# INVESTIGATION OF PROTEIN PROFILES OF *LISTERIA MONOCYTOGENES* IN THE EXISTENCE OF PHENOLIC ACIDS USING MASS SPECTROMETRY

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**by**
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# ABSTRACT


## INVESTIGATION OF PROTEIN PROFILES OF *LISTERIA MONOCYTOGENES* IN THE EXISTENCE OF PHENOLIC ACIDS USING MASS SPECTROMETRY

*Listeria monocytogenes* is one of the foodborne pathogens (FBP), which are a threat to the consumers' health, able to cause listeriosis. *L. monoytgenes* cells, which can easily adapt and survive stresses, can develop resistance to antibiotics used in standard therapy.

Phenolic acids that are a natural defense mechanism against stress conditions in plants, might be used as an antibacterial-candidate in foodborne diseases, so there is a need for a better understanding of the stress-induced responses and mechanisms of bacteria against these substances.

Proteomic approaches are an invaluable method for identifying the stress response in pathogenic bacteria.

For this purpose, in this study firstly, the antibacterial effects of two phenolic acids (3-HPAA and 4-HBA) on bacteria were investigated by determining the minimum inhibitory concentrations (MIC). Subsequently, target changes in the protein profile due to antimicrobial effects of phenolic acids were evaluated using a soft ionization technology and mass spectrometry-based comparative gel-free proteomic approach (Shotgun proteomics).

According to the results, *Listeria monocytogenes* could not develop resistance to both phenolic acids.

This study emphasizes the importance of using of phenolic acids as a novel and natural therapy methods to overcome antibiotic-resistant pathogenic bacteria.

# ÖZET


## *LİSTERİA MONOCYTOGENES* PROTEİN PROFİLLERİNİN KÜTLE SPEKTROMETRESİ KULLANILARAK FENOLİK ASİTLERİN VARLIĞINDA İNCELENMESİ

*Listeria monocytogenes*, tüketicilerin sağlığını tehdit eden ve listeriosis'e neden olabilen gıda kaynaklı patojenlerden (FBP) biridir. Stresleri kolayca adapte edebilen ve hayatta kalabilen *L. monoytgenes* hücreleri, standart tedavide kullanılan antibiyotiklere direnç geliştirebilir.

Bitkilerdeki stres koşullarına karşı doğal bir savunma mekanizması olan fenolik asitler, gıda kaynaklı hastalıklarda antibakteriyel bir aday olarak kullanılabilir, dolayısıyla bakterilerin bu maddelere karşı stres kaynaklı tepkilerinin ve mekanizmalarının daha iyi anlaşılmasına ihtiyaç vardır.

Proteomik yaklaşımlar, patojenik bakterilerdeki stres tepkisini tanımlamak için paha biçilmez bir yöntemdir.

Bu amaçla, bu çalışmada öncelikle, iki fenolik asidin bakteriler üzerindeki antibakteriyel etkileri, minimum inhibitör konsantrasyonları (MIC) belirlenerek araştırılmıştır. Daha sonra, fenolik asitlerin antimikrobiyal etkilerine bağlı olarak protein profilindeki hedef değişiklikler yumuşak bir iyonizasyon teknolojisi ve kütle spektrometrisine dayalı karşılaştırmalı jel içermeyen proteomik yaklaşım (av tüfeği proteomik) kullanılarak değerlendirildi.

Sonuçlara göre, Listeria monocytogenes her iki fenolik aside karşı direnç geliştiremedi.

Bu çalışma, antibiyotiğe dirençli patojenik bakterilerin üstesinden gelmek için fenolik asitlerin yeni ve doğal bir tedavi yöntemi olarak kullanılmasının önemini vurgulamaktadır.

# TABLE OF CONTENTS

LIST OF FIGURES.....viii

LIST OF TABLES.....ix

CHAPTER 1. INTRODUCTION.....1

1.1 Mass Spectrometry.....1

1.1.1 Configurationsand Principles of MS.....1

1.2 Instrumentation.....2

1.2.1 Ions Sources.....2

1.2.2 Mass Analyzers.....6

1.2.3 Detectors.....11

1.3 -Omics Technologyand Proteomics.....12

1.4 Mass Spectrometry-Based Proteomics.....14

1.5. Separation Techniques Coupled with Mass Spectrometry.....14

1.5.1. Electrophoretic Techniques.....14

1.5.2. Chromatographic Techniques.....16

1.6. Protein Identification by Mass Spectrometry.....17

1.6.1 Shotgun Proteomics.....18

1.7. Protein Sequencing.....19

1.8. Database Search Engine.....19

CHAPTER 2. LITERATURE REVIEW.....21

2.1. Food-Borne Diseases.....21

2.2. *Listeria Spp.*.....21

2.2.1. *Listeria monocytogenes*.....22

2.3. Phenolic Compounds.....26

2.4. Aim of the Thesis.....29

CHAPTER 3 EXPERIMENTAL PROCEDURES.....30

3.1. Activation and Single Colony Production of Bacteria.....30

3.2. Antibacterial Activity of Phenolic Acids……30

3.2.1. Preparation of Bacterial Culture…….30

3.2.2. Preparation of Phenolic Acid Solutions……..31

3.3 Total Protein Extraction of Treated Bacteria.....32

3.3.1. Preparation of Bacterial Culture…….32

3.3.2. Phenolic Acids…….32

3.3.3. Treatment with Phenolic acids……....32

3.3.4. Protein Isolation.....33

3.4. Make Sample Suitable for Mass Spectroscopy.....33

3.4.1. Purification, Pre-Concentration of Proteins…….33

3.4.2. Bradford Assay for Quantitative Analyisis…..……...34

3.4.3. In solution digestion…….....34

3.4.4. Fraction Concatenation and LC-MS/MS……..35

3.4.5. Protein Identification over Bioinformatics Analysis……37

CHAPTER 4. RESULTS AND DISCUSSIONS40

4.1 Identified Proteins after 3-HPAA Treatment.....42

4.1.1. DNA Related Proteins after 3-HPAA Treatment…….43

4.1.2. RNA Related Proteins after 3-HPAA Treatment……44

4.1.3. Ribosome and Protein Synthesis Related Proteins after 3-HPAA Treatment.....46

4.1.4. Cell wall, Membrane, and Flagella Related Proteins after3-HPAA Treatment…….....……..49

4.1.5. MetabolismRelatedProteins after3-HPAA Treatment……..….51

4.1.6. Redox Related Proteins after 3-HPAA Treatment…….....55

4.1.7. Virulence Related Proteins after 3-HPAA Treatment…….56

4.2.Identified Proteins after 4-HBA Treatment.....57

4.2.1. DNA Related Proteins after 4-HBA Treatment…….58

4.2.2. RNA Related Proteins after 4-HBA Treatment……..…….....59

4.2.3. Ribosomal and Protein synthesis Related Proteins after 4-HBA Treatment……61

4.2.4. Cell wall, Membrane and Flagella Related Proteins after 4-HBA Treatment……64

4.2.5. Metabolism Related Proteins after 4-HBA Treatment……67

4.2.6. Redox Related Proteinsafter 4-HBA Treatment…….....70

4.2.7. Virulence Related Proteins after 4-HBA Treatment……...71

CHAPTER 5. CONCLUSION.....73

RERERENCES……75

# LIST OF FIGURES

**Figure** **Page**

Figure 1.1 Components of MS....2
Figure 1.2 Process of MALDI....4
Figure 1.3 Commonly used matrixes in MALDI studies....4
Figure 1.4 Working principle of ESI....5
Figure 1.5 Schematic representation of ToF (time-of-flight) analyzer....7
Figure 1.6 Schematic representation of a reflectron-type ToF....7
Figure 1.7 Schematic diagram of quadropole analyzer....8
Figure 1.8 2D and 3D ion trap....9
Figure 1.9 Schematic display of FT-ICR....9
Figure 1.10 The orbitrap....10
Figure 1.11 Working of the EMP....11
Figure 1.12 Schematic and working structure of MCP....12
Figure 1.13 "Ome"s and workspaces called "omic"s'....13
Figure 1.14 Schematic representation of 2D-SDS-PAGE....15
Figure 1.15 Proteomics approaches....18
Figure 2.1 Sorting and structures of phenolic compound....27
Figure 2.2 Structure of 3-HPAA and 4-HBA, respectively....27
Figure 3.1 Collection order of fraction....36
Figure 4.1 Venn diagram showing the numbers of unique and overlapping proteins identified in the presence of 3-HPAA or 4-HBA....50
Figure 4.2 The percentages of proteins for each group of function in the presence of 3-HPAA detected proteins....51
Figure 4.3 The percentages of proteins for each group of function in the presence of 4-HBA detected proteins....57

