DNA replication might be the reason for inhibitory effects of 2-HCA.

Table 3.25. DNA related proteins identified in control but not in 2-HCA treated MRSA.

Protein ID	Protein Name	Function
A0A0H3JML1	Cmp-binding-factor 1	Nucleic acid binding
A0A0H3JUF7	Uncharacterized protein	DNA binding
A0A0H3JM64	Purine nucleoside phosphorylase DeoD-type	Purine-nucleoside phosphorylase activity
P65906	Dihydroorotase	de novo' UMP biosynthetic process. Catalysis of the reversible cyclization of carbamoyl aspartate to dihydroorotate
P99079	Dihydrofolate reductase	Key enzyme in folate metabolism. Catalysis of an essential reaction for de novo glycine and purine synthesis, and for DNA precursor synthesis
P99099	Adenylosuccinate synthetase	Plays an important role in purine nucleotide biosynthesis. Required for the biosynthesis of AMP from IMP
P65237	Ribose-phosphate pyrophosphokinase	Nucleotide biosynthetic process. Involved in the biosynthesis of the phospho-alpha-D-ribosyl-1-pyrophosphate (PRPP)
Q7A5K4	3'-5' exonuclease DinG	DNA replication. 3'-5' exonuclease
Q7A6H4	ATP-dependent helicase/nuclease subunit A	DNA repair. Double-strand break repair. Acts as both DNA helicase and dual-direction single-stranded exonuclease. Recognizes the chi site and makes DNA suitable for the homologous recombination
A0A0H3JM17	DNA repair protein RecN (Recombination protein N)	DNA repair, recombination. May be involved in recombinational repair of damaged DNA
Q7A5S6	Nuclease SbcCD subunit C	DNA repair, recombination, replication. Cleavage of DNA hairpin structures that can inhibit DNA replication

Table 3.26. DNA related proteins identified in 2-HCA treated MRSA but not in control.

Protein ID	Protein Name	Function
A0A0H3JM27	Uncharacterized protein	Nucleobase-containing compound metabolic process
P67047	Thymidylate synthase	Nucleotide biosynthesis. dTTP biosynthetic process. Thymidylate synthase activity
Q99XG5	DNA gyrase subunit A	A type II topoisomerase responsible for negatively supercoiling of closed circular double-stranded (ds) DNA in an ATP-dependent manner. Modulates DNA topology
A0A0H3JW98	Anaerobic ribonucleoside-triphosphate reductase	DNA replication. Ribonucleoside-triphosphate reductase activity
P67109	Nucleotide-binding protein SA0720	Displays ATPase and GTPase activities
A0A0H3JUK3	SA1562 protein	DNA binding, ATP binding
P67425	UvrABC system protein B	Nucleotide excision repair. SOS response. Catalyzes the recognition and processing of DNA lesions. Scans for abnormalities in DNA
P68844	Protein RecA	DNA repair, recombination. SOS response. Catalyzes the hydrolysis of ATP in the presence of single-stranded DNA and the ATP-dependent hybridization of homologous ss-DNAs

3.3.3.2.2. Action of 2-Hydroxycinnamic Acid on RNA

Another functional category was made for RNA-related proteins to display differently identified proteins in control and 2-HCA treated MRSA. Identification of 11 proteins in control but not in 2-HCA treated MRSA suggested the reduction of the production of these proteins (Table 3.27). On the other hand, 2-HCA treatment resulted in identification of 5 proteins that were not identified in control (Table 3.28).

Some of the proteins that were identified in control but not identified in 2-HCA treated MRSA were related with RNA processing (Table 3.27).

Table 3.27. RNA related proteins identified in control but not in 2-HCA treated MRSA.

Protein ID	Protein Name	Function
P60392	Ribosomal RNA small subunit methyltransferase H	rRNA processing. Methylation of the N4 position of cytidine in position 1402 of 16S rRNA
P0A0N7	Ribosomal RNA large subunit methyltransferase H	rRNA processing. Methylation of the pseudouridine at position 1915 in 23S rRNA
Q7A5X7	Polyribonucleotide nucleotidyltransferase	RNA processing. Involved in mRNA degradation
A0A0H3JM44	DEAD-box ATP-dependent RNA helicase CshB	RNA catabolic process. Probable DEAD-box RNA helicase
P66726	DNA-directed RNA polymerase subunit omega	Promotes RNA polymerase assembly
Q99TT5	RNA polymerase sigma factor SigA	Transcription. Promotes the attachment of RNA polymerase to specific initiation sites and are then released
P99156	Transcription elongation factor GreA	Essential for efficient RNA polymerase transcription elongation past template-encoded arresting sites that trap elongating RNA polymerases and resulting in locked ternary complexes. GreA allows the resumption of elongation
A0A0H3JNV1	SA2421 protein	Transcription factor activity
P65578	Transcription antitermination protein NusB	Transcription. Transcription antitermination. Necessary for the transcription of rRNA genes
A0A0H3JTG9	Cold-shock protein C	Regulation of transcription
Q7A5P3	Cold shock protein CspA	Involved in cold stress response. Regulation of DNA-templated transcription

The probable lack of DEAD-box ATP dependent RNA helicase CshB in 2-HCA treated MRSA might indicate the direct effect of 2-HCA on this protein. These helicases are involved in many processes for RNA metabolism in all eukaryotic cells and in many

bacteria (Linder and Jankowsky, 2011). Another reason of unidentifiability of DEAD-box RNA helicase might be the reduction of the energy metabolism in MRSA in the presence of 2-HCA. The reduction in ATP generation was also observed for VA treated MRSA and for membrane related proteins of 2-HCA treated MRSA (Table 3.31). Moreover, DNA directed RNA polymerase subunit omega, RNA polymerase sigma factor SigA, transcription elongation factor GreA and transcription antitermination protein NusB were identified only in control. Unidentifiability of these proteins required for initiation, elongation and proper transcription of genes under 2-HCA stress might indicate the reduction of the transcription of many genes. This reduction might economize the required energy to maintain the expression of essential genes.

The probable lack of polyribonucleotide nucleotidyltransferase that function in the degradation of mRNA might indicate the significant changes in the programming of gene expression. Consistently, one of the proteins identified in 2-HCA treated MRSA but not in control was regulatory protein Spx (Table 3.28). This protein was responsible for reduction of transcription of the genes that induce growth and increase the transcription of homeostasis related genes. Identification of this protein might help explaining the retarded growth of MRSA in the presence of 2-HCA (Chapter 2).

Table 3.28. RNA related proteins identified in 2-HCA treated MRSA but not in control.

Protein ID	Protein Name	Function
P64230	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	tRNA wobble uridine modification. Involved in the addition of a carboxymethylaminomethyl group at the wobble position of certain tRNAs
A0A0H3JKA9	Ribonuclease R	3'-5' exoribonuclease. It takes role in maturation of structured RNAs
A0A0H3JMM9	SA1329 protein	Transcription factor activity
P67182	Probable transcriptional regulatory protein SA0624	Regulation of transcription
P60379	Regulatory protein Spx	Negative regulation of transcription. It may reduce the transcription of genes involved in growth and raise the transcription of genes involved in thiol homeostasis

3.3.3.2.3. Action of 2-Hydroxycinnamic Acid on Ribosome and Protein Synthesis

Treatment of MRSA with 2-hydroxycinnamic acid resulted in identification of 17 ribosome and protein synthesis related proteins only in control (Table 3.29).

Table 3.29. Ribosome and protein synthesis related proteins identified in control but not in 2-HCA treated MRSA.

Protein ID	Protein Name	Function
A0A0H3JKT3	Ribosome biogenesis GTPase A	Assembly of 50S ribosomal subunit
A0A0H3JKS3	Ribosome-binding ATPase YchF	Binds to 70S and the 50S ribosomes
P66440	30S ribosomal protein S16	Structural constituent of ribosome
P66133	50S ribosomal protein L27	Structural constituent of ribosome
P66153	50S ribosomal protein L28	Structural constituent of ribosome
P66231	50S ribosomal protein L33 2	Structural constituent of ribosome
P67015	Aspartate--tRNA ligase	Aspartyl-tRNA aminoacylation
P67513	Leucine--tRNA ligase	Leucyl-tRNA aminoacylation
Q7A537	Tyrosine--tRNA ligase	Tyrosyl-tRNA aminoacylation
P68808	Aspartyl/glutamyl-tRNA amidotransferase subunit C	Allows the formation of correctly charged Asn-tRNA or Gln-tRNA
P99077	Peptide deformylase	Removes the formyl group from newly synthesized proteins
P60748	Foldase protein PrsA	Required in protein secretion
P99104	10 kDa chaperonin	Protein folding
P99082	33 kDa chaperonin	Protein folding
A0A0H3JTY9	Signal recognition particle protein	Targeting of membrane proteins into the cytoplasmic membrane
Q7A468	Protein translocase subunit SecY	Protein transport
Q7A6Q1	Probable protein-export membrane protein SecG	Involved in protein export

Proteins that were not identified in 2-HCA treated MRSA such as ribosome biogenesis GTPase A, ribosome binding ATPase YchF, 30S and 50S ribosomal proteins had important functions in ribosome assembly and stability. Lack of these proteins might indicate the problems in transcription of many genes. Another explanation might be the prevention of transcription of some genes to conserve energy under stress.

Table 3.30. Ribosome and protein synthesis related proteins identified in 2-HCA treated MRSA but not in control.

Protein ID	Protein Name	Function
P64085	GTPase Era	Binds both GDP and GTP .Takes role in 16S rRNA processing and 30S ribosomal subunit biogenesis
P65967	Ribosome-binding factor A	Assists the late maturation steps of the functional core of the 30S ribosomal subunit. Also essential for processing of 16S rRNA
A0A0H3JMR4	Octanoyltransferase LipM	Protein lipoylation. An intermediate carrier of endogenously produced octanic acid
A0A0H3JKZ6	Octanoyl-[GcvH]: protein N-octanoyltransferase	Protein lipoylation. Catalyzes the amidotransfer of the octanoyl moiety
P0A068	Signal peptidase IB	Essential for viability. Cleaves the hydrophobic, N-terminal signal or leader sequences from secreted and periplasmic proteins
P63971	Chaperone protein DnaJ	Protein folding. Takes role in the response to hyperosmotic and heat shock via preventing the aggregation of stress-denatured proteins
Q7A366	Protein translocase subunit SecA 2	Protein import. Interacts with the SecYEG preprotein conducting channel
A0A0H3JNG3	Glycine betaine/carnitine/choline ABC transporter opuCA	Glycine betaine transport
P65797	ATP-dependent protease subunit HslV	Proteolysis involved in cellular protein catabolic process. Plays role in degradation of proteins

Some of the tRNA ligase proteins, namely aspartate-tRNA ligase, leucine-tRNA ligase and tyrosine-tRNA ligase, were not identified in 2-hydroxycinnamic acid treated MRSA. The inhibitory effects on the formation of proteins with acylation functions might suggest the direct interaction between attachment site of amino acid to tRNA and 2-HCA. Unidentifiability of these proteins might indicate the problems in protein translation. Lack of chaperone proteins might indicate the presence of unfolded or misfolded proteins. Also, dependence of 10 kDa chaperonin to ATP was another indicator of ATP depletion in the cells.

Differences in the expression of transporter proteins might be due to reduced production of other proteins that would have been carried to specific locations. Among the proteins identified in the presence of 2-HCA but not in control (Table 3.30), two proteins were responsible for the lipoylation: Octanoyltransferase LipM and octanoyl-(GcvH) protein N-octanoyltransferase. Identification of these two proteins might indicate the requirement of post-translational modifications for the proteins to be functional during metabolic processes.

Signal peptidase IB and ATP-dependent protease subunit HslV might be necessary for the degradation of proteins whose metabolites would be used by other metabolic pathways for formation of biological molecules. In the overall, this might help bacteria to conserve energy.

3.3.3.2.4. Action of 2-Hydroxycinnamic Acid on Cell Wall

When MRSA grown under normal conditions and in the presence of 2-HCA were compared for differences in terms of cell wall and membrane related proteins, 14 proteins in control group and 7 proteins in 2-HCA treated group were identified and these proteins were listed in Table 3.31 and Table 3.32, respectively. Similar to vanillic acid stress, presence of 2-HCA resulted in unidentifiability of ATP synthase epsilon chain and ATP synthase gamma chain. Inadequate production of these proteins would result in reduced ATP production within the cells that might limit the several metabolic processes due to energy depletion. On the other hand, ATP synthase subunit delta was identified in 2-HCA treated MRSA (Table 3.32). Identification of one subunit of ATP synthase in 2-HCA treated bacteria indicated that 2-HCA presence did not result in the complete disruption of this membrane bound enzyme complex. Presence of partial

disruptions on the cell membrane or alterations in the cell membrane might affect the function of ATP synthase complex. Proteins had functions in cell wall biosynthesis such as bifunctional protein GlmU and UDP-N-acetylenolpyruvoylglucosamine reductase were identified in control but not in 2-HCA treated bacteria (Table 3.31) indicated the reduction in the synthesis of membrane related polymers.

Table 3.31. Cell wall and membrane related proteins identified in control but not in 2-HCA treated MRSA.

Protein ID	Protein Name	Function
Q7A7B4	Bifunctional protein GlmU	Cell wall organization. Lipopolysaccharide biosynthesis
P65463	UDP-N-acetylenolpyruvoylglucosamine reductase	Cell wall formation. Peptidoglycan biosynthesis
Q7A615	Cell division protein SepF	Cell septum assembly. Cell division protein
A0A0H3JNG7	Conserved hypothetical protein	Integral component of membrane
A0A0H3JNS3	Uncharacterized protein	Integral component of membrane
A0A0H3JLS9	Uncharacterized protein	Integral component of membrane
A0A0H3JRH6	Uncharacterized protein	Integral component of membrane
A0A0H3JM97	Uncharacterized protein	Integral component of membrane
Q7A5C5	UPF0365 protein SA1402	Integral component of membrane
P67291	UPF0154 protein SA1178	Integral component of membrane
Q7A5I6	Elastin-binding protein EbpS	Promotes binding of soluble elastin peptides to *S.aureus* cells
A0A0H3JND7	SA0168 protein	Transmembrane transport
P63665	ATP synthase epsilon chain	ATP synthesis coupled proton transport. Production of ATP in the presence of a proton gradient
Q7A4E8	ATP synthase gamma chain	ATP synthesis coupled proton transport. Production of ATP in the presence of a proton gradient

Unidentifiability of some of the cell wall formation proteins was reasonable because peptidoglycan structure is the most abundant polymer whose biosynthesis requires too much energy for the cell (Mongodin et al., 2003).

Cell division protein SepF was identified in control, indicating reduced cell division and proliferation in 2-HCA treated MRSA. Lack of this protein might imply that 2-HCA prevented the proper or on scheduled division of bacteria which might contribute to explain delayed growth of bacteria in the presence of phenolic acids (See MIC experiments in chapter 2).