# LIST OF TABLES

**Table** **Page**

Table 1.1 Proporties of different ionization techniques .....3
Table 1.2 Comparision of various mass analyzers.....11
Table 2.1 The highly dangerous some pathogenic bacteria.....22
Table 2.2 The cardinal growth conditions of *L. monocytogenes* .....23
Table 2.3 Cases of Listeriosis .....25
Table 3.1 Concentration of Phenolic Acids .....31
Table 3.2 Minimum inhibitory concentrations of phenolics .....32
Table 3.3 LC gradient programme .....37
Table 3.4 LC-MS/MS time programme and parameters.....38
Table 3.5 Conditions for Mascot server .....39
Table 4.1 Percent inhibition values of phenolic acids.....41
Table 4.2 DNA-Related Proteins .....44
Table 4.3 RNA-Related.....45
Table 4.4 Ribomal and Protein Synthesis Related.....48
Table 4.5 Cell wall, Membrane and Flagella Related .....50
Table 4.6 Metabolism-Related.....52
Table 4.7 Cell homeostasis and Redox-Related .....56
Table 4.8 Virulence related.....56
Table 4.9 DNA-Related.....59
Table 4.10 RNA related.....60
Table 4.11 Ribosomal and Protein Synthesis related.....62
Table 4.12 Cell wall, Membrane, Flagella related.....66
Table 4.13 Metabolism related .....68
Table 4.14 Redox related.....70
Table 4.15 Virulence related.....71

# CHAPTER 1

# INTRODUCTION

## 1.1 Mass Spectrometry

A mass spectrometer (MS) is an analytical device that generates positive or negatively charged gas-phase ions and separates them based on the 'mass-charge ratio' (m/z).
Mass spectrometry was identified for the first time at the end of the 1880s. Eugen Goldstein observed canal rays in 1897 and, then Wilhelm Wien showed that canal rays changing direction under electric and magnetic fields. Sir Joseph John Thomson the particles that are deflecting in the cathode ray tube under the magnetic and electric field. The first mass spectrometry was built by Francis Aston. The first use of commercial mass spectrometers began in the petrochemical in the 1940s, industry. In the early 1950s; it was utilized for the diagnosis of organic substance and to observe the structure of the molecule, on amino acids and peptides firstly in late this year. In the 1980s, applications of mass spectrometry became important with improved methodsof ionized non-volatile and thermally unstable substances.
The utilizing areas of the mass spectrometer can be listed as follows; determination of elemental compositions of substances, qualitative and quantitative analysis of mix, determination of isotopic ratios of atoms, identification of impurities in products, illumination of molecules, forensic medicine, proteomics.

### 1.1.1 Configurations and Principles of MS

A mass spectrometer includes 6 main parts, which are inlet, ion source a mass analyzer, ion detector, data and vacuum system as shown in figure 1.1.
An inlet allows a sample to enter an ion source by high-performance liquid chromatography (HPLC) or gas chromatography (GC). The ion source creates a charged particle. Analyzer sorts the ions based on the mass to charge ratio. The detector records the charge induced by electron multiplier (EM) or multichannel plate (MCP).

The signal processor receives the signal from the detector and it records to create a mass spectrum.A high vacuum system must be used to maintain low pressure. In this way, low pressure reduces the collisions among theions.If there is not a high vacuum, the ions produced in the source will not arrive at the detector.

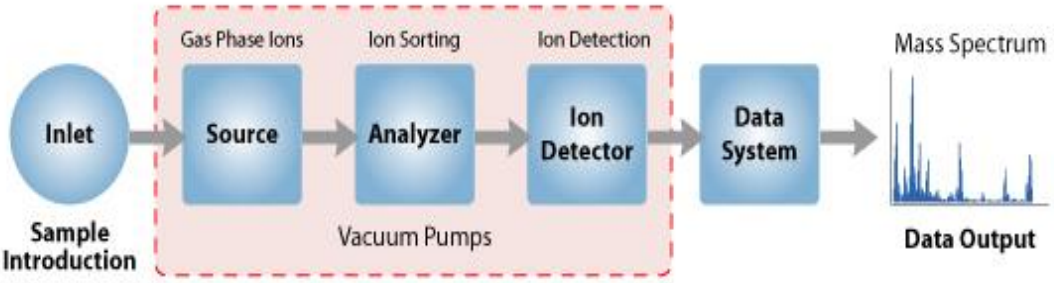



Figure 1.1 Components of MS

(Source:Baghel, 2017)

## 1.2 Instrumentation

### 1.2.1 Ions Sources

The ion source help to convert neutral analyte to positive or negative charge particles. There are several ion sources which are shown in Table 1.1. These are, electron impact (EI), chemical ionization (CI), atmospheric pressure chemical ionization (APCI), field ionization, negative-field ionization (FI, NFI), field desorption (FD), fast atom bombardment (FAB), secondary ion mass spectrometry (SIMS), electrospray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI).

In ion sources, the purpose is to create ions the molecule of the sample without destructively. A spectrum that has a high degree of fragmentation is observed with hard ionization techniques.

On the other hand, ESI and MALDI are known 'soft ionization techniques (Portolés et al. 2011; Hillenkamp et al. 1986). These techniques can help to analyze non-volatile biomolecules and in this way, polar and large substances such as proteins, biopolymers, lipids, etc. can pass to the gas phase without degradation (Creighton 1997; Beck-Sickinger and Mörl 2006; Falconer 1997).

Table 1.1 Proporties of different ionization techniques

| Ionization technique | Typical analytes | Mass range | Sample Introduction | Advantages | Disadvantages |
|---|---|---|---|---|---|
| **EI** | Small,non-polar, thermostable | <1 kDA | GC or liquid/solid | Non-polar analytes, no ion suppression, easily coupled with GC, | Hard ionization needs volatile samples, thermal stability, low MW compounds |
| **ESI** | Polarcompounds | <200 kDa | LC or Solution | Thermolabile compounds, high MW compounds, sensitivity, easy to interface with LC, multi-charged ions | İonizable analyts, sensitive to salts, |
| **MALDI** | Polarcompounds | < 500 kDa | Sample mixed with a solid matrix | Thermolabile compound, sensivity, less sensitive to salts, soft ionization method | Wide range of matrices difficulties in quantitative analysis, ion suppression |
| **APCI** | Neutral Compounds | <1 kDA | LC or Solution | Allows for large scale flow rates, easily to interface with LC, thermostable compounds | Needs solubility in polar solvents, sensitive to salts, ion suppression |

### 1.2.1.1 Matrix-Assisted Laser Desorption/Ionization (MALDI)

Matrix-assisted laser desorption/ionization is known as soft ionization techniques and it is shown in figure 1.2. At first, analyte and matrix mixed into a suitable solvent, and then one microliter analyte-matrix mixture was transferred to the MALDI target. Once the solvent evaporates, uniform co-crystallization observed on the target.

The analyte-matrix spot irradiates with a laser that has a proper frequency. The matrix absorbs the energy of the laser and then helps the analyte to gain extra protons during the adsorption, and then analyte becomes positively charge particle.

Matrix solution is prepared in an organic solvent generally, such as acetonitrile/water and acidifying with trifluoroacetic acid.

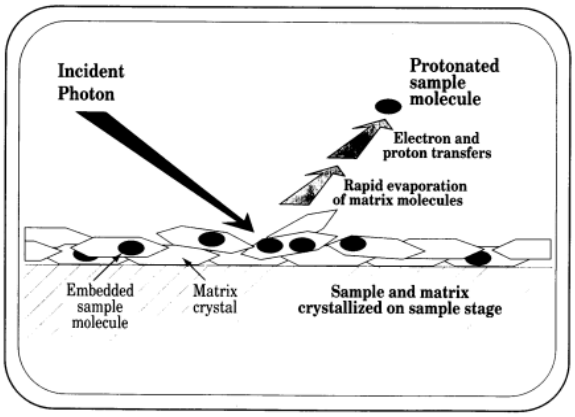



Figure 1.2. Process of MALDI
(Source: Busch, 1995)

Usually, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α-cyano-4-hydroxycinnamic acid (CHCA) are matrixes are the most common matrixes for proteins and peptides. In addition that, 2,5-dihydroxybenzoic acid (DHB) can also used as a matrix. The structre of these matrixes are shown in Figure 1.3. All matrixes used in MALDI system, are generally acts as a proton donor in positive ionization mode or proton acceptor in negative ionization mode. MALDI ionization techniques have a certain tolerance against the impurity in the sample. It is fast and can obtain the results in very short time.

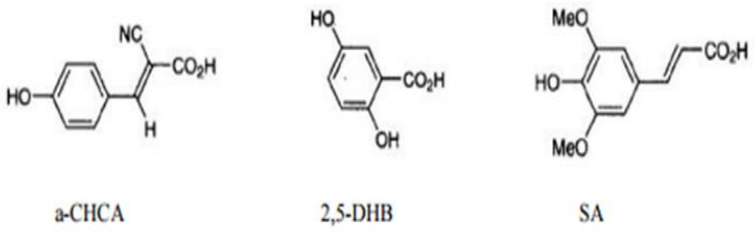



Figure 1.3. Commonly used matrixes in MALDI studies
(Source: Wen. X, 2006)

### 1.2.1.2 Electrospray Ionization (ESI)

Electrospray ionization is another technique that can also use for the non-volatile biological sample. Analyte ionized directly from the liquid environment and that is why on-line separation can be used during the analysis of complex proteins or peptides.

ESI does not tolerate the impurity in the sample. It is also not suitable for uncharged, non-basic, low-polarity compounds.

In this method, first, the sample mixtures are injected into the reverse phase column (C-18 column) and then separated based on their interaction with the stationary phase of the column. Samples separate using the HPLC system and every separated peptide passed along the capillary that holds very high voltage (2- 6 kV). The droplet collides with an inert gas (dry N2). Thus, a better nebulization occurs and droplet becomes smaller, the intensity level of surface charge is increased. After the collision, the droplet reaches to Rayleigh limit when all solvent is evaporated. Next, the coulombic explosion happens, namely smaller droplets happen from the droplet till pure ions have achieved. At the end of all these, spreading ions are accelerated for mass spectrometry (Figure 1.4)

Although ionization materializes under the atmospheric pressure in ESI, in MALDI also under the atmospheric pressure or vacuum can actualize. A complex spectrum obtains with the method of ESI because of multiply-charge. As a result of this situation, larger molecules are analyzed low mass-to-charge range (Ho et al. n.d.; Banerjee and Mazumdar 2012).

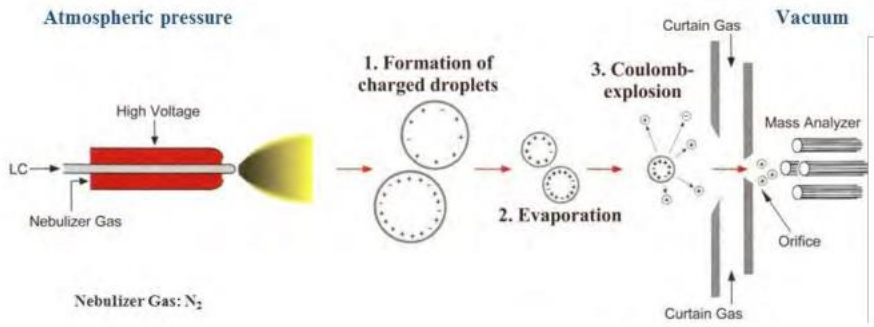



Figure 1.4 Working principle of ESI
(Source: Cheignon, 2016)

### 1.2.2 Mass Analyzers

Gas-phase ions generat in ion sources are accelerated by both electrostatically or magnetically, and transferred to mass analyzers. Mass analyzers separate these ions according to the m/z ratio. Analyzers used some physical characteristics of ions such as momentum, kinetic energy, m/z, flight time to separate them according to m/z ratio (Yates 2000).

Each analyzer can show certain differences in terms of resolution, mass range, transmission, mass accuracy, and MS/MS or $MS^n$capability.The most common mass analyzer aremagnetic sector, electric sector, ion cyclotron resonance (ICR), time-of-flight (TOF), quadrupole (Q), ion trap (IT), and orbitrap,. Certain characteristics of some analyzers are shownin table 1.2.

#### 1.2.2.1 Time-of-Flight (TOF)

A time-of-flight (TOF) mass spectrometerhas been introduced in literature in 1946(Stephens 1946) and to increase the resolution of the mass spectra, some improvement has been developed as reflectron, time lag focusing, and delayed extraction since 1960.A TOF analyzer, establishes a connection between flight time and mass to charge ratio. The relation between flight time and m/e ratio is given below.

$$m/z = t_f^2 2\,Es/(2s + x)$$

The m/z ratio of an ion is measured by determining the time required for it to travel the length of the flight tube in MALDI-TOF system.

Essentially, whole ions directly are given to the analyzer as a pulse from the ion sources with the same kinetic energy. Then, the ions that are carrying the same charge with different mass enter the field-free region at a rate that is inversely proportional to the m / z ratio. As a result, also as seen in figure 1.5, light ions, owing to their high velocity in the field-free, more quickly arrive in the detector. Quite the contrary, heavy ions later reach the device because of having low velocity.

In addition, an energy correction device called reflectron (electrostatic ion mirror) can be used to increase the sensitivity and increase the solubility by homogenizing the kinetic energy.

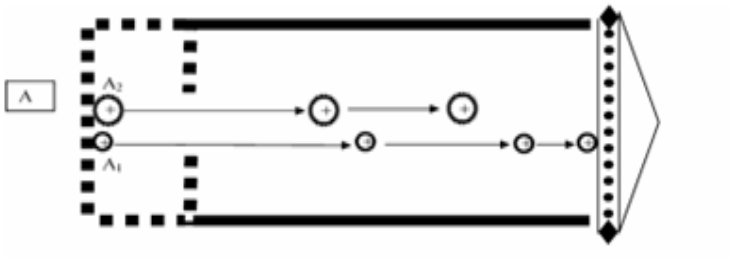



Figure 1.5 Schematic representation of ToF (time-of-flight) analyzer
(Source: Aneed, et al. 2009)

Reflectron generates a retarding area. In this way, ions that have higher kinetic energy as shown in figure 1.6 penetrate far down in the reflectron so that mass resolution is improved(El-Aneed, Cohen, and Banoub 2009).

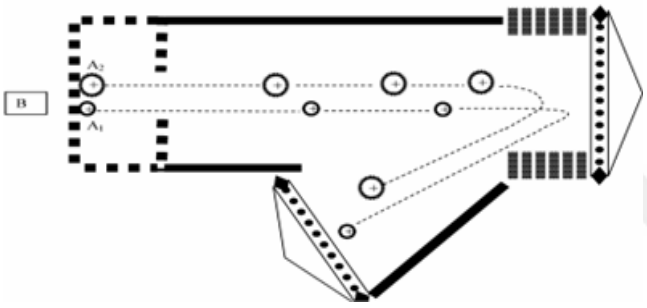



Figure 1.6 Schematic representation of a reflectron-type To
(Source: Aneed, et al. 2009)

### 1.2.2.2 Quadrupole (Q)

The working principle of the quadrupole mass analyzer belongs to Paul Wolfgang and is described in the 1950s. The analyzer consists of four parallel circular-shaped metal rods as shown in the figure 7.

Two opposite rods are charged by either a positive or a negative direct-current (DC) potential and the other two are linked to an alternating radio-frequency (RF) potential. Ions that get out from the source passes in between quadrupole rods by an accelerating potential and reaches a detector.

Each m/z ratio equals a constant DC/RF rate. The ions that have a stable trajectory in certain m/z values transfers with applied

DC/RF voltage between the rods. RF voltage consistently changes, in order to deliver the ions to the detector.(El-Aneed, Cohen, and Banoub 2009; Niessen and Falck 2015).

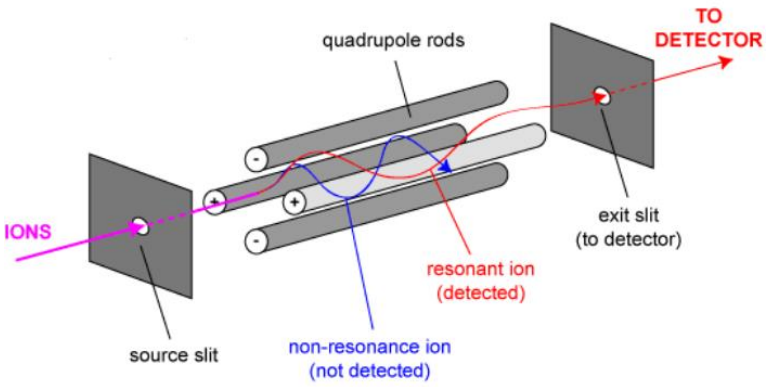



Figure 1.7 Schematic diagram of quadropole analyzer
(Source: Biemann, 1987)

### 1.2.2.3 Ion Trap (IT)

An ion trap analyzer captures ions in two (2D ion trap) or three dimensions (3D ion trap) by using a combination of electric or magnetic fields.3D ion traps that known as also Paul ion trap include four electrodes, two ring electrodes, and two ellipsoid end-cap electrodes, as shown in Figure 1.8. Even if configuration different, actually it works with the same principle of a quadrupole mass analyzer.