Probable transglycosylase SceD (Table 3.32) had ability for peptidoglycan cleavage and had effect on clumped bacterial cells. Identification of such hydrolytic enzyme processing in cell wall might be involved in the processes providing insertion of the subunits of the cell wall to corresponding locations (Mongodin et al., 2003). Another possibility was correlated with the decreased quorum sensing ability of bacteria which together would result in less biofilm production in MRSA due to its ability to separate bacterial clumps (Table 3.37).

Table 3.32. Cell wall and membrane related proteins identified in 2-HCA treated MRSA but not in control.

Protein ID	Protein Name	Function
Q99TF4	Alanine dehydrogenase 2	Cell wall biosynthesis
Q7A4F2	Probable transglycosylase SceD	Is able to cleave peptidoglycan. Affects clumping and separation of bacterial cells
A0A0H3JNL8	SA2103 protein	Integral component of membrane
A0A0H3JN07	SA1559 protein	Integral component of membrane
A0A0H3JN15	SA1575 protein	Integral component of membrane
P99109	ATP synthase subunit delta	ATP synthesis coupled proton transport. Production of ATP in the presence of a proton gradient
A0A0H3JNQ8	SA2156 protein	Lactate transmembrane transporter activity

Identification of different uncharacterized proteins that had functioned as integral components of the cell membrane both in control (Table 3.31) and in 2-HCA treated MRSA (Table 3.32) pointed the presence of alterations in the membrane composition of the cell.

3.3.3.2.5. Action of 2-Hydroxycinnamic Acid on Metabolism

Treatment of MRSA with 2-HCA resulted in identification of 90 differently expressed proteins with roles in several metabolic reactions. In Table 3.33 identified proteins that belonged to control (48 proteins), and in Table 3.34 identified proteins that belonged to 2-HCA treated MRSA (42 proteins) were listed. According to the proteins listed in Table 3.33, presence of 2-HCA caused the alterations in amino acid metabolism, lipid metabolism, carbohydrate and iron metabolisms. Alterations in the TCA cycle were important to provide precursors to amino acid and nucleic acid metabolisms (Somerville and Proctor, 2009). Identification of different TCA enzymes in control (Table 3.33) and 2-HCA treated MRSA (Table 3.34) were consistent with this information and might be required to produce metabolic intermediates.

Ability of *S. aureus* to live in aerobic and anaerobic environments and to use oxygen, nitrate or nitrite as acceptors during electron transport chain provides variability in the metabolic pathways. In the presence of oxygen, *S. aureus* produces menaquinone to be used in the electron transport chain for transfer of electrons to cytochrome c. Transfer of electrons to oxygen generates a proton gradient across the cell membrane which subsequently results in generation of ATP. However, this transfer process of electrons might also result in formation of reactive oxygen species (Somerville and Proctor, 2009). A protein responsible in menaquinone biosynthesis was identified only in control MRSA (Table 3.33). It might indicate the preference of bacteria to use non-oxidative metabolic pathways to protect cells from oxidative damage. The lack of the proteins with functions in iron metabolism might support the decrease of enzymatic reactions that needed iron as cofactors and also to protect cells from oxidative damage which was highly related with iron metabolism as mentioned previously. Different electron acceptor preference might also affect the formation of proton gradient across the membrane which then would result in less energy production within the cell.

Table 3.33. Proteins related with several metabolic processes identified in control but not in 2-HCA treated MRSA.

Protein ID	Protein Name	Function
P66695	Ribose-5-phosphate isomerase A	Pentose-phosphate shunt, non-oxidative branch. Catalysis of the conversion of ribose-5-phosphate to ribulose 5-phosphate
A0A0H3JNI1	Ribulose-5-phosphate reductase	Polyteichoic acid biosynthesis. Catalysis of the reduction of D-ribulose 5-phosphate to D-ribitol 5-phosphate
P66767	S-adenosylmethionine synthase	S-adenosylmethionine biosynthetic process. Catalysis of the formation of S-adenosylmethionine
Q7A807	PTS system glucose-specific EIICBA component	The phosphoenolpyruvate-dependent sugar phosphotransferase system. Catalysis of the phosphorylation of incoming sugar substrates
A0A0H3JP54	Tagatose-6-phosphate kinase	D-tagatose 6-phosphate catabolic process. Lactose metabolic process. 1-phosphofructokinase activity
Q7A699	Probable quinol oxidase subunit 1	Oxidative phosphorylation pathway. Catalyzes quinol oxidation
P68779	Adenine phosphoribosyltransferase	Purine ribonucleoside salvage. Catalyzes a reaction that forms AMP
P65936	Uridylate kinase	CTP biosynthesis via de novo pathway
P99176	Guanylate kinase	Essential for recycling GMP
A0A0H3JNH8	Alpha-acetolactate decarboxylase	Acetoin biosynthetic process. Acetolactate decarboxylase activity
A0A0H3JL28	Aldehyde-alcohol dehydrogenase	Acetaldehyde and alcohol dehydrogenase activities in alcohol metabolism
P99119	L-lactate dehydrogenase 2 (L-LDH 2)	Carbohydrate metabolism. Catalyzes the conversion of lactate to pyruvate
P99174	Deoxyribose-phosphate aldolase 2	Deoxyribose phosphate catabolic process. Catalysis of an aldol reaction between acetaldehyde and D-glyceraldehyde 3-phosphate

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Table 3.33. (cont.)

A0A0H3JSP4	SA0255 protein	Phosphoenolpyruvate-dependent sugar phosphotransferase system. Kinase activity
A0A0H3JMN5	Glucose-6-phosphate 1-dehydrogenase	Pentose-phosphate shunt. Catalysis of the oxidation of glucose 6-phosphate to 6-phosphogluconolactone
P99153	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	Gluconeogenesis. Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate
P99093	3-oxoacyl-[acyl-carrier-protein] reductase FabG	Catalysis of the reaction required for the elongation cycle of fatty acid biosynthesis
Q7A557	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	Fatty acid biosynthetic process. Catalyzes the carboxylation of biotin on its carrier protein
P65739	Phosphate acyltransferase	Fatty acid biosynthetic process. Phospholipid biosynthetic process
P99148	Aconitate hydratase A	TCA. Catalyzes the isomerization of citrate to isocitrate
A0A0H3JMN4	SA2200 protein	Amino acid-transporting ATPase activity
Q7A5L8	L-threonine dehydratase	Catalyzes the anaerobic formation of alpha-ketobutyrate and ammonia
P64218	Probable glycine dehydrogenase subunit 1	The glycine cleavage system. Required for degradation of glycine
P63554	Arginine deiminase	Arginine catabolic process to ornithine
Q7A627	Carbamate kinase 1	Arginine metabolic process
A0A0H3JQQ7	Aminopeptidase ampS	Aminopeptidase activity
P99172	Isopentenyl-diphosphate delta-isomerase	Isoprenoid biosynthetic process
P60120	Putative pyridoxal phosphate-dependent acyltransferase	Biosynthetic process. Pyridoxal phosphate binding. Transferase activity
P65656	3-methyl-2-oxobutanoate hydroxymethyltransferase	Pantothenate biosynthetic process. Catalyzes the transfer of hydroxymethyl

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Table 3.33. (cont.)

P65659	Pantothenate synthetase	Pantothenate biosynthetic process
A0A0H3JQN7	Hit-like protein involved in cell-cycle regulation	Catalytic activity
A0A0H3JNU5	SA2416 protein	ATPase activity. ATP binding
A0A0H3JP40	Cytokinin riboside 5'-monophosphate phosphoribohydrolase	Cytokinin biosynthetic process. Hydrolase activity
Q7A5P6	Uncharacterized hydrolase SA1230	Hydrolase activity
A0A0H3JLB1	Uncharacterized protein	Hydrolase activity
Q7A3E8	Putative acetyltransferase SA2342	Acetyltransferase activity
A0A0H3JQG2	SA1548 protein	Transferase activity
A0A0H3JNB0	Probable ss-1,3-N-acetylglucosaminyltransferase	Transferase activity, transferring glycosyl groups
P67420	Uroporphyrinogen decarboxylase (UPD)	Protoporphyrinogen IX synthesis. Decarboxylation of acetate groups
P99096	Glutamate-1-semialdehyde 2,1-aminomutase 1	Protoporphyrinogen IX synthesis. Glutamate-1-semialdehyde 2,1-aminomutase
A0A0H3JUQ8	Protoporphyrinogen oxidase	Catalyzes 6-electron oxidation of protoporphyrinogen-IX
P64334	Delta-aminolevulinic acid dehydratase	Protoporphyrin IX biosynthesis. Biosynthesis of tetrapyrroles
Q7A428	Urease accessory protein UreF	Nitrogen compound metabolic process. Required for maturation of urease
P67404	Urease subunit alpha	Urea degradation
A0A0H3JNG6	Uncharacterized protein	Siderophore biosynthetic process
A0A0H3JNJ9	Probable molybdate-binding protein	Molybdate ion transport
A0A0H3JM26	Uncharacterized protein	Iron-sulfur cluster assembly
Q7A6B1	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase	Menaquinone biosynthetic process. Catalyzes the decarboxylation of 2-oxoglutarate

Some of the proteins identified in control (Table 3.33) but not identified in 2-hydroxycinnamic acid treated MRSA implied the defects in iron metabolism. Proteins such as uroporphyrinogen decarboxylase, glutamate-1-semialdehyde 2,1-aminomutase 1, protoporphyrinogen oxidase and delta-aminolevulinic acid dehydratase had functions in the formation of protoporphyrin IX (Sachar et al., 2016), a final intermediate in the synthesis of heme, were not identified in 2-HCA treated MRSA.

Identification of uncharacterized proteins (Table 3.33) that functioned in siderophore biosynthetic process and iron-sulfur cluster assembly in control might indicate the lack of iron uptake in 2-HCA treated MRSA, because iron uptake mediated by siderophores. Since presence of iron induces the OH radicals that have negative effects on cellular processes (Chang et al., 2006), reduction of iron uptake in 2-hydroxycinnamic acid treated cells might be required to prevent generation of more oxidative damage.

Consistently with the differences observed in iron metabolism of control MRSA, ferrodoxin and SA0797 proteins were identified in 2-hydroxycinnamic acid treated MRSA (Table 3.34). These two proteins were responsible for iron ion binding. Their increased levels might be required to decrease the free iron levels under the phenolic acid stress to protect cells from oxidative stress.

One of the most striking response proteins was GTP pyrophosphokinase (ppGpp synthase 1). This protein was the only protein in all differently identified proteins that responsible for the stringent response in bacteria. It mediates the several cellular activities in response to changes in nutriments in the environment (Traxler et al., 2008). Therefore, its identification in 2-HCA treated MRSA (Table 3.34) might indicate the less access of bacteria to the nutrients or inability of bacteria to utilize the present nutrients within the media.

Identification of proteins having roles in gluconeogenesis might indicate the glucose need of cells to produce energy or metabolites. The requirement of glucose might be due to need of ATP production within the cell. In the presence of glucose, cells might produce ATP and also NADH to be used in other metabolic pathways. Differences in carbohydrate metabolism as response to 2-HCA treatment might indicate the shift of cells between metabolic pathways to use and to produce required macromolecules to stay alive.

Table 3.34. Proteins related with several metabolic processes identified in 2-HCA treated MRSA but not in control.

Protein ID	Protein Name	Function
Q99TL8	GTP pyrophosphokinase (ppGpp synthase I)	Guanosine tetraphosphate (ppGpp) biosynthetic process. Mediator of the stringent response
Q7A584	GTPase Obg	Binds GTP, GDP and possibly (p)ppGpp with moderate affinity. Takes roles in control of the cell cycle, stress response, ribosome biogenesis
Q7A441	Molybdopterin synthase sulfur carrier subunit	Molybdopterin biosynthesis (Part of cofactor biosynthesis)
Q7A6T6	Putative lipid kinase SA0681	Phospholipid biosynthetic process. Catalysis of the phosphorylation of lipids other than diacylglycerol
Q7A3G4	PTS system glucoside-specific EIICBA component	The phosphoenolpyruvate-dependent sugar phosphotransferase system. Catalysis of the phosphorylation of incoming sugar substrates and their translocation
P63756	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	Phosphatidylglycerol biosynthetic process. Plays role in the synthesis of the acidic phospholipids
P60701	HPr kinase/phosphorylase	Carbohydrate metabolic process. Phosphorylation of a serine residue in HPr
P99128	Phosphoenolpyruvate carboxykinase	Gluconeogenesis. Catalyzes the conversion of oxaloacetate to phosphoenolpyruvate
A0A0H3JRW0	SA2318 protein	Gluconeogenesis. L-serine ammonia-lyase activity
A0A0H3JMA3	Pyruvate carboxylase	Gluconeogenesis. Catalyzes the carboxylation of the biotin and the transfer of the carboxyl group to pyruvate
P99167	Isocitrate dehydrogenase	TCA. Isocitrate dehydrogenase activity
A0A0H3JTV5	Succinate dehydrogenase iron-sulfur protein subunit	TCA. Electron transfer activity, iron-sulfur cluster binding
A0A0H3JMB8	Citrate synthase	Tricarboxylic acid cycle. Transferase activity

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Table 3.34. (cont.)

A0A0H3JRQ6	SA2244 protein	Aminopeptidase activity
Q99SN6	Probable succinyl-diaminopimelate desuccinylase	Lysine biosynthetic process via diaminopimelate. Diaminopimelate biosynthetic process
P60298	Ornithine aminotransferase 2	L-proline biosynthesis. Interconversion of ornithine to glutamate semialdehyde
P63740	Carbamoyl-phosphate synthase large chain	Arginine biosynthetic process. de novo' UMP biosynthetic process
P99076	1-pyrroline-5-carboxylate dehydrogenase	Proline catabolic process to glutamate.
A0A0H3JNT1	Glycine betaine aldehyde dehydrogenase gbsA	Glycine betaine biosynthetic process from choline. Carotenoid biosynthesis
P99168	Probable glycine dehydrogenase subunit 2	The glycine cleavage system catalyzes the degradation of glycine
A0A0H3JQH3	SA1566 protein	Aminopeptidase activity. Metal ion binding
A0A0H3JVA7	Peptidase M20 domain-containing protein 2	Metabolic process. Hydrolase activity
P60798	Pyridoxal 5'-phosphate synthase subunit PdxS	Pyridoxal phosphate biosynthetic process. Pyridoxal 5'-phosphate synthase activity
A0A0H3JK03	Mevalonate kinase	Isoprenoid biosynthetic process
Q7A427	Urease accessory protein UreG	Facilitates the incorporation of the urease nickel metallocenter
A0A0H3JMM0	Respiratory nitrate reductase alpha chain	Nitrate metabolic process. Nitrate reductase activity
A0A0H3JNR2	Uncharacterized protein	Kinase activity
Q99UT4	Uncharacterized N-acetyltransferase SA1019	Transferase activity. Transferring acyl groups
A0A0H3JNN5	SA2327 protein	Thiamine pyrophosphate binding
P99140	Mannitol-1-phosphate 5-dehydrogenase	Mannitol metabolic process
A0A0H3JQE7	SA1524 protein	Malate dehydrogenase (NAD+) activity

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Table 3.34. (cont.)

A0A0H3JTR1	SA0895 protein	Isochorismate synthase activity
A0A0H3JL68	SA0670 protein	Metabolic process. Catalytic activity
A0A0H3JS27	Sulfite reductase [NADPH] flavoprotein alpha-component	Hydrogen sulfide biosynthetic process. Cysteine biosynthetic process. Catalyzes the 6-electron reduction of sulfite to sulfide
Q7A782	FMN-dependent NADPH-azoreductase	Catalysis of the cleavage of azo bond in aromatic azo compounds
A0A0H3JU75	Ferrodoxin	Iron ion binding. Electron transfer activity
A0A0H3JM38	SA0797 protein	Iron-sulfur cluster assembly. Iron ion binding
A0A0H3JNW5	Uncharacterized protein	Hydrolase activity
A0A0H3JLV1	Uncharacterized protein	Hydrolase activity
A0A0H3JRV2	Uncharacterized protein	Hydrolase activity
Q7A3C4	Uncharacterized hydrolase SA2367	Hydrolase activity
A0A0H3JRT1	SA2278 protein	Hydrolase activity

3.3.3.2.6. Action of 2-Hydroxycinnamic Acid on Cell Homeostasis

Proteins related with the cell homeostasis and redox also displayed differences in control and 2-HCA treated MRSA. However, these differences were mostly for alterations of proteins having similar functions within the cell. According to identified proteins in control and 2-HCA treated MRSA, while some proteins with oxidoreductase activities were only identified in the control (Table 3.35), some of them were only identified in 2-HCA stressed MRSA (Table 3.36).

Similar to presence of VA, catalase protein was also identified in MRSA grown in the presence of 2-hydroxycinnamic acid (Table 3.36). Identification of this protein was consistent with the literature indicating the upregulation of stres response proteins under stress conditions (Blair et al., 2009). Alongside of the catalase, identification of glutathione peroxidase, peroxide responsive repressor PerR and alkyl hydroperoxide

reductase AhpD might indicate the increased peroxidase activity. Their presence might be required to protect cells from detrimental effects of oxidative damage.

Table 3.35. Proteins related with cell homeostasis and redox identified in control but not in 2-HCA treated MRSA.

Protein ID	Protein Name	Function
A0A0H3JVE7	SA1989 protein	Oxidoreductase activity
A0A0H3JNK2	SA2080 protein	Oxidoreductase activity
Q7A3L9	Uncharacterized oxidoreductase SA2266	Oxidoreductase activity
P99101	Thioredoxin reductase	Removal of superoxide radicals

Table 3.36. Proteins related with cell homeostasis and redox identified in 2-HCA treated MRSA but not in control.

Protein ID	Protein Name	Function
Q7A5T2	Catalase	Protect the cells from hydrogen peroxide by decomposing it to water and oxygen
P99097	Glutathione peroxidase homolog BsaA	Response to oxidative stress. Glutathione peroxidase activity
Q7A4T8	Peroxide-responsive repressor PerR	Controls a regulon of oxidative stress resistance and iron-storage proteins
A0A0H3JMU4	Alkyl hydroperoxide reductase AhpD	Antioxidant protein with alkyl hydroperoxidase activity
A0A0H3JMB7	Succinate dehydrogenase flavoprotein subunit	Oxidoreductase activity
A0A0H3JKC7	Uncharacterized protein	Oxidoreductase activity
A0A0H3JNH3	SA2001 protein	Oxidoreductase activity
A0A0H3JTJ9	SA0791 protein	Oxidoreductase activity

3.3.3.2.7. Action of 2-Hydroxycinnamic Acid on Pathogenicity

When differently identified proteins related with the pathogenicity were examined, eight different proteins in control and five different proteins in 2-HCA treated MRSA could be seen in Table 3.37 and Table 3.38, respectively.

Table 3.37. Proteins related with pathogenicity identified in control but not in 2-HCA treated MRSA.

Protein ID	Protein Name	Function
P65330	S-ribosylhomocysteine lyase	Quorum sensing. Required for the synthesis of autoinducer 2
Q7A7R8	ESAT-6 secretion system extracellular protein B	Pathogenesis. Virulence factor that is essential for the infection in the host
Q99SU9	Staphylococcal complement inhibitor	Required to counter the first line of host defense. Inhibits phagocytosis and killing of *S.aureus* by human neutrophils
P67278	Ribonuclease Y (Conserved virulence factor A)	Pathogenesis. mRNA catabolic process. Endonuclease activity
Q7A514	HTH-type transcriptional regulator rot	Global regulator with both positive and negative effects that mediates the modulation of several genes involved in virulence
Q7A5Q1	Conserved virulence factor B	Contributes to the expression of virulence factors and the production of hemolysin, DNase, protease and protein A
Q7A4R9	Response regulator protein VraR	Response to antibiotic. Member of the two-component regulatory system VraS/VraR. Promotes expression of beta-lactam and glycopeptide resistance. Activator of the genes encoding beta-lactam-inducible penicillin-binding protein 2, UDP-N-acetylglucosamine enolpyruvyl transferase, monofunctional glycosyltransferase, methicillin resistance-related protein and teicoplanin resistance-related protein
A0A0H3JLY5	PBP2	Penicillin binding activity

Similar to vanillic acid treatment (Table 3.21), 2-HCA treatment did not allow the identification of proteins (Table 3.37) such as S-ribosylhomocysteine lyase, ESAT-6 secretion system extracellular protein B, Staphylococcal complement inhibitor, conserved virulence factors A and B, HTH-type transcriptional regulator rot, response regulator protein VraR and PBP2. These proteins had functions in quorum sensing, maintenance of infection, fighting against host defense system and response to antibiotics. Identification of these proteins only in the control might indicate the significant effect of 2-HCA on the virulence of pathogenic bacteria.

On the other hand, as seen from Table 3.38, treatment with 2-HCA did not diminish the virulence of MRSA. Pathogenicity related proteins clumping factor A, sensor protein SrrB, serine-aspartate repeat containing protein C, response regulator protein GraR and lipidII glycine glycyltransferase protein were identified as response to 2-HCA stress. Clumping factor A had roles in increasing the bacterial clump formation and adherence to human fibrinogens.

Table 3.38. Proteins related with pathogenicity identified in 2-HCA treated MRSA but not in control.

Protein ID	Protein Name	Function
Q99VJ4	Clumping factor A	Pathogenesis. Promotes bacterial attachment to human fibrinogens. Induces formation of bacterial clumps
Q7A781	Serine-aspartate repeat containing protein C	Cell adhesion. Mediates the interactions of *S.aureus* with the extracellular matrix components of higher eukaryotes
Q7A5H7	Sensor protein SrrB (Staphylococcal respiratory response protein B)	Member of the two-component regulatory system SrrA/SrrB. Global regulation of staphylococcal virulence factors in response to environmental oxygen levels
Q99VW2	Response regulator protein GraR	Pathogenesis. Response to antibiotic. Member of the two-component regulatory system GraR/GraS
Q7A447	Lipid II:glycine glycyltransferase (Factor essential for expression of methicillin resistance X)	Response to antibiotic. Responsible for the catalysis of the incorporation of the first glycine of the pentaglycine interpeptide bridge that is characteristic of the *S.aureus* peptidoglycan

Together with the serine-aspartate repeat containing protein C, identification of clumping factor A in 2-HCA treated cells implied the maintenance of bacterial clump formation and adhesion to the host cells conversely to decrease of S-ribosylhomocysteine lyase observed in control MRSA. One reason for this converse effect on the production of virulence related proteins might be the expression of the responsible genes under the direction of different regulatory regions within the cell. Moreover, identification of lipid II glycine glycyltransferase might support the alterations in the cell wall of MRSA in the presence of 2-HCA.

3.3.3.2.8. Action of 2-Hydroxycinnamic Acid on Other Proteins

The identified proteins of control and 2-HCA treated MRSA that were categorized under this group were mainly predicted proteins with uncharacterized functions and listed in Table 3.39 and Table 3.40, respectively.

Table 3.39. Other proteins identified in control but not in 2-HCA treated MRSA.

Protein ID	Protein Name	Function
A0A0H3JK15	Uncharacterized protein	Predicted protein
A0A0H3JPD8	Uncharacterized protein	Predicted protein
A0A0H3JUM1	Uncharacterized protein	Predicted protein
A0A0H3JVC0	Uncharacterized protein	Predicted protein
A0A0H3JNW8	SA0522 protein	Predicted protein
A0A0H3JSJ2	SA0165 protein	Predicted protein
A0A0H3JMZ4	SA2317 protein	Predicted protein
A0A0H3JU44	SA1243 protein	Predicted protein
A0A0H3JRD4	Hydroxamate siderophore binding lipoprotein	Predicted protein
P60359	UPF0297 protein SA1445	Experimental evidence at protein level
Q7A4P4	Uncharacterized protein SA1737	Belongs to the cycloisomerase 2 family
Q7A3Q5	Protein flp	Its precise function is unknown

Table 3.40. Other proteins identified in 2-HCA treated MRSA but not in control.

Protein ID	Protein Name	Function
A0A0H3JNQ3	Uncharacterized protein	Predicted protein
A0A0H3JLV6	Uncharacterized protein	Predicted protein
A0A0H3JLE7	Uncharacterized protein	Predicted protein
A0A0H3JNZ4	Uncharacterized protein	Predicted protein
A0A0H3JPK0	Uncharacterized protein	Predicted protein
A0A0H3JL43	Uncharacterized protein	Predicted protein
A0A0H3JMX1	Uncharacterized protein	Predicted protein
A0A0H3JU52	Uncharacterized protein	Predicted protein
A0A0H3JLQ0	Uncharacterized protein	Predicted protein
A0A0H3JT54	Uncharacterized protein	Predicted protein
A0A0H3JL46	Uncharacterized protein	Predicted protein
A0A0H3JM08	Uncharacterized protein	Predicted protein
A0A0H3JP11	Uncharacterized protein	Predicted protein
A0A0H3JKU5	Uncharacterized protein	Predicted protein
A0A0H3JL06	SA0569 protein	Predicted protein
Q99UZ6	UPF0637 protein SA0957	Predicted protein
A0A0H3JP65	SA0672 protein	Predicted protein
Q7A593	UPF0337 protein SA1452	Belongs to the UPF0337 family
Q7A4T2	UPF0374 protein SA1684	Belongs to the UPF0374 family
Q7A5M8	UPF0346 protein SA1254	Belongs to the UPF0346 family
Q7A339	UPF0312 protein SA2479	Belongs to the UPF0312 family
Q7A598	UPF0473 protein SA1443	Belongs to the UPF0473 family
Q7A5S4	Uncharacterized protein SA1186	Belongs to the HesB/IscA family
A0A0H3JL51	SA0646 protein	Protein-cromophore linkage

To summarize the action of 2-hydroxycinnamic acid on MRSA, the percentages of proteins belonging to each functional category were displayed in Figure 3.9 for the proteins that identified only in control and only in 2-HCA treated MRSA. Many proteins identified only in the control and only in the 2-HCA treated MRSA belonged to metabolism related proteins as obtained in the VA treated MRSA group (Figure 3.7). When metabolism related of control group and 2-HCA treated MRSA were compared similar percentages of identified proteins could be seen: 38% of identified control proteins and 39% of identified 2-HCA treated MRSA proteins. The lowest percentages of proteins belonging to functional categories were cell homeostasis for control with 3% and pathogenicity for 2-HCA treated MRSA with 5%. Interestingly, a considerable percentage of the identified proteins that categorized under other proteins were obtained for both control (10%) and 2-HCA treated MRSA (22%). This might display the incontrovertible effect of those proteins in the action mechanism of 2-hydroxycinnamic acid.

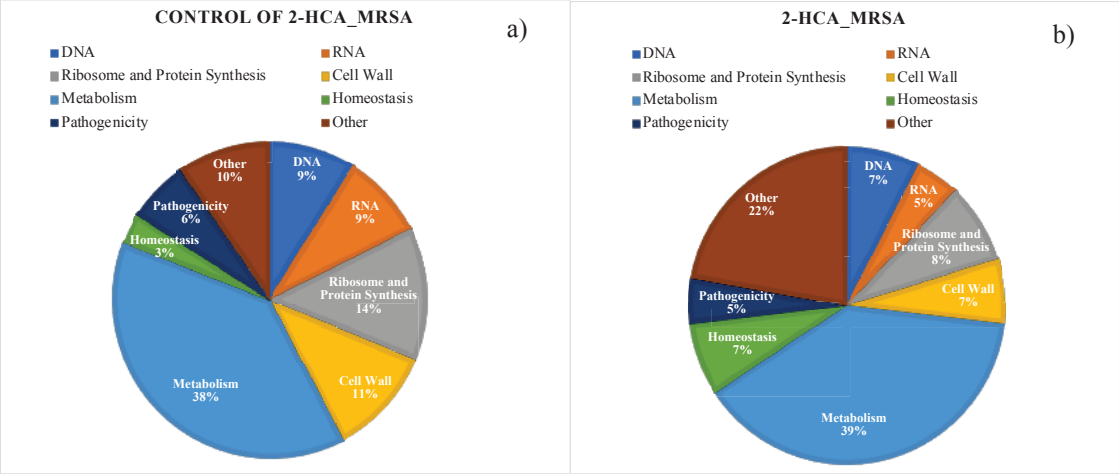

Figure 3.9. The percentages of the identified proteins for each functional category (a) only in control MRSA and (b) only in 2-HCA treated MRSA.

Different from the percentages of proteins related with pathogenicity in VA treated MRSA, the percentages of identified pathogenicity proteins in control and 2-HCA treated MRSA were similar.

3.3.3.3. Comparison of MRSA Proteomes under Vanillic Acid and 2-Hydroxycinnamic Acid Stresses

For comparison of vanillic acid and 2-hydroxycinnamic acid effect on the proteome, MRSA proteins identified in the presence and absence of each phenolic acid were examined (Figure 3.10). The Venn diagram shown in Figure 3.10 was drawn by using a web tool named as 'Bioinformatics and Evolutionary Genomics'. Database search improved presence of differently expressed proteomes in VA and 2-HCA treated MRSA samples.

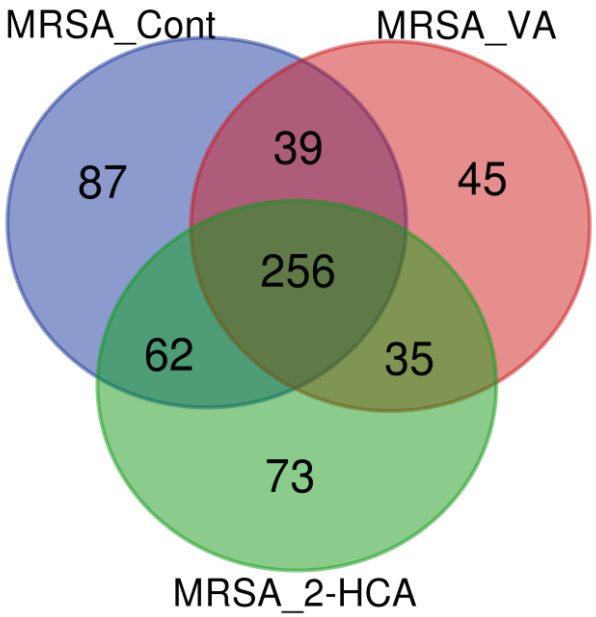

Figure 3.10. Numbers of identified proteins under control conditions (MRSA_Cont), in the presence of 2-hydroxycinnamic acid (MRSA_2-HCA) and in the presence of vanillic acid (MRSA_VA).