All ions in analyzer are trapped thanks to oscillated electric three-dimensional quadrupole fields but ions that have the specific mass to charge ratio after applied certain voltage to the ring electrode are delivered to a detector. When the voltage is changed different ions reach the detector. Then, the 2D ion trap that knows as a linear ion trap was developed. Here, in order to confine the ions stopping potentials are applied to electrodes and the ions are trapped via two-dimensional radiofrequency (Marshall, Hendrickson, and Jackson 1998).

Both of these analyzers include helium gas. Thus, it checks the orbit of ions in the trap. As well as, $MS^n$ analyses are taken place thanks to He gas. So, detailed information has achieved about the fragmentation mechanism.

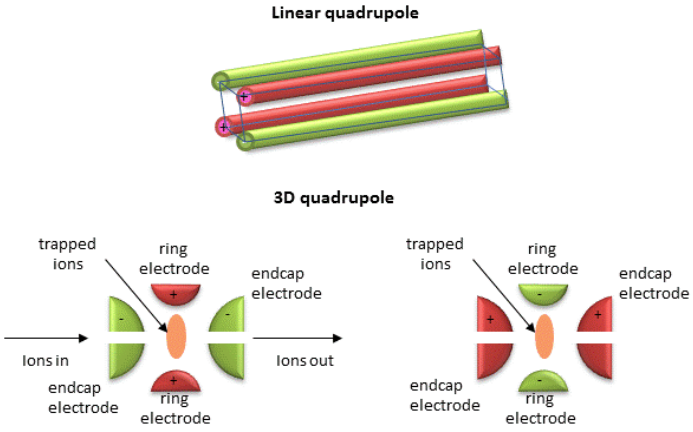



Figure 1.8 2D and 3D ion trap

### 1.2.2.4 Fourier Transform Ion Cyclotron Resonance Mass Analyzer

A Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR -MS) is an ion trap system but, in here ions are trapped by force of high magnetic area of a conducting magnet rather than in an electric field. The ion cyclotron resonance (ICR)cell includes two trapping plates, two excitation plates, and two receiver plates. Also, whole plates are positioned as opposed, and is shown in Figure 1.9. In principle, ions with m/z are injected into the cell and then they move with cyclotron motion (circular orbit).By the way, cyclotron frequency is inversely correlated with m/z. Then an RF pulse is applied to excite trapped ions in their cyclotron loop in the cell. The ions that have the same m/z start moving due to increasing of a radius of the cyclotron motion and consistent motion of ions generate an image currently at receiver plates. Lastly, obtained from these plates the time-domain signal is transformed to the frequency-domain signal by using Fourier transformation. As a result, a mass spectrum is obtained (Niessen and Falck 2015; Marshall, Hendrickson, and Jackson 1998; Hoffmann Edmond de 2007).

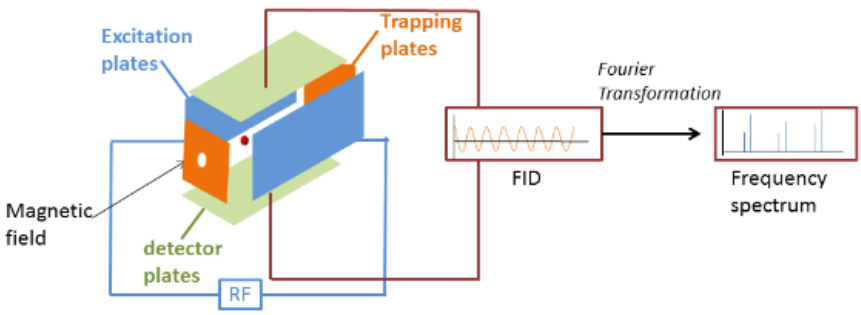



Figure 1.9 Schematic display of FT-ICR

### 1.2.2.5 Orbitrap

The Orbitrap mass analyzer was invented by Makarov in 1999 (Makarov 2000), in 2005 Hu et al. and reported suitabilityfor proteomic analysis(Savaryn, Toby, and Kelleher 2016).

Orbitrap has 100–1000 times greater resolving power and mass accuracy than other quadrupoles. They measure m/z as a function of ion frequency along with the oscillatory motion.

In contrast, quadrupole (IT and resolving quadrupole) analyzers measure m/z by using the stability of ions in the electromagnetic field. As illustrated in Figure 1.10. After charged ions are injected into the orbitrap, the oscillatory motion of each population is measured at the same time. Because of electromagnetic forces, the ions oscillate in the axial dimension and they orbit around the central electrode simultaneously. When the ions oscillate in the axial dimension, they move from the region of one electrode to the other. So, electrodes perceive moving charge that is achieving signal for ion oscillatory frequency. Then, time-domain oscillation data firstly is converted to the frequency domain by Fourier transformation, secondly, to m/z spectral data and a mass spectrum can obtain. (Hu et al. 2005).

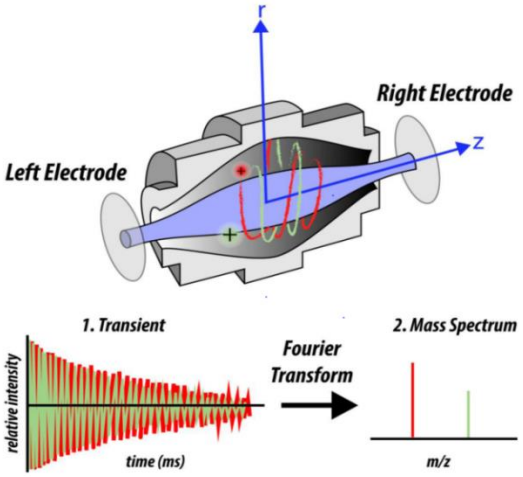



Figure 1.10 The orbitrap
(Source: Savaryn, 2017)

Table 1.2 Comparision of various mass analyzers

| PROPORTIES | QUADRUPOLE | ION TRAP | TOF REFLECTRON | MAGNETIC SECTOR | FT-ICR | Q-TOF |
|---|---|---|---|---|---|---|
| **Accuracy** | 100 ppm | 100 ppm | <5 ppm | < 3ppm | < 5ppm | <1 ppm |
| **Resolution** | 2,000 | 4,000 | 15,000 | 30,000 | 100,000 | 80,000 |
| **m/z range** | 4,000 | 4-6,000 | 10,000 | 8,000 | 10,000 | 10,000 |
| **Scan rate** | ~ 2Hz | ~5 Hz | 50 Hz | 0,1 Hz | ~ 1 Hz | ~20 Hz |
| **MS/MS** | $MS^2$ (QqQ) | $MS^n$ | MS | $MS^2$ | $MS^n$ | $MS^2$ |

## 1.2.3 Detectors

End of the mass analyzer, ion currents reach the last part of mass spectrometry for the detection of their mass. Detectors detect the charge of induced when an ion passes by hits a surface and it transforms into a usable signal. All mass spectrometers, apart from FT-ICR, have a detector. Detector shows vary depending on device and experiment. The most common detectorsused in mass spectrometry are electron multiplier, and microchannel plates.

### 1.2.3.1. Electron multiplier (EMP)

Electron multiplier (EMP) is the most common detector in the mass spectrometer.
Within of device, shown inFigure 1.12, ions pass the transform dynode, then first amplification generates secondary electrots. At last, a waterfall of ions occurs and they are collected and detected in a metal cup.

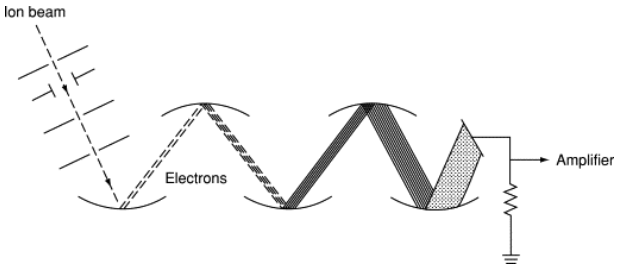



Figure 1.11. Working of the EMP
(Source: F.A. Mellon, 2003)

## 1.2.3.2. Multichannel plate (MCP)

A microchannel plate includes a series of glass capillaries. When the ion strikes the surface of channel one of the capillaries creates a huge number of secondary electrons and produces a current pulse at the output and is shown in Figure 1.13.