When FDR was kept below 1%, 45 proteins were identified as expressed only in VA treated MRSA, 73 proteins were identified as expressed only in 2-HCA treated MRSA. The number of the proteins that identified in only control but not in VA-treated or 2-HCA-treated MRSA was 87. On the other hand, 256 proteins were mutual in

control, VA treated and 2-HCA treated MRSA (Mutually expressed proteins under all conditions were listed in Appendix C). When identified proteins were examined from the point of phenolic acid stress, 35 mutually expressed proteins were seen. It could also be seen that 39 of the proteins that could not been identified in 2-HCA stress were common for VA treated and control MRSA; and 62 of the proteins that could not been identified in VA stress were common for 2-HCA treated and control MRSA.

For detailed comparison of VA and 2-HCA responses, 87 proteins that were identified only in control but not in phenolic acid treated MRSA and 35 proteins that were mutually expressed in VA and 2-HCA treated cells were categorized and discussed. Proteins that could not be identified in both phenolic acid stress but identified in control cells could be assumed as disappeared proteins or highly downregulated proteins in response to both phenolic acid stresses (Tables 3.41 to 3.47).

3.3.3.3.1. Unidentified DNA Related Proteins of Phenolic Acid Treated MRSA

The DNA related proteins that were not identified in vanillic acid treated and 2-hydroxycinnamic acid treated MRSA were listed in Table 3.41. The proteins listed in this category implied the effect of both phenolic acids on inhibition of DNA precursors, DNA replication and DNA repair enzymes.

Identification of DNA repair proteins such as ATP-dependent helicase/nuclease subunit A and nuclease SbcCD subunit C in control but not in phenolic acid stressed bacteria might suggest the increase of error-prone DNA synthesis in the presence of both VA and 2-HCA, because lack of these proteins indicates the DNA repair without homologous recombination during replication.

The lack of nucleic acid binding proteins or the proteins such as ribose-phosphate pyrophosphokinase and adenylosuccinate synthetase that function in nucleotide biosynthesis might suggest the problems in the formation and stability of DNA precursors.

Moreover, unidentifiability of dihydrofolate reductase protein in MRSA treated with vanillic acid and 2-hydroxycinnamic acid might confirm the problems in the synthesis of DNA precursors. Identification of 3’-5’exonuclease DinG in control but not in phenolic acid treated MRSA might support the idea of defective DNA replication.

Table 3.41. DNA related proteins that were identified only in control but were not identified in the presence of both phenolic acid stresses.

Protein ID	Protein Name	Function
A0A0H3JML1	Cmp-binding-factor 1	Nucleic acid binding
A0A0H3JUF7	Uncharacterized protein	DNA binding
A0A0H3JM64	Purine nucleoside phosphorylase DeoD-type	Purine-nucleoside phosphorylase activity
P99079	Dihydrofolate reductase	Catalysis of a reaction for glycine and purine synthesis, and for DNA precursor synthesis
P99099	Adenylosuccinate synthetase	Purine nucleotide biosynthesis
P65237	Ribose-phosphate pyrophosphokinase	Nucleotide biosynthetic process
Q7A5K4	3'-5' exonuclease DinG	DNA replication. 3'-5' exonuclease
Q7A6H4	ATP-dependent helicase/nuclease subunit A	Double-strand break repair. Functions in the initiation of homologous recombination
Q7A5S6	Nuclease SbcCD subunit C	DNA repair, recombination, replication. Cleavage of DNA hairpin structures

3.3.3.3.2. Unidentified RNA Related Proteins of Phenolic Acid Treated MRSA

The effects of both phenolic acids on RNA processing and transcription could be seen in Table 3.42. One of the most striking differences was DEAD-box ATP dependent RNA helicase CshB. This highly conserved protein among all organisms had functions in RNA metabolism (Linder and Jankowsky, 2011). Its absence might represent the strategy of bacteria for conservation of the energy. DNA directed subunit omega and RNA polymerase sigma factor SigA were not identified in both phenolic acid stresses which might cause delay in the initiation of transcription considering their hampering effect on RNA polymerase assembly and attachment to DNA.

Table 3.42. RNA related proteins that were identified only in control but were not identified in the presence of both phenolic acid stresses.

Protein ID	Protein Name	Function
P60392	Ribosomal RNA small subunit methyltransferase H	rRNA processing. Methylation of the N4 position of cytidine in position 1402 of 16S rRNA
P0A0N7	Ribosomal RNA large subunit methyltransferase H	rRNA processing. Methylation of the pseudouridine at position 1915 in 23S rRNA
A0A0H3JM44	DEAD-box ATP-dependent RNA helicase CshB	RNA catabolic process. Probable DEAD-box RNA helicase
P66726	DNA-directed RNA polymerase subunit omega	Promotes RNA polymerase assembly
Q99TT5	RNA polymerase sigma factor SigA	Transcription. Promotes the attachment of RNA polymerase to specific initiation sites
P99156	Transcription elongation factor GreA	Necessary for efficient RNA polymerase transcription elongation past template-encoded arresting sites.
A0A0H3JNV1	SA2421 protein	DNA-binding transcription factor activity
P65578	Transcription antitermination protein NusB	Functions in antitermination of transcription by binding specifically to the boxA antiterminator sequence of the ribosomal RNA operons
A0A0H3JTG9	Cold-shock protein C	Regulation of transcription

3.3.3.3.3. Unidentified Ribosome and Protein Synthesis Related Proteins of Phenolic Acid Treated MRSA

Among the ribosome and protein synthesis related proteins, 10 of them were identified in the presence of neither VA nor 2- HCA (Table 3.43). Lack of these proteins was probably related with the impaired transcription of many genes under the stress conditions and the economical use of possessed energy. Moreover,

unidentifiability of chaperones and transporters might indicate the increase of misfolded, unfolded and mislocated proteins within the cell. Both phenolic acids hindered the aminoacylation of leucine and tyrosine that were required to initiate protein translation. The absence of these proteins might be related with the effect of phenolic acids on the contact regions of amino acids and tRNAs.

Table 3.43. Ribosome and proteins synthesis related proteins that were identified only in control but were not identified in the presence of both phenolic acid stresses.

Protein ID	Protein Name	Function
A0A0H3JKS3	Ribosome-binding ATPase YchF	Binds to both the 70S ribosome and the 50S ribosomal subunit
P66153	50S ribosomal protein L28	Structural constituent of ribosome
P67513	Leucine--tRNA ligase	Leucyl-tRNA aminoacylation for protein translation
Q7A537	Tyrosine--tRNA ligase	Tyrosyl-tRNA aminoacylation for protein translation
P99077	Peptide deformylase	Functions in protein biosynthesis by removing the formyl group from the N-terminal Met of newly synthesized proteins
P60748	Foldase protein PrsA	Functions in protein secretion by helping the post-translocational folding of secreted proteins
P99082	33 kDa chaperonin	Prevents aggregation of thermally unfolding and oxidatively damaged proteins
A0A0H3JTY9	Signal recognition particle protein	Responsible for targeting and insertion of nascent membrane proteins into the cytoplasmic membrane
Q7A468	Protein translocase subunit SecY	Protein transport. The central subunit of the protein translocation channel SecYEG
Q7A6Q1	Probable protein-export membrane protein SecG	Involved in protein translocation

3.3.3.3.4. Unidentified Cell Wall Related Proteins of Phenolic Acid Treated MRSA

According to the proteins listed in Table 3.44, treatment of bacteria with VA or 2-HCA did not allow the identification of some of the cell wall related proteins. Lack of proteins such as bifunctional protein GlmU and UDP-N-acetylenolpyruvoylglucosamine reductase might indicate the disruptions and alterations on the peptidoglycan structure. Unidentifiability of ATP synthase gamma chain and epsilon chain in the presence of the phenolic acid stress confirmed the loss of membrane potential probably due to disruptions on the membrane. According to the literature, phenolic acids cause leak of the ions from the cell and disruption of the proton gradient across the cell membrane (Borges et al., 2013). Our findings were consistent with the literature and also with the findings in metabolism related proteins indicating energy depletion within the cell.

Table 3.44. Cell wall and membrane related proteins that were identified only in control but were not identified in both phenolic acid stresses.

Protein ID	Protein Name	Function
Q7A7B4	Bifunctional protein GlmU	Cell wall organization
P65463	UDP-N-acetylenolpyruvoyl glucosamine reductase	Cell wall formation. Peptidoglycan biosynthesis
A0A0H3JNG7	Conserved hypothetical protein	Integral component of membrane
A0A0H3JNS3	Uncharacterized protein	Integral component of membrane
A0A0H3JLS9	Uncharacterized protein	Integral component of membrane
A0A0H3JM97	Uncharacterized protein	Integral component of membrane
P67291	UPF0154 protein SA1178	Integral component of membrane
A0A0H3JND7	SA0168 protein	Transmembrane transport
P63665	ATP synthase epsilon chain	Production of ATP
Q7A4E8	ATP synthase gamma chain	Production of ATP

3.3.3.3.5. Unidentified Metabolism Related Proteins of Phenolic Acid Treated MRSA

When metabolism related proteins of the control and the phenolic acid treated MRSA were compared, unidentifiability of tagatose 6-phosphate kinase in the presence of both phenolic acids (Table 3.45) might indicate the differences in carbon source preferences of bacteria under stress conditions. This loss might indicate the lack of ability of bacteria to use galactose and lactose as carbon sources (Kuroda et al., 2001). Differences in metabolic pathways under stress conditions were also observed for other carbohydrate metabolic pathways. Lack of enzymes such as ribose-5-phosphate isomerase A, ribulose-5-phosphate reductase, PTS system glucose specific EIICBA component, deoxyribose phosphate aldolase and SA0255 protein indicated the different metabolism of sugars in the presence of phenolic acids.

Moreover, the control specific proteins such as acetyl coenzyme A carboxylase carboxyl transferase subunit beta, phosphate acyltransferase and aconitate hydratase A were the enzymes of fatty acid metabolism. Their lack might support the alterations in the cell membrane. According to the proteins listed in Table 3.45, both VA and 2-HCA prevented the expression of some proteins playing roles in iron metabolism and nitrogen metabolism. However, since the used concentrations of phenolic acids were not bactericidal, bacteria responded with the formation of different proteins for maintenance of metabolic reactions.

3.3.3.3.6. Unidentified Cell Homeostasis Related Proteins of Phenolic Acid Treated MRSA

Presence of either VA or 2-HCA did not hinder the identification of a great number of proteins related with the cell homeostasis. Only two proteins were specific to control MRSA which were SA2080 protein (Protein ID: A0A0H3JNK2) and cold shock protein CspA (Protein ID: Q7A5P3). SA2080 protein is responsible for displaying oxidoreductase activities. It was reasonable because cells grown in the presence of several inhibitors are usually tended to increase the proteins responsible for oxidative stress response and maintenance of homeostasis (Blair et al., 2009). Cold shock protein CspA was involved in the stress response.

Table 3.45. Proteins related with several metabolic processes that were identified only in control but were not identified in the presence of both phenolic acid stresses.

Protein ID	Protein Name	Function
P66695	Ribose-5-phosphate isomerase A	Pentose-phosphate shunt. Catalysis of the conversion of ribose-5-phosphate to ribulose 5-phosphate
A0A0H3JNI1	Ribulose-5-phosphate reductase	Polyteichoic acid biosynthesis. Catalysis of the reduction of D-ribulose 5-phosphate to D-ribitol 5-phosphate
Q7A807	PTS system glucose-specific EIICBA component	The phosphoenolpyruvate-dependent sugar phosphotransferase system. Catalysis of the phosphorylation of incoming sugar substrates and their translocation
A0A0H3JP54	Tagatose-6-phosphate kinase	D-tagatose 6-phosphate catabolic process. Lactose metabolic process.
P65936	Uridylate kinase	CTP biosynthesis via de novo pathway. Part of pyrimidine metabolism
P99176	Guanylate kinase	Essential for recycling GMP and cGMP
A0A0H3JNH8	Alpha-acetolactate decarboxylase	Acetoin biosynthetic process. Acetolactate decarboxylase activity
P99174	Deoxyribose-phosphate aldolase 2	Catalysis of an aldol reaction between acetaldehyde and D-glyceraldehyde 3-phosphate
A0A0H3JSP4	SA0255 protein	Phosphoenolpyruvate-dependent sugar phosphotransferase system
Q7A557	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	Fatty acid and malonyl-CoA biosynthesis. Carboxylation of biotin
P65739	Phosphate acyltransferase	Fatty acid biosynthetic process. Phospholipid biosynthetic process
P99148	Aconitate hydratase A	TCA. Catalysis of the isomerization of citrate to isocitrate
P64218	Probable glycine dehydrogenase subunit 1	The glycine cleavage system catalyzes the degradation of glycine

(cont. on next page)

Table 3.45. (cont.)

A0A0H3JQQ7	Aminopeptidase ampS	Aminopeptidase activity
P99172	Isopentenyl-diphosphate delta-isomerase	Catalysis of the arrangement of the homoallylic substrate isopentenyl to dimethylallyl diphosphate
P60120	Putative pyridoxal phosphate-dependent acyltransferase	Biosynthetic process. Pyridoxal phosphate binding
P65659	Pantothenate synthetase (PS)	Catalysis of the condensation of pantoate
A0A0H3JNU5	SA2416 protein	ATPase activity. ATP binding
A0A0H3JP40	Cytokinin riboside 5'-monophosphate phosphoribohydrolase	Cytokinin biosynthetic process. Hydrolase activity
Q7A5P6	Uncharacterized hydrolase SA1230	Hydrolase activity
A0A0H3JLB1	Uncharacterized protein	Hydrolase activity
Q7A3E8	Putative acetyltransferase SA2342	Acetyltransferase activity
A0A0H3JNB0	Probable ss-1,3-N-acetylglucosaminyltransferase	Transfer of glycosyl groups
A0A0H3JUQ8	Protoporphyrinogen oxidase	Protoporphyrin-IX biosynthesis. Catalysis of the oxidation of protoporphyrinogen-IX to form protoporphyrin-IX
P64334	Delta-aminolevulinic acid dehydratase	Protoporphyrin IX biosynthesis. Catalysis of a reaction in tetrapyrrole biosynthesis
Q7A428	Urease accessory protein UreF	Nitrogen metabolism. Functions in maturation of urease by incorporating urease nickel metallocenter
P67404	Urease subunit alpha	Urea degradation
A0A0H3JNG6	Uncharacterized protein	Siderophore biosynthetic process
Q7A6B1	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase	Menaquinone biosynthetic process. Catalysis of the decarboxylation of 2-oxoglutarate

3.3.3.3.7. Unidentified Pathogenicity Related Proteins of Phenolic Acid Treated MRSA

The unidentifiability of the proteins related with pathogenicity of MRSA was one of the most significant outcomes obtained in proteomic part of this study. Since *S. aureus* was normally a commensal microorganism, even impaired virulence might help to prevent diseases caused by antibiotic resistant bacteria. All of the pathogenicity related proteins identified in control MRSA were listed in Table 3.46.