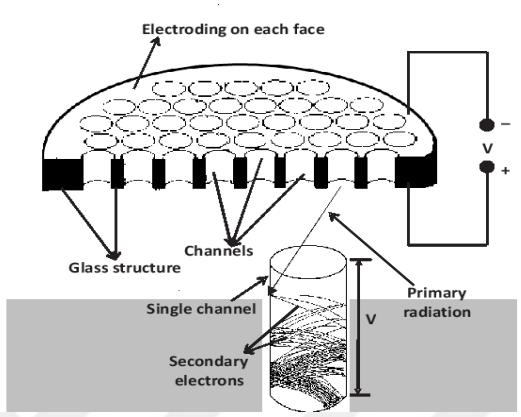



Figure 1.12 Schematic and working structure of MC
(Source: Mrıdula Ranı Jana, 2014)

## 1.3 -Omics Technology and Proteomics

Omics technologies are a new way of measuring families of cellular molecules with a holistic approach. Sequence differences and some functional activities are obtained among the individuals and species owing to these methods.

Omics technologies that known as commonly are genomic, transcriptomic, proteomic and metabolic and these are at a level of protein, gene, mRNA, and metabolite, respectively. Within this scope, genomics(Stover 2004) examines the structural and functional functions of DNA, which carries the genetic information of an organism. Transcriptomics (Trujillo, Davis, and Milner 2006)investigates the mRNA produced by transcription of DNA as a follow up of genomics. Proteomics (Carbonaro 2004) illuminates structure, placement, amounts, modifications, functions of the total protein the interaction with other proteins and macromolecules in a tissue, cell or organism. Metabolomics (Wishart 2008)refers to the determination and quantification of metabolites which are the products of biochemical processes in cells or physiological fluids at a certain time. All omic technologies are associated with each other in a layout (Figure 1.14)(Horgan and Kenny 2011; Debnath, Prasad, and Bisen 2010).

Due to the different and diverse structures and functions of proteins, different cell types are formed in organisms with the same genetic information.

With the Human Genome Project, it was observed that the understanding of biological activity could not be understood only with genes. Because the resulting DNA sequence does not include information of the time and the rate at which a gene is used. At the end of the project, it was focused firstly on RNA then on proteins to eliminate the lack of this information(Gygi et al. 1999).

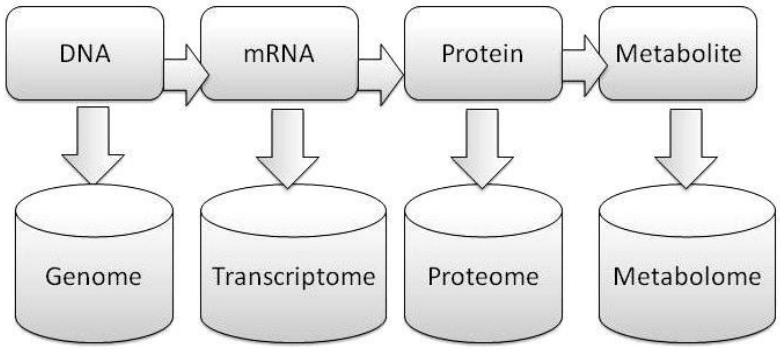



Figure 1.13 "Ome"s and workspaces called "omic"s'
(Source: Mousumi Debnath, 2010)

According to estimates the amount of gene encoding 40,000 proteins in humans is close to that number, but with RNA splicing and post-translational modification (PTM), this count may be increased up to 2,000,000 proteins. This situation explains proteins are more dynamic and complex as structurally than genes(Kosak, Science, and 2004 n.d.). They also need to interact with other proteins and molecules to perform their functions. All this can be explained by the proteomic approach (Chandrasekhar et al. 2014).

In fact, genomic and proteomic supports each other. Together with the two approaches, disease markers and drug targets can be identified and design products to prevent, and treat diseases(Genomics, bioinformatics, and 2007 n.d.).

Based on the protein response under different conditions proteomics are classified into three groups.

A typic proteomic analysis starts with expression proteomics. Compares the protein expression levels of a cell under the stress and control group and it provides information about physiology and pathogenic mechanisms, however, singly expression proteomic does not include functional knowledge. Structural proteomics can help identification of

three-dimensional structure and complexities of proteins. Functional proteomic can find the role of proteins as biologically with protein-protein interactions and PTM analyses. Besides, functional proteomics is necessary for disease mechanısm and drug-protein interactions(Chandrasekhar et al. 2014).

## 1.4 Mass Spectrometry-Based Proteomics

Mass spectrometry has an important place in proteomic studies. Because it provides a profile of global proteins and information about the expression of the protein. These types of studies, with mass spectrometry, are known as mass spectrometry-based proteomics (Cravatt, Simon, and Yates Iii n.d.; Creighton 1997).

In a general proteomic analysis, the typical progression is as follows. After the proteins are isolated, protein separation is done via one of 2-DE or LC techniques. Next, enzymatic digestion is performed by a protease (usually it is trypsin). Then steps go on peptide extraction and, MS analysis. Lastly, endings with a database search. If peptides describe with MALDI, the protocol of peptide mass fingerprinting will use. But, if identification occurs thanks to LC, ESI-MS will connect. This approach provides information about the sequence of the protein(Darie 2013).

## 1.5. Separation Techniques Coupled with Mass Spectrometry

MS-based proteomic importantly depends on separation techniques after mass analysis for the simplifying of complex peptide and protein mixtures.

Before proteomic applying, a suitable separation technique is chosen. Commonly using separation operations are two-dimensional polyacrylamide gel electrophoresis (2D-GE) known as gel-based and liquid chromatography (LC) familiar as the gel-free method(Yates, Ruse, and Nakorchevsky 2009).

### 1.5.1. Electrophoretic Techniques

Two-dimensional gel electrophoresis (2DE) is a technique that was introduced by O'Farrell (O'Farrell 1975) and is used to remove biological confusion. Spots are formed in 2DE analysis and here each spot represents specific a protein, a comment can do

about the abundance of this protein produced in the cell, parallel as the intensity of the spot.

This method works by combining the separation technique in 2 different directions (Figure 1.15).In the first direction, a charge-dependent separation is realized in a pH gradient with isoelectric focusing (IEF). Charged molecules immigrate to pH gradient until it sees the spot of isoelectric and exactly at this point electrical charge is zero, namely, it is non-motility.In the second direction, according to molecular weight (MW) separation is occurred via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS is an anionic detergent. It severs protein molecules from each other and also it has a negative charge so, peptides collect a highly negative charge. As a result, the electrical charge of all protein is equalized and separation is happened by increasing the intensity of the gel and in the direction of electric.The following step is the visualizing of separated proteins. This is done with various staining methods. Lastly, spots are excised from the gel and analyzed with generally MALDI-TOF.

Although a powerful tool for protein analysis there are some drawbacks. One of them is time-consuming because of cutting spots of proteins and sections of staining. It has a small dynamic range. Therefore, some protein that has high molecular weight cannot be detected low soluble proteins cannot appear on the gel. In order to overcome all this challenge, new proteomic approaches are researched(Berkelman and Stenstedt 2002).

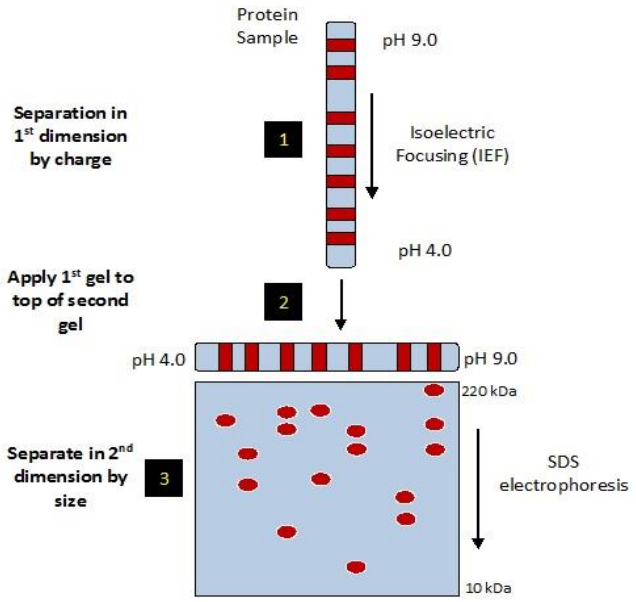



Figure 1.14. Schematic representation of 2D-SDS-PAGE
(Source: N.C. Kendrick,2008)

### 1.5.2. Chromatographic Techniques

The main event in chromatographic techniques based on separations is the interaction of components with a stationary and mobile phase. The molecules are held up according to their affinity to the stationary phase and are dragged along the stationary phase with the flow of the mobile phase. Namely, the work of typic a liquid chromatography depends on the retarding time of the compounds in the column.

### 1.5.2.1 High-Performance Liquid Chromatography(HPLC)

Gel-based proteomics has been used as off-line sampled with a pulsed ionization tool after spots are excised from the gel. But, the high-pressure liquid chromatography (HPLC) is combined with ESI. In this way, an on-line procedure is generated. Asa result, the ion-suppression effect is reduced, the intensity of low abundance peptides are increased, and impurities are removed(Eckerskorn et al. 1992).