Table 3.46. Proteins related with pathogenicity that were identified in control but were not identified in the presence of both phenolic acid stresses.

Protein ID	Protein Name	Function
P65330	S-ribosylhomocysteine lyase	Quorum sensing. Required for the synthesis of autoinducer 2
Q7A7R8	ESAT-6 secretion system extracellular protein B	Virulence factor that is important for the establishment of infection in the host
Q99SU9	Staphylococcal complement inhibitor	Required for the countering the first line of host defense. Inhibition of phagocytosis and killing of *S.aureus* by human neutrophils
P67278	Ribonuclease Y	Pathogenesis. mRNA catabolic process
Q7A514	HTH-type transcriptional regulator rot	Pathogenesis. Modulates the expression of several genes involved in virulence
Q7A5Q1	Conserved virulence factor B	Functions in the expression of virulence factors. Required for DNase, hemolysin, protease and protein A production
Q7A4R9	Response regulator protein VraR	Induces resistance to beta-lactam and glycopeptides. Activator of the genes encoding beta-lactam-inducible PBP2, UDP-N-acetylglucosamine enolpyruvyl transferase, monofunctional glycosyltransferase, methicillin and teicoplanin resistance-related proteins
A0A0H3JLY5	PBP2	Penicillin binding activity

In terms of inhibition of quorum sensing, both phenolic acids inhibited the expression of S-ribosylhomocysteine lyase. The decreased expression of this protein might affect the other pathogenicity related proteins due to less number of cells or impaired communication between the cells. Considering their functions, lack of ESAT-6 secretion system extracellular protein B and staphylococcal complement inhibitor might indicate the decreased ability of bacteria to cause infection on the host. Together with HTH-type transcriptional regulator rot, conserved virulence factor A and conserved virulence factor B were not identified in phenolic acid treated cells implying the direct effect of phenolic acids on the expression of virulence factors. Moreover, response regulator VraR was only identified in control MRSA. This protein had importance for promoting resistance to several antibiotics such as beta lactams and glycopeptides. The possible downregulation or disappearance of this protein might matter in terms of decreased resistance ability of MRSA against multiple drugs.

3.3.3.3.8. Unidentified Other Proteins of Phenolic Acid Treated MRSA

Among control MRSA specific proteins that were not identified in the presence of both phenolic acids, 9 of them were assigned as predicted proteins and listed in Table 3.47.

3.3.3.3.9. Phenolic Acid Responsive DNA Related Proteins

When Figure 3.8 was examined, identification of 35 proteins that were mutual for the presence of both vanillic acid and 2-HCA but not identified in control MRSA could be seen. These proteins might reflect the common response of MRSA to the presence of phenolic acids regardless of the type of the phenolic acid. Mutually identified DNA related proteins were shown in Table 3.48. According to findings, both phenolic acids induced the expression of thymidylate synthase protein that responsible for formation of DNA precursors. This might be an important response for bacteria to compensate the lack of several DNA precursors that were not identified in the phenolic acid stress. Interestingly, DNA gyrase subunit A was identified in the presence of both phenolic acid stress while subunit B was identified only in the presence of VA stress.

The function of DNA gyrase is the introduction of negative supercoils to adjust the DNA topology for replication, recombination and repair processes (Hawkey, 2003). Presence of gyrases in phenolic acid stressed MRSA but not in control MRSA might be the increased need for DNA recombination required to repair damaged DNA.

Table 3.47. Other proteins that were identified only in control but were not identified in the presence of both phenolic acid stresses.

Protein ID	Protein Name	Function
A0A0H3JPD8	Uncharacterized protein	Predicted protein
A0A0H3JUM1	Uncharacterized protein	Predicted protein
A0A0H3JVC0	Uncharacterized protein	Predicted protein
A0A0H3JNW8	SA0522 protein	Predicted protein
A0A0H3JSJ2	SA0165 protein	Predicted protein
A0A0H3JMZ4	SA2317 protein	Predicted protein
A0A0H3JU44	SA1243 protein	Predicted protein
A0A0H3JRD4	Hydroxamate siderophore binding lipoprotein	Protein predicted
Q7A3Q5	Protein flp	Its precise function is unknown

Table 3.48. DNA related proteins identified in the presence of phenolic acids but not in control MRSA.

Protein ID	Protein Name	Function
P67047	Thymidylate synthase (TS)	Catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate for DNA biosynthesis
Q99XG5	DNA gyrase subunit A	DNA topological change. Favors strand separation, DNA replication, transcription, recombination and repair

3.3.3.3.10. Phenolic Acid Responsive RNA Related Proteins

Two RNA related proteins were identified in MRSA as mutual phenolic acid response proteins and they were probable transcriptional regulatory protein SA0624 (Protein ID: P67182) and SA1329 protein (Protein ID: A0A0H3JMM9). The functions of these proteins were regulation of transcription and transcription factor activity, respectively. Their identification might indicate the requirement of reprogramming of transcription under the both phenolic acid stresses.

3.3.3.3.11. Phenolic Acid Responsive Ribosome and Protein Synthesis Related Proteins

There were not many mutually identified ribosome and protein synthesis related proteins as response to phenolic acid stress. The only identified protein under this category was SA2156 protein (Protein ID: A0A0H3JNQ8) that provided transport of lactate. Identification of this protein might support the differences in carbohydrate metabolism observed in the presence of phenolic acids and might be required to alter carbohydrate preferences of the MRSA.

3.3.3.3.12. Phenolic Acid Responsive Cell Wall Related Proteins

The proteins related with the cell wall and membranes identified as response to phenolic acids were SA1559 protein (Protein ID: A0A0H3JN07) and SA2103 protein (Protein ID: A0A0H3JNL8). These proteins were integral components of the membrane. Although both phenolic acids resulted in the alterations in the composition of the cell membrane, only a few identical response proteins were identified.

Identification of only two mutual proteins might suggest the different actions on the cell membrane through targeting different proteins or structures. This might oblige cells to respond to phenolic acids with different changes.

Different moieties of vanillic acid and 2-hydroxycinnamic acid might be the cause of different effect on the cell membrane.

3.3.3.3.13. Phenolic Acid Responsive Metabolism Related Proteins

When the identified phenolic acid stress response proteins that related with metabolic processes were examined, identification of proteins with roles in tricarboxylic acid cycle could be seen (Table 3.49).

The main function of TCA is to produce metabolic intermediates and reducing power for the cellular processes (Somerville and Proctor, 2009). Citrate synthase and isocitrate dehydrogenase were TCA enzymes that also contribute to glutamate forming pathway. Consistently, both phenolic acids induced the formation of glutamate in the amino acid metabolism. Identified proteins such as ornithine aminotransferase 2, 1-pyrrolline-5-carboxylate dehydrogenase were responsible for conversion of different amino acids to glutamate. The possible reason of this induction might be the function of glutamate. Since it functions as a donor for nitrogen for all biological molecules (Somerville and Prector, 2009) the cells under phenolic acid stress might try to increase the nitrogen metabolism to produce nitrogen containing essential molecules. Identification of the proteins responsible for iron ion binding might be due to prevent oxidative damage as mentioned earlier.

Moreover, identification of proteins responsible for catalyses of reactions with aminopeptidase activities (SA1566 protein and SA2244 protein) might suggest the alterations in the amino acid metabolism in the presence of phenolic acids.

3.3.3.3.14. Phenolic Acid Responsive Cell Homeostasis Related Proteins

The identical response of bacteria to the presence of both vanillic acid and 2-hydroxycinnamic acid was to increase catalase production (Table 3.50). Although *S. aureus* is catalase positive, the unidentifiability of this protein in the control could be the low levels of protein under normal conditions.

Identification FMN-dependent NADPH azoreductase and alkylhydroperoxide reductase AhpD might imply the increased oxidative stress in the phenolic acid treated cells. Identification of FMN-dependent NADPH-azoreductase might also suggest attend of cells to cleave azo bonds in the aromatic compounds.

Table 3.49. Proteins related with several metabolic processes identified in the presence of phenolic acids but not in control MRSA.

Protein ID	Protein Name	Function
Q7A584	GTPase Obg	Control of the cell cycle, stress response, ribosome biogenesis
A0A0H3JMB8	Citrate synthase	TCA. Transferase activity
P99167	Isocitrate dehydrogenase	TCA. Isocitrate dehydrogenase activity
A0A0H3JQE7	SA1524 protein	Malate dehydrogenase activity
Q7A427	Urease accessory protein UreG	Nitrogen metabolism. Incorporation of the urease nickel metallocenter
A0A0H3JQH3	SA1566 protein	Aminopeptidase activity
A0A0H3JRQ6	SA2244 protein	Aminopeptidase activity
P60298	Ornithine aminotransferase 2	Interconversion of ornithine to glutamate
A0A0H3JS27	Sulfite reductase flavoprotein alpha-component	Catalysis of the reduction of sulfite to sulfide
P99076	1-pyrroline-5-carboxylate dehydrogenase	Proline catabolic process to glutamate
A0A0H3JNT1	Glycine betaine aldehyde dehydrogenase gbsA	Betaine-aldehyde dehydrogenase activity
P63740	Carbamoyl-phosphate synthase large chain	L-arginine biosynthesis, UMP biosynthesis
Q7A3C4	Uncharacterized hydrolase SA2367	Hydrolase activity
A0A0H3JRT1	SA2278 protein	Hydrolase activity
A0A0H3JLV1	Uncharacterized protein	Hydrolase activity
Q99UT4	Uncharacterized N-acetyltransferase SA1019	Transferase activity
A0A0H3JNN5	SA2327 protein	Magnesium ion binding
A0A0H3JU75	Ferrodoxin	Iron ion binding
A0A0H3JM38	SA0797 protein	Iron-sulfur cluster assembly. Iron ion binding

Table 3.50. Proteins related with cell homeostasis and redox identified in the presence of phenolic acids but not in control MRSA.

Protein ID	Protein Name	Function
Q7A5T2	Catalase	Protection of cells from the toxic effects of H_2O_2 by decomposing H_2O_2 into H_2O and oxygen
Q7A782	FMN-dependent NADPH-azoreductase	Oxidoreductase activity. Cleaves the azo bond in the aromatic azo compounds
A0A0H3JMU4	Alkyl hydroperoxide reductase AhpD	Antioxidant protein with alkyl hydroperoxidase activity

3.3.3.3.15. Phenolic Acid Responsive Pathogenicity Related Proteins

When mutual response to phenolic acids was examined in terms of pathogenicity, it was observed that both vanillic acid and 2-hydroxycinnamic acid stresses allowed identification of sensor protein SrrB and clumping factor A (Table 3.51). Their presence might suggest that although the phenolic acids significantly decreased the levels of the proteins related with pathogenicity, their presence at tested concentrations were not enough to completely diminish bacterial virulence.

Table 3.51. Proteins related with pathogenicity identified in the presence of phenolic acids but not in control MRSA.

Protein ID	Protein Name	Function
Q7A5H7	Sensor protein SrrB	Member of two-component regulatory system SrrA/SrrB. Functions in regulation of virulence factors in response to oxygen levels
Q99VJ4	Clumping factor A (Fibrinogen receptor A)	Promotes attachment of bacteria to the human fibrinogen. Increases formation of bacterial clumps, and decreases phagocytosis by macrophages

3.3.3.3.16. Phenolic Acid Responsive Other Proteins

The predicted and uncharacterized proteins that were mutual for vanillic acid and 2-hydroxycinnamic acid stresses were listed in Table 3.52.

Table 3.52. Other proteins identified in the presence of phenolic acids but not in control.

Protein ID	Protein Name	Function
A0A0H3JLE7	Uncharacterized protein	Predicted protein
A0A0H3JM08	Uncharacterized protein	Predicted protein
Q7A593	UPF0337 protein SA1452	Belongs to UPF0337 protein family
Q99UZ6	UPF0637 protein SA0957	Belongs to the UPF0637 family

The percentages of the proteins from different functional categories that identified only in control MRSA (87 proteins) and in the presence of both phenolic acids (35 proteins) were displayed as a pie chart in Figure 3.11.

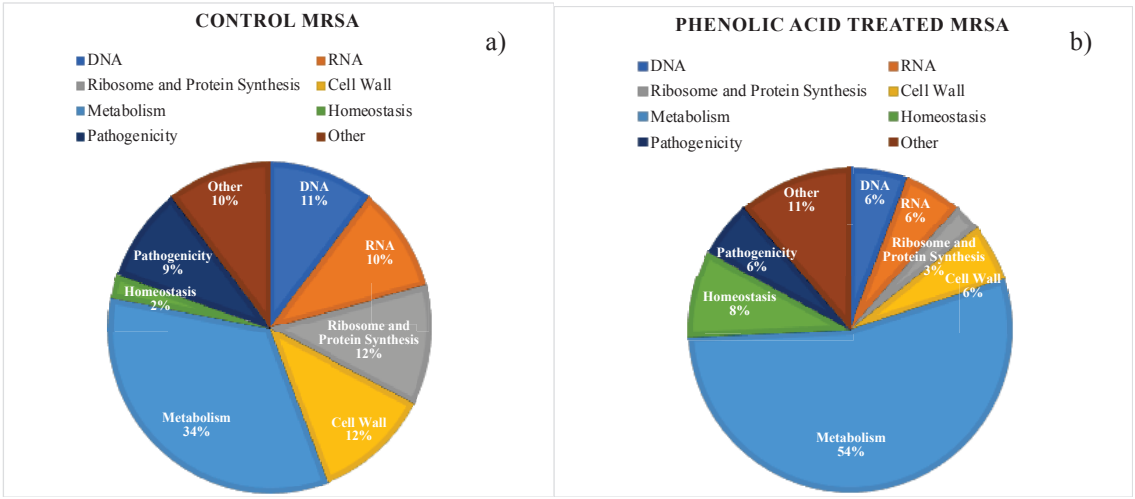

Figure 3.11. The percentages of the identified proteins for each functional category (a) only in control MRSA and (b) only in phenolic acid treated MRSA.

As expected, higher percentage of the proteins for the functional categories was observed in metabolism. The percentage of the metabolism related proteins identified as mutual for both phenolic acids was 54% that represented the more than half of the identified proteins.