This tool has very advantages such as reproducibility, speed, high recovery, and high resolution when compared with the gel-based method. Thanks to all these properties it became the main method for biological applications. Ion exchange (IEX), reverse phase (RP), hydrophilic interaction chromatography (HILIC), affinity, and gel filtration are chromatographic techniques using in the MS-based proteomics. RP from among these is essential to LC/MS (Aguilar 2004)

### 1.5.2.2 Reversed-Phase Chromatography (RP)

There are two situations that effect separation in this mode. The first of these, different hydrophobicities in components of the mixture. Another sitıation is the interaction of the sample with a liquid mobile phase (polar) and a stationary phase (non-polar) and in here polar firstly are eluted.

The stationary phase of reversed-phase columns consists of long-chain hydrocarbons that bonded to the silica surface. The most common RP stationary phase is C18. The solvent system in RP includes water or a buffer solution, and organic solvents. Methanol or acetonitrile frequently are used as an organic solvent.

### 1.5.2.3 Multi-dimensional Separation

The idea of multi-dimensional separation connects more than one separation techniques in order to develop the resolving power. This approach is intersected individual separation methods which use different molecular properties of molecules, so orthogonality is provided(Giddings 1984).After the enzymatic digestion, more than one peptides appearance from each protein in the proteomic studies. For this reason, 1D-LC is unsatisfying due to low peak capacity to decrease obtained complexity so, thanks to properties of higher resolution(it increase dynamic range and proteome coverage) 2D-LC is preferred rather than one dimensional LC.

In multidimensional techniques, the first dimension separation for peptides and protein has happened via ion-exchange chromatography (IEX). Then, fractions are separated with RP-HPLC and they analyzed by ESI-MS(Opiteck et al. 1997).

## 1.6 Protein Identification by Mass Spectrometry

The bottom-up proteomics (BUP) and top-down proteomics (TDP) are dissimilar and complementary flows which for identify the proteins by using mass spectrometry (Figure 1.16).

In the top-down approach, proteins are introduced into the ESI without enzymatic digestion and they are recognized by high-resolution mass spectrometry. This identification includes masses of both protein and its fragment ions. According to the quality of the obtained spectrum, all possible modifications present on the molecule can characterize. By contrast to top-down proteomics, with the bottom-up proteomics, proteins are first hydrolyzed into smaller peptides using an enzyme such as trypsin. Trypsin cleaves peptide bonds on the C terminal of arginine or lysine residues. These smaller peptide chains are separated by liquid chromatography (LC). Then, separated peptides convert to the gas phase via ESI.

The resulting ions are profiled to determine the mass of precursor ion (MS), next, these molecules are fragmented and second, mass spectrometry is obtained that is mass of the fragmentation (MS/MS). Then, the collected data is compared with a protein database. This provides identification of amino acid sequence from the pattern with helping of peptide mass fingerprint (PMF) of each peptide.

In addition to all think, gel-free methods mean the shotgun approach in which protein mixture is not pretreated for the separation, digesting more than one protein together in a sample solution (Wolters, Washburn, and Yates 2001).

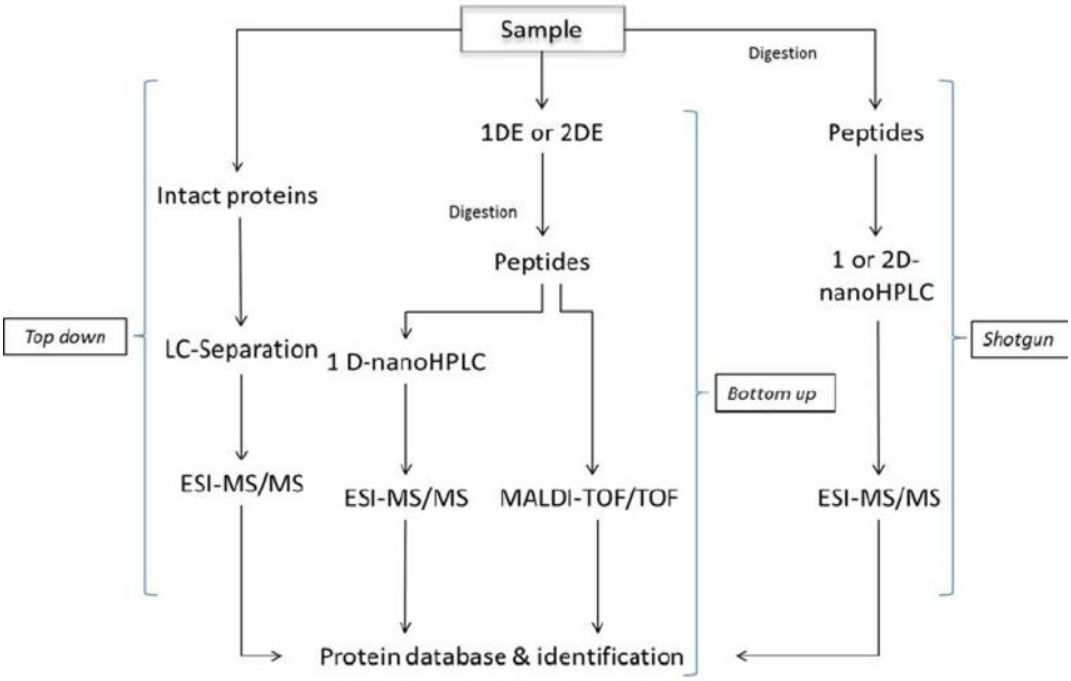



Figure 1.15. Proteomics approaches
Source: (E. Razzazi-Fazeli, 2011)

## 1.6.1 Shotgun Proteomics

Mass spectrometry allows for the identification and characterization of biological samples with fragmentation in shotgun methods. After this step, the isolated protein mixture is digested via an enzyme that generally is trypsin. Later, the acquired peptide mix is separated with a one or multi-dimensional HPLC, then automatically analyzed for MS and MS/MS data, respectively. Next, this data is compared with a suitable protein database to obtain peptide sequence match.

As in all proteomic technologies, sample preparation is an important section in the shotgun approach. The too complex of the peptide mixture is obtained because of enzymatic digestion, so it can result in the same peptide sequences in different proteins or isoforms. This causes loss of the connectivity between the peptides and associated proteins. For preventing this, peptides countable degree should be decreased before the section of LC-MS/MS. In this purpose, gel-free multi-dimensional methods that link with MS are used. As for the off-line and the on-line there are two kinds.

In off-line situation, fractions of samples are collected after the first dimensional and then they are reinjected again into the second dimensional. But, two chromatographic methods are coupled to each other; eluted samples are transferred directly into the other column. Fractions collection is not happening. The column-switching system or multidimensional protein identification technology (MUDPIT) helps in here.

The multi-dimensional chromatographic technique realizes to separation in line with orthogonality. Thereby, RP is not chosen for both dimensions of 2D-LC. Due to orthogonality in proteomic approaches, different RP that have not the same pH values are chosen. High-pH RPLC is a semi-orthogonal compared to low-pH. High-pH RPLC–low pH RPLC is an option to increase proteome coverage for shotgun analysis(Wang et al. 2011).

## 1.7 Protein Sequencing

Protein sequencing is involving the amino acid sequence of protein or peptide. Edman degradation is one of the traditional techniques for this. In this progress, firstly phenyl isocyanate is reacted with the N-terminal amino group of the polypeptide chain; at the result, the derivatized residue is obtained that hydrolyzes amide bond. Then, removed N-terminal amino acid is determined by HPLC and comparison with standards. Edman degradation is not preferred proteomics studies. Because peptides that have moreover nearly fifty amino acids long cannot be sequenced reliably with it(Smith n.d.). Another technique is Western blot that uses the interaction of antigen-antibody to find a target protein (Towbin, Staehelint, and Gordon 1979). Now, mass spectrometry methods have been using as a modern technique for protein or peptide sequencing. Tandem mass spectrometry has a key role in proteomic studies. In these type spectrometries that combined the same kind of analyzers (QqQ), as first, an ion is selected as the parent ion, then, in the second analyzer, the collision with inert gas and daughter ions occur.Next, the last analyzer measures m/z value of occurred ions. Actually, all these steps are collision-induced dissociation (CID) that has been known as MS/MS technique.