Summary of what have been found in terms of possible inhibitory mechanisms of phenolic acids according to eight specified categories:

- Action on DNA: Phenolic acids affected the presence and/or amount of DNA gyrases and topoisomerases apart from the proteins taking roles in general DNA metabolism. Especially in vanillic acid treatment both topoisomerases were not identified indicating possible prevention of the expression of these proteins. This finding was important because there are currently used drugs targeting DNA topoisomerases to fight against pathogenic bacteria. Advantage of vanillic acid is the inability of bacteria to develop resistance upon continuous exposure to phenolic acids as proved in the previous chapter. On the other hand, main reason of inhibitory effect of 2-HCA on DNA might be induction of SOS response for damaged DNA. This error-prone DNA replication might be the cause of retarded growth of bacteria in the presence of subinhibitory 2-HCA concentrations.
- Action on RNA: Phenolic acids affected the RNA processing and transcription regulation. Prolonged or early termination of transcription of genes might affect the subsequent processes and inhibit the growth of bacteria as observed in MIC experiments.
- Action on ribosomes and protein synthesis: Phenolic acids affected the ribosome structure and assembly, also prevented the expression of proteins taking roles in protein synthesis. One of the reasons of downregulation in the expression of protein biosynthesis related proteins could be the matter of energy conservation in the stressful environments.
- Action on cell wall and membranes: Phenolic acids resulted in the changes in organization of the peptidoglycan structure indicating disruptions. Moreover, loss of membrane permeability and electrochemical across the cell membrane was proved by the defects in energy production through proton motive force.
- Action on metabolic pathways: Phenolic acids resulted in several alterations in the enzymes of almost all metabolic pathways. The most common tendency was to increase the amounts of reducing equivalents to be used in all metabolic reactions. Delayed growth of bacteria in the presence of subinhibitory concentrations of phenolic acids

might be due to alterations in the preferences or obligation of bacteria for different metabolic pathways.

- Action on cell homeostasis: Phenolic acids increased the formation of oxidative stress in the bacteria. According to the alterations in the iron metabolism, preference of metabolic pathways and production of many antioxidant proteins, bacteria tried to maintain cell homeostasis. However, increase of reactive oxygen species within the cells might help the inhibitory effects of phenolic acids on bacterial growth.
- Action on pathogenicity: Phenolic acids impaired the quorum sensing ability of bacteria and also decreased the expression of virulence factors and genes promoting antibiotic resistance. When compared with 2-HCA, vanillic acid was more effective in the impairing of expression of the virulence genes. An advantage of using phenolic acids may contribute to overall better community health.
- Action through the alterations in uncharacterized proteins: Although many identified proteins were uncharacterized, their probable effect on inhibition of bacteria might be incontrovertible.

3.4. Conclusion

According to the outcomes obtained from shotgun proteomic approach both phenolic acids affected the expression of proteins having functions in DNA synthesis and structure; RNA structure and transcription; ribosome stability, processing, and protein synthesis; cell wall associated proteins and cell membrane composition; carbohydrate, lipid, energy, iron, amino acid metabolisms; proteins maintain the cell homeostasis, pathogenicity of the organism and many uncharacterized proteins. Moreover, this technique allowed the comparison of the action mechanisms of a hydroxycinnamic acid and a hydroxybenzoic acid. Although 2D-PAGE technique did not allow the identification of many proteins as shotgun proteomics did, it allowed the comparison of MRSA and MSSA treated with the same phenolic acids. All the proteins identified in 2D-PAGE protein profiling, were also covered by shotgun proteomic technique.

Overall, use of proteomic approach provided the identification of the protein profiles of bacteria under specified physiological conditions. It allowed the identification of primary or secondary targets on the bacteria.

The significance of the information obtained from this study was the determination of the possible ways to combat with pathogenic bacteria that resistant to multiple drugs but not able to develop resistance against neither vanillic acid nor 2-hydroxycinnamic acid.

3.5. Future Aspects

Although this study remarked the some of the action mechanisms of vanillic acid and 2-hydroxycinnamic acid on two *S. aureus* strains and also showed the potential targets on one of the most notorious pathogens, further studies may be performed to confirm the obtained results. For confirmation of the proteins that identified only in the control and only in the presence of each phenolic acid, transcriptome studies may be carried out. Global transcript analysis by microarray technologies or next-generation sequencing may be helpful to understand the global response of bacteria to the presence of phenolic acids in the transcriptome levels which then may contribute to comparison of protein response with the transcript response. Moreover, for the confirmation of the changes on specifically chosen target proteins, such as virulence or DNA related proteins, RT-PCR technique may be used. After determination of the phenolic acid affected gene regions, specific mutants that defected on the gene of interest can be created to understand the direct effect of phenolic acids. However, the results obtained from this study preserve their scientific significance in terms of indicating the maintenance of phenolic acid susceptibility in MRSA and MSSA upon continuous exposure and the potential targets on one of the most notorious pathogens.

REFERENCES

Alves, M. J.; Ferreira, I. C F. R.; Froufe, H. J. C.; Abreu, R. M. V.; Martins, A.; Pintado, M. Antimicrobial Activity of Phenolic Compounds Identified in Wild Mushrooms, SAR Analysis and Docking Studies. *J. Appl. Microbiol*, **2013**, *115*(2), 346-357.

Akiyama, H.; Fujii, K.; Yamasaki, O.; Oono, T.; Iwatsuki, K. Antibacterial action of several tannins against *Staphylococcus aureus*. *J.Antimicrob. Chemother*. **2001**, *48*, 487-491.

Andjelkovic, M.; Vancamp, J.; Demeulenaer, B.; Depaemelaere, G.; Socaciu, C.; Verloo, M.; Verhe, R. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chem.* **2006**, *98*(1), 23-31.

Aslam, B.; Basit, M.; Nisar, M. A.; Khurshid, M.; Rasool, M. H. Proteomics: Technologies and Their Applications. *J.Chromatogr. Sci.* **2017**, *55*(2), 182-196.

Baggerman, G.; Vierstraete, E.; De Loof, A.; Schoofs, L. Gel-Based versus Gel-Free Proteomics: A Review. *Comb. Chem. High Throughput Screening*. **2005**, *8*, 669-677.

Bandow, J. E.; Brötz, H.; Leichert, L. I. O.; Labinschinski, H.; Hecker, M. Proteomic Approach to Understanding Antibiotic Action. *Antimicrob. Agents Chemother*. **2003**, *47*(3), 948-955.

Bioinformatics and Evolutionary Genetics Web Tool. http://bioinformatics.psb.ugent.be /webtools/Venn/ (accessed Oct 22, 2018).

Blair, S. E.; Cokcetin, N. N.; Harry, E. J.; Carter, D. A. The unusual antibacterial activity of medical-grade *Leptospermum* honey: antibacterial spectrum, resistance and transcriptome analysis. *Eur. J. Clin. Microbiol.Infect. Dis*. **2009**, *28*, 1199-1208.

Boisset, S.; Geissmann, T.; Huntzinger, E.; Fechter, P.; Bendiridi, N.; Possedko, M.; Chevalier, C.; Helfer, A. C.; Benito, Y.; Jacquier, A.; Gaspin, C.; Vandenesch, F.; Romby, P. *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcriptional regulator Rot by an antisense mechanism. *Genes Dev.* **2007**, *21*, 1353-1366.

Borges, A.; Ferreira, C.; Saavedra, M. J.; Simges, M. Antibacterial Activity and Mode of Action of Ferulic and Gallic Acids against Pathogenic Bacteria. *Microb. Drug Resist*. **2013**, *19*(4), 256-265.

Carrera, M.; Böhme, K.; Gallardo, J. M.; Barros-Velazquez, J.; Canas, B.; Calo-Mata, P. Characterization of Foodborne Strains of Staphylococcus aureus by Shotgun Proteomics: Functional Networks, Virulence Factors and Species-Specific Peptide Biomarkers. *Front. Microbiol*. **2017**, *8*, 2458.

Chandra, H.; Bishnoi, P.; Yadav, A.; Patni, B.; Mishra, A. P.; Nautiyal, A. R. Antimicrobial Resistance and the Alternative Resources with Special Emphasis on Plant-Based Antimicrobials-A Review. *Plants*. **2017**, *6*(16), 1-11.

Chang, W.; Small, D. A.; Toghrol, F.; Bentley, W. E. Global Transcriptome Analysis of *Staphylococcus aureus* Response to Hydrogen Peroxide. *J. Bacteriol*. **2006**, *188*(4), 1648-1659.

Cirz, R. T.; Jones, M. B.; Gingles, N.A.; Minogue, T. D.; Jarrahi, B.; Peterson, S. N.; Romesberg, F. E. (2007). Complete and SOS-mediated response of *Staphylococcus aureus* to the antibiotic ciprofloxacin. *J. Bacteriol*. **2007**, *189*(2), 531-539.

Cueva, C.; Moreno-Arribas, M. V.; Martin-Alvarez, P. J.; Bills, G.; Vicente, M. F.; Basilio, A.; Rivas, C. L.; Requena, T.; Rodriguez, J. M.; Bartolome, B. Antimicrobial Activity of Phenolic Acids against Commensal, Probiotic and Pathogenic Bacteria. *Res. Microbiol*. **2010**, *161*, 372-382.

Condell, O.; Sheridan, A.; Power, K. A.; Bonilla-Santiago, R.; Sergeant, K.; Reanut, J.; Burgess, C.; Fanning, S.; Nally, J.E. Comparative proteomic analysis of Salmonella tolerance to the biocide active agent triclosan. *J. Proteomics*. **2012**, *75*, 4505-4519.

Daglia, M. Polyphenols as Antimicrobial Agents. *Curr. Opin. Biotechnol.* **2012**, *23*, 174-181.

Dantes, R.; Belflower, R.; Aragon, D.; Dumyati, G.; Harrison, L. H.; Lessa, F. C.; Lynfield, R.; Nadle, J.; Petit, S.; Ray, S. M.; Schaffner, W.; Townes, J.; Fridkin, S. National burden of invasive methicillin-resistant *Staphylococcus aureus* infections, United States, 2011. *JAMA Intern. Med*. **2013**, *173*(21), 1970-1978.

Davies, J.; Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* **2010**, *74*(3), 417-433.

DeLeo, F. R.; Otto, M.; Barry, N. K.; Chambers, H. Community-associated methicillin-resistant *Staphylococcus aureus*. *Lancet*. **2010**, *37*, 1557-1568.

Deurenberg, R. H.; Vink, C.; Kalenic, S.; Friedrich, A. W.; Bruggeman, C. A.; Stobberingh, E. E. The molecular evolution of methicillin-resistant *Staphylococcus aureus*. *Clin. Microbiol. Infect*. **2007**, *13*, 222-235.

Dung, N. T.; Chi, D. H.; Thao, L. T. B.; Dung, N. T. K.; Nhi, N. B.; Chi, P. V. Identification and Characterization of Membrane Proteins from Mouse Brain Tissue. *J. Proteomics Bioinf*. **2013**, *6*(6), 142-147.

El-Aneed A.; Cohen, A.; Banoub, J. Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers. *Appl. Spectrosc. Rev*. **2009**, *44*, 210-230.

Etebu, E.; Arikekpar, I. Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *Int. J. Appl. Microbiol. Biotechnol. Res*. **2016**, *4*, 90-101.

Ferguson, P. L.; Smith, R. D. Proteome Analysis by Mass Spectrometry. *Annu. Rev. Biophys. Biomol. Struct*. **2003**, *32*, 399-424.

Fernandez-Reyes, M.; Rodriguez-Falcon, M.; Chiva, C.; Panchon, J.; Andreu, D.; Rivas, L. The cost of resistance to colistin in *Acinetobacter baumannii*: a proteomic perspective. *Proteomics*. **2009**, *9*, 1632-1645.

Glish, G. L.; Vachet, R. W. The Basics of Mass Spectrometry in the Twenty-First Century. *Nat. Rev. Drug Discov*. **2003**, *2*, 140-150.

Hawkey, P. M. The origins and molecular basis of antibiotic resistance. *BMJ*, **1998**, *317*, 657-660.

Hawkey, P. M. Mechanisms of quinolone action and microbial response. *J. Antimicrob. Chemother*. **2003**, *51*, 29-35.

Hecker, M.; Engelmann, S.; Cordwell, S. J. Proteomics of *Staphylococcus aureus*-current state and future challenges. *J. Chromatogr. B*. **2003**, *787*, 179-195.

Heleno, S. A.; Martins, A.; Queiroz, M. J. R. P.; Ferreira, I. C. F. R. Bioactivity of phenolic acids: Metabolites versus parent compounds. *Food Chem*. **2015**, *173*, 501-513.

Hesketh, A.; Deery, M. J.; Hong, H-J. High-Resolution Mass Spectrometry Based Proteomic Analysis of the Vancomycin-Induced Cell Wall Stress in *Streptomyces coelicolor* A3(2). *J. Proteome Res*. **2015**, *14*, 2915-2928.

Hiramatsu, K.; Ito, T.; Tsubakishita, S.; Sasaki, T.; Takeuchi, F.; Morimoto, Y.; Katayama, Y.; Matsuo, M.; Kuwahara-Arai, K.; Hishinuma, T.; Baba, T. Genomic Basis for Methicillin Resistance in *Staphylococcus aureus*. *Infect. Chemother*. **2013**, *45*(2), 117-136.

Howes, M-J. R.; Perry, N. S. L.; Houghton, P. J. Plants with Traditional Uses and Activities Relevant to the Management of Alzheimer's Disease and Other Cognitive Disorders. *Phytother. Res*. **2003**, *17*, 1-18.

Karpievitch, Y. V.; Polpitiya, A. D.; Anderson, G. A; Smith, R. D.; Dabney, A. R. Liquid Chromatography Mass Spectrometry-Based Proteomics: Biological and Technological Aspects. *Ann. Appl. Stat*. **2010**, *4*(4), 1797–1823.

Khadem, S.; Marles, R. Monocyclic Phenolic Acids; Hydroxy- and Polyhydroxybenzoic acids: Occurrence and Recent Bioactivity Studies. *Molecules*. **2010**, *15*, 7985-8005.

Kinter, M.; Sherman, N. E. *Protein Sequencing and Identification Using Tandem Mass Spectrometry*. Wiley-Interscience, Inc: New York, 2000; pp 153-159.

Kohanski, M. A.; DePristo, M. A.; Colins, J. J. Sublethal Antibiotic Treatment Leads to Multidrug Resistance via Radical-Induced Mutagenesis. *Mol. Cell*. **2010**, *37*, 311-320.

Kuroda, M.; Ohta, T.; Uchiyama, I.; Baba, T.; Yuzawa, H.; Kobayashi, I.; Cui, L.; Oguchi, A.; Aoki, K-i.; Nagai, Y.; Lian, J.; Ito, T.; Kanamori, M.; Matsumaru, H.; Maruyama, A.; Murakami, H.; Hosoyama, A.; Mizutani-Ui, Y.; Takahashi, N. K.; Sawano, T.; Inoue, R-i.; Kaito, C.; Sekimizu, K.; Hirakawa, H.; Kuhara, S.; Goto, S.; Yabuzaki, J.; Kanehisa, M.; Yamashita, A.; Oshima, K.; Furuya, K.; Yoshino, C.; Shiba, T.; Hattori, M.; Ogasawara, N.; Hayashi, H.; Hiramatsu,

K. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet*, **2001**, *357*, 1225-1240.

Laureti L.; Matic I.; Gutierrez A. Bacterial Responses and Genome Instability Induced by Subinhibitory Concentrations of Antibiotics. *Antibiotics*. **2013**, *2*(1), 100-114.