## 1.8 Database Search Engine

The last requirement is to identify the proteome is search engines. Achieved spectral data from tandem MS measurement is searched a sequence database using search

engines like Mascot, the desired database is selected usually as NCBI or UniProt and it can be obtained in FASTA format. Lastly, some parameters are privatized for the search. These are enzyme type, the number of allowed miscleavages, fixed and variable modifications, peptide, and MS/MS tolerances and peptide charges, data format, and ınstrument. Also, another important parameter is the decoy search. It presents sequences from databases for the search that have been reversed or randomized. At the same time, it refers to the false discovery rate (FDR) is an assessment of data quality.So low values than %5 should select for proteomic studies.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1. Food-Borne Diseases

Foodborne pathogenic bacteria can grow and multiply on food that is produced and stored in non-hygienic environments causing foodborne diseases. Thus, the consumption of contaminated food can cause serious health problems resulting in hospitalization and even death. The food-borne diseases that also known as food poisoning includes also zoonotic diseases that transmitted between animals and humans. The foodborne diseases can be fatal for some groups of people; especially the newborns, the elderly, pregnant women and also people with a weaker immune system. Even today, foodborne pathogens can cause outbreaks and result in economic losses as well as health problems. If two or more people become ill or die after the ingestion of food from the same source, food-borne diseases are defined as an outbreak. According to the Foodborne Disease Outbreak Monitoring System (FDOSS), 5,760 outbreaks, 100,939 sicknesses, 5,699 hospital treatment, and 145 deaths were reported between 2009 and 2015 ("Surveillance for Foodborne Disease Outbreaks--United States, 2009-2010." n.d.). L.*monocytogenes*, *Salmonella*, and E.*coli* O157: H7 strains are the most common agents with the highest share in outbreaks by deaths (82%) and hospitalizations (82%) There are lots of pathogen microorganisms known to cause of the food-borne diseases however *Listeria monocytogenes* is one of the important bacteria among the others (Table2.1) (Adley, Packaging, and 2016 n.d.).

## 2.2. Listeria Spp.

The genus *Listeria* is an etiologic agent of food poisoning that belongs to the class Bacilli. This genus right now includes twenty species which are *L.aquatica, L.booriae, L.cornellensis, L.costaricensis, L.goaensis, L.fleischmannii, L.floridensis, L.grandensis, L.grayi, L.innocua, L. ivanovii, L. marthii, L. monocytogenes, L. newyorkensis,*

*L.riparia, L.rocourtiae, L.seeligeri, L.thailandensis, L.weihenstephanensis* and *L.welshimer.*

*L.monocytogenes* is one of the known pathogenic species to humans among all *Listeria* species. Several serotypes belonging to this species were identified via somatic (O) and flagellar (H) antigens of factor antiserum as 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7. Among all, the serotype 4b has the highest pathogenicity level. In addition, the mortality rate is very high in patients infected with serotype 4b than any other serotypes (Uçar et al. n.d.).

Table 2.1. The highly dangerous some pathogenic bacteria

| **PATHOGEN** | **ILLNES** | **SYMTOMS** |
|---|---|---|
| *Bacillus cereus (B.cereus)* | *B. cereus* food poisoning | Abdominal cramps, watery diarrhea, nausea |
| *Brucella spp.* | Brucellosis | Profuse sweating, joint and muscle pain |
| *Clostridium botulinum* | Botulism | Vomiting, diarrhea, muscle weakness; can result in respiratory failure and death |
| *Listeria monocytogenes* | Listeriosis | Fever, muscle aches, and nausea or diarrheaIn pregnant women; premature delivery or stillbirt |
| *Salmonella spp.* | Salmonellosis | Diarrhea, fever, abdominal cramps, vomiting |
| *Staphylococcus aureus* | Staphylococcal food poisoning | Nausea, vomiting; abdominal cramps, diarrhea and fever |

### 2.2.1. *Listeria monocytogenes*

*L. monocytogenes* is a Gram-positive, intracellular, non-spore forming, small food-borne pathogen bacteria that can grow best in microaerophilic to anaerobic

environments. It can be motile and actively moves at 25ºC, but this movement is almost non-existent at 37ºC. It is a halotolerant bacterium that can grow at high NaCl concentrations (10-12% NaCl). For example, Jensen, A. et al. showed cells of *L. monocytogenes* that improved biofilm and aggregation in the presence of NaCl (Jensen et al. 2007). It can also grow at broad pH range (3,0-9,5). Even though the optimum grow temperature is 30-37ºC, its psychrophilic nature is the most important feature of *L. monocytogenes* which allows the bacteria to grow even at refrigeration temperatures. The cardinal growth temperatures of *L. monocytogenes* is presented in Table2.2.

Table 2.2. The cardinal growth conditions of *L. monocytogenes*

| FACTOR | MINIMUM | OPTIMAL | MAXIMUM |
|---|---|---|---|
| **Temperature** | 0,1 ºC | 37 ºC | 45 ºC |
| **PH** | 3,0 | 6,0-8,0 | 9,5 |

*L. monocytogenes* commonly present in nature. Various kinds of meat, water grass, soil and feces of human or animals are the most important sources. Animal-to-animal transmission can be via fecal-oral route. Animal-to-human transmission may occur by the contaminated food of animal origins such as meat, eggs, cheese or milk as well as the produce. (Lakicevic, Nastasijevic, and Raseta 2015). Consumption of the contaminated food may cause a lethal gastrointestinal disease called as listeriosis. Listeriosis is a serious food-borne disease caused by opportunistic pathogen *L. monocytogenes* which causes death via food poisoning. Symptoms of this disease appear between 3-14 (up to 70) days after incubation. They can include fever, muscle aches, headache, stiff neck, confusion, loss of balance, and convulsions. Besides listeriosis, *L. monocytogenes* may give rise to some other dangerous conditions such as meningitis and septicemia. Infections during pregnancy can lead to miscarriage, premature delivery, as well as life-threatening infection of the newborn. This pathogen is able to pass some host barriers: the intestinal barrier, the blood-brain barrier and the maternofetal barrier. The disease starts after this transition. Firstly, the bacteria cross the intestinal barrier then it reaches the liver and also the spleen with a pathway of lymph and the blood.

Then the bacteria by hematogenous spreading can arrive at the brain and the placenta. The effect of these conditions increases in high-risk groups consist of pregnant women, newborns, elderly, and immunodeficient patients (McMullen and Freitag 2015). Additionally, listeriosis has zoonotic importance. This disease generally affects to goats, birds, fish and sheep. In adult ruminants, it causes inflammation of the brain as well as neurologic symptoms. Listeriosis is also common in rabbits and rodents and results in a blood infection in these animals (D'Orazio 2014). The foods that are involved with listeriosis outbreaks mainly are unpasteurized milk, ice-cream, raw meats, ready-to-eat products, smoke-dried fishery products and shelled sea products, soft cheeses, frozen vegetables, and fruits. The outbreaks of listeriosis related to the ingestion of even at low doses of *L. monocytogenes* with contaminated foods or drink ($10^2$-$10^3$ cfu/mg or cfu/ml). The outbreaks have been reported in different countries such as The United States of America, Germany, Austria, The United Kingdom, Swedish, Czech Republic, Japan. (Table 2.3) In addition to these, between early 2017 and May 2018 in South Africa totally 1,034 events were reported. There were more than 600 deaths and 400 of them was newborn. The biggest and well-known listeriosis outbreak took place in August 2008, Canada. Nearly 40% of patients have lost their lives (Attaran et al, 2008). According to data of Center for Disease Control and Prevention (CDC) (1999), *L. monocytogenes* was the second pathogen with %20 mortality ratio ("BAM: Detection and Enumeration of *Listeria Monocytogenes*FDA" n.d.). Each year the listeriosis is observed approximately 1,600 people and nearly 260 dies. In fact, it can be collaborated with public health regulators to minimize the size and possible consequences of the outbreak. For example, in March 2018 there was an outbreak in Australia with melon, but the impact was limited by the source was quickly identified and the help of the International Network of Food Safety Authorities (INFOSAN) (Salama et al. 2018). In spite of all these consciousness and precautionary warnings, the CDC reported a new outbreak in April 2019 which was caused by sliced delicatessen meat and cheese. A total of 8 people were reported to be infected and one of them died. A high dose of penicillin or ampicillin or amoxicillin has been utilized in the treatment of listeriosis. Dosage is important for each drug. Patient with an allergy to penicillin and its derivatives can be treated by trimethoprim/sulfamethoxazole (TMP/SMX). Today, the treatment of bacterial infections is becoming increasingly difficult due to the development of antibiotic resistance in bacteria.