Linder, P.; Jankowsky, E. From unwinding to clamping – the DEAD box RNA helicase family. *Nat. Rev. Mol. Cell Biol*. **2011**, *12*, 505-516.

Liu, G. Y. Molecular Pathogenesis *of Staphylococcus aureus* Infection. *Pediatr. Res*. **2009**, *65*(5), 71R-77R.

Liu, Z.; Wang, W.; Zhu, Y.; Gong, Q.; Yu, W.; Lu, X. Antibiotics at subinhibitory concentrations improve the quorum sensing behavior of *Chromobacterium violaceum*. *FEMS Microbiol. Lett*. **2013**, *341*, 37-44.

Lodovici, M.; Guglielmi, F.; Meoni, M.; Dolara, P. Effect of natural phenolic acids on DNA oxidation in vitro. *Food Chem. Toxicol*. **2001**, *39*(12), 1205-1210.

Lou, Z.; Wang, H.; Zhu, S.; Ma, C.; Wang, Z. Antibacterial Activity and Mechanism of Action of Chlorogenic Acid. *J. Food Sci*. **2011**, *76*(6), M398-M403.

Lou, Z.; Wang, H.; Rao, S.; Sun, J.; Ma, C.; Li, J. *p*-Coumaric acid kills bacteria through dual damage mechanisms. *Food Control*. **2012**, *25*(2), 550-554.

Lv, L-X.; Yan, R.; Shi, H-Y.; Shi, D.; Fang, D-Q.; Jiang, H-Y.; Wu, W-R.; Guo, F-F.; Jiang, X-W.; Gu, S-L.; Chen, Y-B.; Yao, J.; Li, L-J. Integrated transcriptomic and proteomic analysis of the bile stress response in probiotic *Lactobacillus salivarius* LI01. *J. proteomics*. **2016**, *150*, 216-229.

Meissner, F.; Mann, M. Quantitative shotgun proteomics: considerations for a high-quality workflow in immunology. *Nat. Immunol*. **2014**, *15*(2), 112-117.

Mongodin, E.; Finan, J.; Climo, M. W.; Rosato, A.; Gill, S.; Archer, G. L. Microarray Transcription Analysis of Clinical *Staphylococcus aureus* Isolates Resistant to Vancomycin. *J. Bacteriol*. **2003**, *185*(15), 4638-4643.

Morgan, J.; Smith, M.; Mc Auley, M. T.; Enrique Salcedo-Sora J. Disrupting folate metabolism reduces the capacity of bacteria in exponential growth to develop persisters to antibiotics. *Microbiology*. **2018**, *164*(11), 1432-1445.

Nair, C. G.; Chao, C.; Williams, H. D. Sub-lethal concentrations of antibiotics increase mutation frequency in the cystic fibrosis pathogen *Pseudomonas aeruginosa*. *Lett. Appl. Microbiol*. **2013**, *56*(2), 149-154.

Nohynek, L. J.; Alakomi, H-L.; Kähkönen, M. P.; Heinonen, M.; Helander, I. M.; Oksman-Caldentey, K-M.; Puupponen-Pimiä, R. H. Berry Phenolics: Antimicrobial Properties and Mechanisms of Action Against Severe Human Pathogens. *Nutr. Cancer*. **2006**, *54*(1), 18-32.

Nostro, A.; Roccaro, A. S.; Bisignano, G.; Marino, A.; Cannatelli, M. A.; Pizzimenti, F. C.; Cioni, P. L.; Procopio, F.; Blanco, A. N. Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *J. Med. Microbiol*. **2007**, *56*, 519-523.

Oliveira, C. E. V.; Stamford, T. L. M.; Neto, N. J. G.; Souza, E. L. Inhibition of *Staphylococcus aureus* in broth and meat broth using synergies of phenolics and organic acids. *Int. J.Food Microbiol*. **2010**, *137*, 312-316.

Pasqua, R. D.; Mamone, G.; Ferranti, D.; Ercolini, D.; Mauriello, G. Changes in the proteome of *Salmonella enterica* serovar Thompson as stress adaptation to sublethal concentrations of thymol. *Proteomics*. **2010**, *10*, 1040-1049.

Patel, A.; Calfee, R. P.; Plante, M.; Fischer, S. A.; Arcand, N.; Born, C. Methicillin-resistant *Staphylococcus aureus* in orthopaedic surgery. *J. Bone Joint Surg.* **2008**, *90*(11), 1401-1406.

Pillai, S. K.; Wennersten, C.; Venkataraman, L.; Eliopoulos, G. M.; Moellering, R. C. Jr.; Karchmer, A. W. Development of Reduced Susceptibility in Methicillin-Susceptible *Staphylococcus aureus*. *Clin. Infect. Dis*. **2009**, *49*, 1169-1174.

Reddy, P. J.; Ray, S.; Sathe, G. J.; Prasad, T. S. K.; Rapole, S.; Panda, D.; Srivastava, S. Proteomic Analysis of *Bacillus subtilis* after Treatment with Plumbagin, a plant-Derived Naphtoquinone. *OMICS*. **2015**, *19*(1), 12-23.

Rehm, S. J.; Tice, A. *Staphylococcus aureus*: Methicillin-Susceptible *S. aureus* to Methicillin-Resistant *S. aureus* and Vancomycin-Resistant *S. aureus*. *Clin. Infect. Dis*. **2010**, *51*(S2), S176-S182.

Resing, K. A.; Ahn, N. G. Proteomics strategies for protein identification, *FEBS Lett.* **2005**, *579*, 885-889.

Rivas-Sendra, A.; Landete, J. M.; Alcantara, C.; Zuniga, M. Response of *Lactobacillus casei* BL23 to phenolic compounds. *J. Appl. Microbiol*. **2011**, *111*, 1473-1481.

Rodriguez Vaquero, M. J.; Alberto, M. R.; de Manca de Nadra, M. C. Antibacterial effect of phenolic compounds from different wines. *Food Control*. **2007**, *18*, 93-101.

Sachar, M.; Anderson, K. E.; Ma, X. Protoporphyrin IX: the Good, the Bad, and the Ugly. *J. Pharmacol. Exp. Ther*. **2016**, *356*(2), 267-275.

Shin, J.; Prabhakaran, V-S.; Kim, K-S. The multi-faceted potential of plant-derived metabolites as antimicrobial agents against multidrug-resistant pathogens. *Microb. Pathog*. **2018**, *116*, 209-214.

Sianglum, W.; Srimanote, P.; Wonglumsom, W.; Kittiniyom, K.; Voravuthikunchai, S. P. Proteome Analyses of Cellular Proteins in Methicillin-Resistant *Staphylococcus aureus* Treated with Rhodomyrtone, a Novel Antibiotic Candidate. *Plos One*. **2011**, *6*(2), 1-10.

Sigma-Aldrich Web Page. https://www.sigmaaldrich.com/catalog/search?term=2-Hydroxycinnamic+acid%2C+predominantly+trans&interface=Product%20Name&N=0+&mode=mode%20matchpartialmax&lang=en&region=TR&focus=productN=0%20220003048%20219853286%20219853261 (accessed Nov 7, 2018).

Somerville, G. A.; Proctor, R. A. At the Crossroads of Bacterial Metabolism and Virulence Factor Synthesis in Staphylococci. *Microbiol. Mol. Biol. Rev*. **2009**, *73*(2), 223-248.

Takahashi, H.; Takada, K.; Tsuchiya, T.; Miya, S.; Kuda, T.; Kimura, B. *Listeria monocytogenes* develops no resistance to ferulic acid after exposure to low concentrations. *Food Control*. **2015**, *47*, 560-563.

Traxler, M. F.; Summers, S. M.; Nguyen, H-T.; Zacharia, V. M.; Smith, J. T., Conway, T. The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. *Mol. Microbiol*. **2008**, *68*(5), 1128-1148.

Visutthi, M.; Srimanote, P.; Voravuthikunchai, S. P. Responses in the Expression of Extracellular Proteins in Methicillin-Resistant *Staphylococcus aureus* Treated with Rhodomyrtone. *J. Microbiol*. **2011**, *49*(6), 956-964.

Walsh, C. Molecular mechanisms that confer antibacterial drug resistance. *Nature*. **2000***, 406*, 775-781.

Walters, M. S.; Mobley, H. L. T. Identification of uropathogenic *Escherichia coli* surface proteins by shotgun proteomics. *J. Microbiol. Methods*. **2009**, *78*, 131–135.

Wang, Y.; Yang, F.; Gritsenko, M. A.; Wang, Y.; Clauss, T.; Liu, T., Shen, Y.; Monroe, M. E.; Lopez-Ferrer, D.; Reno, T.; Moore, R. J.; Klemke, R. L.; Camp, D. G.; Smith, R. D. Reversed-phase chromatography with multiple fraction concatenation strategy for proteome profiling of human MCF10A cells. *Proteomics*. **2011**, *11*(10), 2019-2026.

Yang, F.; Shen, Y.; Camp II, D. G.; Smith, R. D. High pH reversed-phase chromatography with fraction concatenation as an alternative to strong-cation exchange chromatography for two-dimensional proteomic analysis. *Expert Rev. Proteomics*. **2012**, *9*(2), 129-134.

Zhang, Y.; Fonslow, B. R.; Shan, B.; Baek, M-C.; Yates III, J. R. Protein Analysis by Shotgun/Bottom-up Proteomics. *Chem. Rev*. **2013**, *113*(4), 2343-2394.

Zheng, L. F.; Dai, F.; Zhou, B.; Yang, L.; Liu, Z. L. Prooxidant activity of hydroxycinnamic acids on DNA damage in the presence of Cu(II) ions: mechanism and structure-activity relationship. *Food Chem. Toxicol*. **2008**, *46*(1), 149-156.

APPENDIX A

EFFECT OF ETHANOL ON MRSA AND MSSA

The antimicrobial experiment to determine the effect of ethanol on MRSA and MSSA growth were performed as it was done for antimicrobial experiments performed for DMSO (Chapter 2). Table A.1 showed the percent inhibitions of tested concentrations of ethanol.

Table A.1. Inhibitory effect of ethanol concentrations on MRSA and MSSA.

Concentration of Ethanol (vol/vol) (%)	MRSA Percent Inhibition (%)	MSSA Percent Inhibition (%)
25	54	84
20	60	83
15	70	85
10	76	89
5	41	22
1	25	13
0.6	-9	2
0.5	-33	11
0.4	-2	0
0.3	-4	13
0.2	-24	15
0.1	-23	-19

APPENDIX B

BRADFORD PROTEIN ASSAY

Bradford assay was used for protein quantification. Standard graphic was formed by measuring absorbance of different concentrations of bovine serum albumin (BSA). For gel-based approach, the BSA was prepared as 1 mg/ml stock and diluted to the concentrations of 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, 250 µg/ml and 500 µg/ml for assay. For gel-free proteomic approach (shotgun proteomics), concentrations of BSA were prepared as 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml and 250 µg/ml by dissolving BSA in resuspension buffer (7 M Urea, 2 M thiourea and 0.1 M Tris-HCl pH: 7.8). Bradford reagent at the amount of 180 µl (1X) was added into the wells of 96-well plate and mixed with 20 µl of each concentration of BSA. As a blank, 20 µl of PBS or resuspension buffer was mixed with reagent. After 10 minutes of incubation in dark, absorbance measurement was done at 595 nm. BSA standard graphic was plotted as absorbance (595 nm) against BSA concentration (µg/ml).

Figure B.1 corresponded to BSA standard graphic used for calculations of isolated protein concentrations during (A) SDS-PAGE and 2D-PAGE analyses and (B) shotgun proteomic analyses.

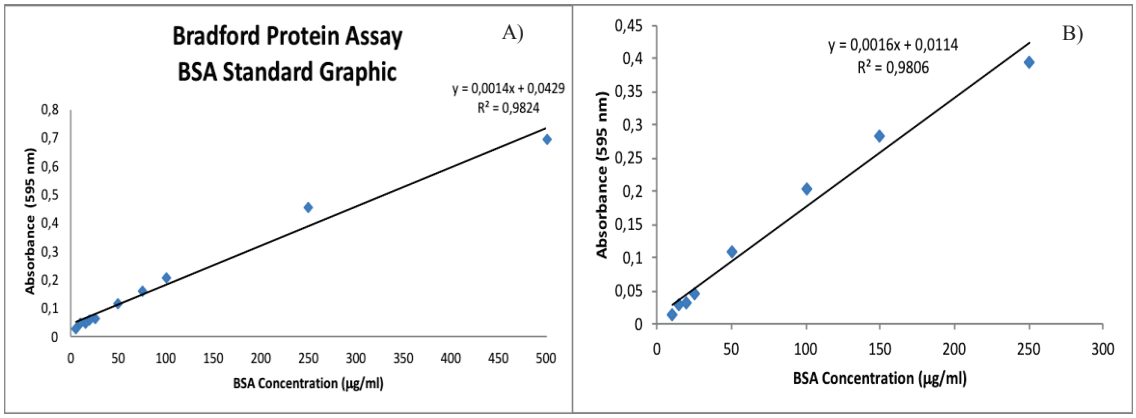

Figure B.1. BSA standard graphic for (A) gel-based and (B) gel-free proteomics.

APPENDIX C

MUTUAL PROTEINS THAT IDENTIFIED UNDER ALL CONDITIONS OBTAINED VIA SHOTGUN PROTEOMICS

Mutually identified proteins of control, VA treated and 2-HCA treated MRSA obtained in shotgun proteomic studies were listed in Table C.1.

Table C.1. Mutual proteins of control and phenolic acid treated MRSA.

Protein ID	Protein Name
P99090	D-alanine aminotransferase
P99169	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B
A0A0H3JR78	Uncharacterized protein
P65457	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2
P63765	Cell division protein FtsA
Q7A4Q3	Adenylosuccinate lyase (ASL)
P65134	Translation initiation factor IF-2
A0A0H3JPB7	SA0774 protein
A0A0H3JQG7	SA1558 protein
P99136	Glyceraldehyde-3-phosphate dehydrogenase 1 (GAPDH 1)
Q7A4V3	UPF0342 protein SA1663
P67579	Methionine--tRNA ligase
A0A0H3JMF1	Signal recognition particle receptor FtsY
Q7A698	Probable quinol oxidase subunit 2
Q820A6	Pyruvate dehydrogenase E1 component subunit alpha
P0A002	Acyl carrier protein (ACP)
P99110	Chaperone protein DnaK (Heat shock protein 70)
A0A0H3JLS3	Uncharacterized protein
A0A0H3JMS9	Glucokinase
A0A0H3JPS6	Coenzyme A biosynthesis bifunctional protein CoaBC
Q99W05	Arginine--tRNA ligase
Q7A4G0	DEAD-box ATP-dependent RNA helicase CshA
A0A0H3JMC8	Uncharacterized protein
A0A0H3JU93	SA1343 protein
P66706	DNA-directed RNA polymerase subunit alpha

(cont. on next page)

Table C.1. (cont.)

P67041	Phenylalanine--tRNA ligase beta subunit
P0A0D1	Streptomycin 3"-adenylyltransferase
P99177	Probable cysteine desulfurase
P67572	Asparagine--tRNA ligase
A0A0H3JVF5	Alpha-acetolactate synthase
A0A0H3JM15	SA0759 protein
Q7A5J0	30S ribosomal protein S1
A0A0H3JMP6	Nicotinate phosphoribosyltransferase
Q7A780	Serine-aspartate repeat-containing protein D
P66108	50S ribosomal protein L20
P63892	D-alanine--D-alanine ligase
P99087	Phosphoglucosamine mutase
A0A0H3JUD9	Uncharacterized protein
P99095	Glutamine synthetase
P99091	Serine hydroxymethyltransferase
Q7A6A9	1,4-dihydroxy-2-naphthoyl-CoA synthase
A0A0H3JU25	Uncharacterized protein
Q7A732	Transcriptional regulator SarA (Staphylococcal accessory regulator A)
A0A0H3JLU5	Fructose specific permease
A0A0H3JMQ4	2-oxoisovalerate dehydrogenase subunit alpha
P99083	60 kDa chaperonin (GroEL protein)
A0A0H3JS65	Kanamycin nucleotidyltransferase
P66318	50S ribosomal protein L9
P99155	50S ribosomal protein L10
P67396	Uracil phosphoribosyltransferase
A0A0H3JNH4	SA0231 protein
A0A0H3JPB1	SA0758 protein
P66553	30S ribosomal protein S3
Q7A5J1	DNA-binding protein HU
P99137	Molybdenum cofactor biosynthesis protein B
Q7A7B3	50S ribosomal protein L25 (General stress protein CTC)
P65256	L-lactate dehydrogenase 1 (L-LDH 1)
Q7A551	Putative universal stress protein SA1532
P63871	Cysteine synthase (CSase)
P99062	Adenylate kinase (AK)
P99178	Serine--tRNA ligase
Q7A473	50S ribosomal protein L13
Q7A7S4	ESAT-6 secretion system extracellular protein A
P60432	50S ribosomal protein L2
Q7A7V0	Ribitol-5-phosphate cytidylyltransferase 1

(cont. on next page)

Table C.1. (cont.)

Q7A4T5	Glutamate-1-semialdehyde 2,1-aminomutase 2
P64060	GTPase Der (GTP-binding protein EngA)
P99102	Deoxyribose-phosphate aldolase 1
A0A0H3JMJ3	Uncharacterized protein
P64225	Aminomethyltransferase
Q7A460	50S ribosomal protein L22
Q7A6R5	Protein translocase subunit SecA 1
P99171	Elongation factor Ts (EF-Ts)
P99134	Immunoglobulin G-binding protein A (Staphylococcal protein A) (SpA)
Q99S93	UPF0457 protein SA1975.1
P66646	30S ribosomal protein S9
P99098	Superoxide dismutase [Mn/Fe] 1
P99074	Alkyl hydroperoxide reductase C
P99117	Fructose-bisphosphate aldolase class 1
Q7A463	50S ribosomal protein L14
A0A0H3JK70	SA0102 protein
P66630	30S ribosomal protein S8
P66616	30S ribosomal protein S7
Q7A5X6	Ribonuclease J 2 (RNase J2)
P66715	Probable DNA-directed RNA polymerase subunit delta
A0A0H3JP27	Uncharacterized protein
A0A0H3JM60	SA0859 protein
Q7A7U6	Iron-sulfur cluster repair protein ScdA
P67327	UPF0176 protein SA2481
Q7A6H3	Uncharacterized protein SA0829
Q7A742	Alcohol dehydrogenase (ADH)
P99135	Phosphoglycerate kinase
P65272	Elongation factor 4 (EF-4)
Q7A5R3	Aminoacyltransferase FemA (Essential for methicillin resistance A)
A0A0H3JTT8	SA0959 protein
P66334	30S ribosomal protein S10
P0A019	D-alanyl carrier protein (DCP)
P99112	ATP synthase subunit beta
P63806	Cytidylate kinase (CK)
P99170	Glutamate--tRNA ligase
Q7A4D0	Pyrimidine-nucleoside phosphorylase
Q7A697	Bifunctional protein FolD
A0A0H3JLK5	SA0455 protein
P99122	Thioredoxin (Trx)
Q7A6G6	Chaperone protein ClpB

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Table C.1. (cont.)

Q7A559	Pyruvate kinase (PK)
P60278	DNA-directed RNA polymerase subunit beta
Q7A469	50S ribosomal protein L17
A0A0H3JKF0	Uncharacterized protein
P99129	Glycine--tRNA ligase
P0A0F2	50S ribosomal protein L11
A0A0H3JM18	Xaa-Pro dipeptidase
A0A0H3JL75	Ribonucleoside-diphosphate reductase
P99160	Probable transglycosylase IsaA (Immunodominant staphylococcal antigen A)
P99075	Fructose-bisphosphate aldolase (FBP aldolase)
P64003	Septation ring formation regulator EzrA
P63844	GTP-sensing transcriptional pleiotropic repressor CodY
P99161	Transketolase (TK)
Q7A7I6	UPF0355 protein SA0372
Q7A3H8	Putative NAD(P)H nitroreductase SA2311
P99072	CTP synthase
Q7A4D8	Putative aldehyde dehydrogenase
A0A0H3JRE5	Uncharacterized protein
Q7A7X6	Formate acetyltransferase
P99139	Molybdopterin molybdenumtransferase
P99111	ATP synthase subunit alpha
P66173	50S ribosomal protein L29
A0A0H3JL08	SA1311 protein
P67593	Tryptophan--tRNA ligase
P60285	DNA-directed RNA polymerase subunit beta'
P99065	Peptide methionine sulfoxide reductase MsrB
A0A0H3JLJ2	Uncharacterized protein
P65475	UDP-N-acetylmuramate--L-alanine ligase
P0A0F6	50S ribosomal protein L15
P99121	Methionine aminopeptidase (MAP) (MetAP)
P60735	50S ribosomal protein L24
P64108	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ
A0A0H3JLK6	Uncharacterized protein
P99165	ATP-dependent 6-phosphofructokinase (ATP-PFK)
Q7A7B5	Putative septation protein SpoVG
P60857	PTS system glucose-specific EIIA component (EIIA-Glc) (EIII-Glc)
P66579	30S ribosomal protein S5
A0A0H3JNV0	SA2202 protein
P99107	D-alanine--D-alanyl carrier protein ligase (DCL)
A0A0H3JNP0	3-hydroxy-3-methylglutaryl CoA synthase

(cont. on next page)

Table C.1. (cont.)

Q7A6U1	Lipoteichoic acid synthase
P0A0D8	Mannitol-specific phosphotransferase enzyme IIA component (EIIA)
P63334	6-phosphogluconate dehydrogenase, decarboxylating
P99089	ATP-dependent Clp protease proteolytic subunit
P68789	Elongation factor G (EF-G)
A0A0H3JLB6	DNA polymerase I
P99115	Probable malate:quinone oxidoreductase 2
P61059	50S ribosomal protein L4
P99078	Glucose-6-phosphate isomerase (GPI)
P61598	Putative surface protein SA2285
P99080	Trigger factor (TF) (PPIase)
P99157	Alkaline shock protein 23
P99154	50S ribosomal protein L7/L12
Q99V41	Bifunctional autolysin
Q7A5H6	Transcriptional regulatory protein SrrA
A0A0H3JK90	Ribonucleoside-diphosphate reductase minor subunit
P99066	Elongation factor P
P65636	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex
A0A0H3JTJ0	SA0777 protein
Q99TF2	Acetate kinase
Q7A6L4	UPF0051 protein SA0778
P66544	30S ribosomal protein S2
P99151	Alanine dehydrogenase 1
P99120	Diacetyl reductase [(S)-acetoin forming]
A0A0H3JMH7	Uncharacterized protein
P99133	Triosephosphate isomerase
Q7A461	50S ribosomal protein L16
Q7A774	3-hexulose-6-phosphate synthase (HPS)
A0A0H3JLH9	Enoyl-[acyl-carrier-protein] reductase [NADPH]
A0A0H3JKT1	Uncharacterized protein
A0A0H3JLE0	SA1565 protein
A0A0H3JN86	SA1749 protein
P66563	30S ribosomal protein S4
P60855	Uncharacterized protein SA0370
P99073	Ornithine carbamoyltransferase
Q7A531	UPF0478 protein SA1560
A0A0H3JUN2	Uncharacterized protein
P66838	Anti-sigma-B factor antagonist
A0A0H3JKY5	SA1224 protein
P64313	Protein/nucleic acid deglycase HchA

(cont. on next page)

Table C.1. (cont.)

Q7A797	ATP-dependent Clp protease ATP-binding subunit ClpC
A0A0H3JLE5	Uncharacterized protein
P99116	D-lactate dehydrogenase (D-LDH)
P99088	Enolase
Q7A467	50S ribosomal protein L18
P99084	Dihydrolipoyl dehydrogenase
Q7A583	50S ribosomal protein L21
P63790	ATP-dependent Clp protease ATP-binding subunit ClpX
P99108	Cell division protein FtsZ
Q7A6H1	Coenzyme A disulfide reductase
A0A0H3JMG3	SA2119 protein
A0A0H3JJS8	Uncharacterized protein
Q7A6J4	NADH dehydrogenase-like protein SA0802
P65140	Translation initiation factor IF-3
P63538	Probable endonuclease 4
P99100	Phosphopentomutase
A0A0H3JJZ6	Uncharacterized protein
P99152	Elongation factor Tu
P63797	ATP-dependent protease ATPase subunit HslU
A0A0H3JNB5	SA2170 protein
P99142	30S ribosomal protein S6
P64228	Glutamine--fructose-6-phosphate aminotransferase
P64270	2,3-bisphosphoglycerate-independent phosphoglycerate mutase
P66388	30S ribosomal protein S13
Q7A6K4	Acid sugar phosphatase
Q7A4E7	ATP synthase subunit b
P99138	Probable branched-chain-amino-acid aminotransferase
A0A0H3JTZ1	Transcription termination/antitermination protein NusA
P99105	GMP synthase [glutamine-hydrolyzing]
P99113	Glycerol kinase
Q7A5V7	Aerobic glycerol-3-phosphate dehydrogenase
Q7A465	50S ribosomal protein L5
A0A0H3JMK7	Uncharacterized protein
Q7A5C0	30S ribosomal protein S20
Q7A552	Uncharacterized peptidase SA1530
P0A0G8	30S ribosomal protein S12
P99132	Probable tautomerase SA1195.1
P60449	50S ribosomal protein L3
Q99W68	50S ribosomal protein L1
P99106	Inosine-5'-monophosphate dehydrogenase

(cont. on next page)

Table C.1. (cont.)

Q7A6D4	Putative phosphoesterase SA0873
Q99V14	Phosphoenolpyruvate-protein phosphotransferase
P63489	Glutamyl-tRNA(Gln) amidotransferase subunit A
Q7A6L9	UPF0337 protein SA0772
Q7A3F4	ATP-dependent Clp protease ATP-binding subunit ClpL
P99143	Phosphocarrier protein HPr
Q7A466	50S ribosomal protein L6
Q7A8D1	Bleomycin resistance protein
P0A0H7	Serine-protein kinase RsbW (Anti-sigma-B factor)
A0A0H3JN49	Uncharacterized protein
P99071	Succinate--CoA ligase [ADP-forming] subunit beta
Q99RW4	Putative formate dehydrogenase SA2102
P67509	Isoleucine--tRNA ligase
Q7A459	50S ribosomal protein L23
P99063	Pyruvate dehydrogenase E1 component subunit beta
Q7A5M6	UPF0403 protein SA1261
P66357	30S ribosomal protein S11
Q99TJ8	Valine--tRNA ligase
P99086	Protein GrpE (HSP-70 cofactor)
A0A0H3JMR6	Uncharacterized protein
P63645	Argininosuccinate synthase
Q7A5Y3	Proline--tRNA ligase
Q7A522	Putative dipeptidase SA1572
P66083	50S ribosomal protein L19
P99103	Beta sliding clamp (Beta clamp) (Sliding clamp)
P67585	Threonine--tRNA ligase
P0A0G0	50S ribosomal protein L30
Q7A6R6	Ribosome hibernation promotion factor (HPF)
P0A0H2	rRNA adenine N-6-methyltransferase
P67610	Lysine--tRNA ligase

VITA

PERSONAL INFORMATION

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EDUCATION

2012-2018 Doctor of Philosophy (Ph. D.)
Izmir Institute of Technology, Department of Molecular Biology and Genetics. Thesis Title: Investigation of Molecular Effect of Phenolic Acids on Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* in Comparison to their Phenolic Acid Resistant Mutants

2009-2012 Master of Science (M. Sc.)
Izmir Institute of Technology, Department of Molecular Biology and Genetics. Thesis title: Outer Membrane Protein Profiling of *Escherichia coli* O157:H7 in Response to Phenolic Acid Stress

2005-2009 Bachelor of Science (B. Sc.)
Ege University, Department of Biology

WORK and RESEARCH EXPERIENCE

Research and Teaching Assistant: Izmir Institute of Technology, Department of Molecular Biology and Genetics, January 2013 - June 2018.

Researcher with scholarship in the project PIRSES-GA-2010-269211 at CINDEFI-CONICET, July 2014 - October 2014, La Plata, Argentina.

CONFERENCE PRESENTATIONS

Poster Presentations

1. Keman*, D., Ozdemir*, O., Fratebianchi de la Parra, D., Cavalitto, S. 2014. Study of the Pectolytic Activities Produced by *Aspergillus sojae* Utilizing Agroindustrial Residues. 3rd Argentina Symposium on Biotechnological Processes (SAProBio 2014), Santa Fe, Argentina.
2. Keman, D., Ozdemir, O., Soyer, F. 2011. Outer Membrane Protein Profiling of *Escherichia coli* O157:H7 in Response to Caffeic Acid Stress. International Symposium on Secondary Metabolites: Biological, Chemical and Biotechnological Properties (ISSMET2011), Denizli, Turkey. p:68.
3. Ozdemir, O. O., Keman, D., Soyer, F. Profiling of Outer Membrane Proteins of Salmonella Enteritidis in Response to 3-Hydroxyphenylacetic Acid. International Symposium on Secondary Metabolites: Biological, Chemical and Biotechnological Properties (ISSMET2011), Denizli, Turkey p.105.

Oral Presentations

1. Keman, D., Soyer, F., Yalçın, T. 2017. Metisiline Dirençli *Staphylococcus aureus*'un *o*-Kumarik Asit Varlığındaki Protein Profilinin İncelenmesi. 2. Ulusal Proteomik Kongresi, İstanbul, Turkey.
2. Keman, D., Soyer, F., Atbaşı, Z. Effect of phenolic acid-mixed bone cement against methicillin-resistant *Staphylococcus aureus*. 2015. Symposium on Powdered and Porous Materials, Aydın, Turkey.

Submitted Papers

1. Keman, D., Soyer, F. Paper submitted with the title ''Subinhibitory Phenolic Acid Concentrations do not Lead to Resistance in *Staphylococcus aureus* on the Contrary to Subinhibitory Antibiotic Concentrations''.