This undesirable situation encouraged the exploration of natural and new antimicrobial agents as an alternative to ones in use for bacterial infection treatment (Janakiraman 2008; Felipe. 2012). The listeriosis begins with the invasion of the bacteria to human cells and becomes intracellular. During the invasion, many different proteins play role especially the internalin family (InlA and InlB). Internalin proteins affect the virulence of the pathogen (Dussurget, Pizarro-Cerda, and Cossart 2004). In recent years, some new internalin proteins such as InlJ, InlI, and InlK were identified in literature (Sabet et al. 2005; Carbonaro 2004). *L.monocytogenes* mainly completes the intracellular life cycle in five steps. Firstly, it invades the host cells by the interaction of InlA and InlB with surface receptors (E-cadherin and Met) which is special to them. Next, *Listeria* escapes from vacuole by the action of pore-forming toxin (listeriolysin-O, LLO) and phosphatidylinositide phospholipase C (PI-PLC). Then it spreads by actin polymerization in which ActA promoting cell to cell spreading of the pathogen. Lastly, contact with neighboring host cells occurs and bacteria localizes in a double membrane vacuole (Luque-Sastre et al. 2018).

Table 2.3. Cases of Listeriosis

| YEAR | REGION | SOURCE | CASES/DEATHS |
|---|---|---|---|
| 1988 | İngiltere | Pâté | 355/94 |
| 1992 | France | Jellied pork tongue | 101/21 |
| 2006 | Germany | Soft Cheese | 189/26 |
| 2009 | Austria | Curd Cheese | 34/8 |
| 2011 | USA | Cantaloupe | 147/33 |
| 2012 | USA | Ricotta Cheese | 22/4 |
| 2013- 2014 | Denmark | Pork, meat products | 41/17 |
| 2015 | USA | Ice-Cream | 10/3 |
| 2016 | USA | Packaged Salads | 19/1 |
| 2017 | USA | Raw Milk Cheese | 8/2 |
| 2018 | USA | Deli Ham | 4/1 |

## 2.3. Phenolic Compounds

Phenolic compounds are the second metabolites produced by all plants, are the chemicals that have various roles in their plant metabolisms as well as the natural defense mechanism against stressed conditions, predators and pathogenic organisms. (Duthie, Gardner, and Kyle 2003).

Phenolic compounds have received much attention recently due to their biological activations such as antimicrobial, antioxidant, antidiabetic, anticarcinogenic, cardio-protective and anti-inflammatory properties (Rao et al. 2007). For instance, baicalin that is a flavone glycoside has been shown to anti-inflammatory effect on the treatment of Human Immunodeficiency Virus, HIV (Li et al. 2000). Quercetin that is a flavanol derivative can be used to therapy of a patient with asthma and reported helpful to the person that has infectious HIV and diabetes (Yao et al. 2004).

According to the carbon skeleton, phenolic compounds are classified as simple phenols and polyphenols (Hurtado-Fernández et al. 2010). Phenolic acids that belong to the simple phenols sorted into two groups which are hydroxybenzoic acids and hydroxycinnamic acid and these are having the chemical structure C6-C1, C6-C3, respectively (Tripoli et al. 2005). (Figure 2.1.) In hydroxycinnamic acids, there is the carboxylic group that linked to an ethylene group to the benzene ring, but in hydroxybenzoic acids, the carboxylic group is directly attached. Hydroxybenzoic acids are more polar than hydroxycinnamic acids due to having side chains. Chains can help the transfer of acid through the cell membrane, this is important for inhibition (Vaquero, Alberto, and de Nadra 2007). 4-hydroxybenzoic acid, gallic acid, vanillic acid are some of the hydroxybenzoic acids. 3-hydroxyphenyl acetic acid, caffeic acid, ferulic acid, p-coumaric acid, and o-coumaric acid are sorts of cinnamic acids.

In this study, the antimicrobial activity on *L. monocytogenes* of 3-hydroxyphenyl acetic acid (3-HPAA) and 4-hydroxybenzoic acid (4-HBA) that belongs to the different groups were investigated in this study. 3-HPAA with a molecular weight of 152,15 g/mole (Sigma-Aldrich), has a 2-carbon side chain and one hydroxy group on the benzene ring, 4-HBA has a hydroxy at C-4 of the benzene ring.

The antimicrobial and antioxidant properties of the phenolic compounds can change with the number and the position of the hydroxyl groups on the benzene ring (Lima et al., 2019; Heleno et al., 2015) (Figure 2.2). Thereby, at the same time, phenolic acids also were compared among themselves.

Literature studies presented that phenolic compounds and phenolic-containing plant extracts may be potential antimicrobial agents against Gram-negative or Gram-positive pathogenic bacteria.

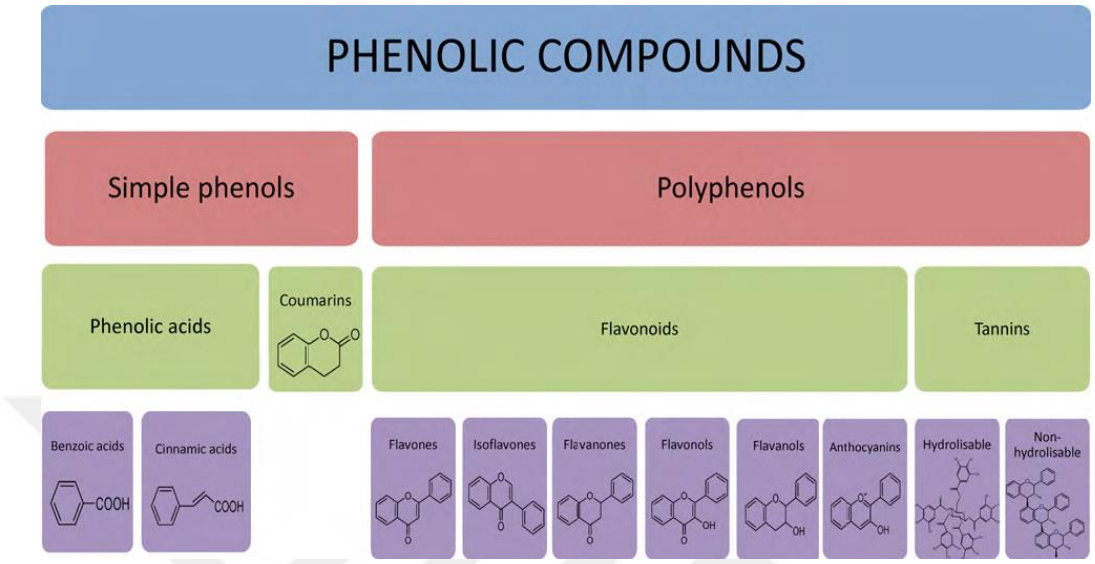
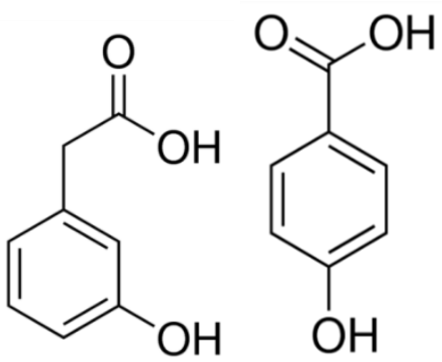



Figure 2.1 Sorting and structures of phenolic compound
(Source: Hurtado-Fernandez et al., 2010)

In some study is shown that Gram-positive bacteria are more susceptible than gram-negative ones to aromatic substances due to lack of outer membrane (Zhang et al. 2011).Cueva et al. tested the effects of 13 different compounds (two of them are 3-HPAA and 4-HBA) on some pathogenic bacteria. Results indicated that the benzoic and phenylacetic acids have the highest effect on pathogens. In addition, was shown that the strain of bacteria andexposure to dose is important. (Cueva et al., 2010).



Figüre 2.2. Structure of 3-HPAA and 4-HBA, respectively

Phenolic acids also have significant roles in the prevention of diseases (Cueva et al. 2010).The researchers have been lots of studies to an understanding of the effect of phenolic compounds or essential oils on microorganisms.

Prior studies proposed that phenolics inhibit the growth by firstly targeting the membrane of the cell. After the integration of phenolic acid to the membrane, selective permeability is disrupted, acidity increases in the cytoplasm, so it exerts antimicrobial activity by denaturation of enzymes and proteins. A.O. Gill et al. demonstrated in a study the disruption of some bacteria of cellular membranes by plant oil aromatics. Bactericidal activity of eugenol, carvacrol, and cinnamaldehyde on pathogens which are *E. coli O157: H7* and *L. monocytogenes* of serotype 1 found. While at 10 mM eugenol or carvacrol increased the taking of propidium iodide by E. coli and *L. monocytogenes*, at 10 mM of cinnamaldehyde caused a slight increase in propidium iodide for E. coli, on *L. monocytogenes* it had no effect. As a result, carvacrol and eugenol with bactericidal concentration disrupted the cytoplasmic membrane and increased permeability (Gill and Holley 2006).

In another study, Lv et al. explored the impact of a mix of essential-oil that are oregano, basil, bergamot and perilla oils on *Staphylococcus aureus* and *Bacillus subtilis* (gram-positive bacteria), *Escherichia coli* (gram-negative bacteria), and *Saccharomyces cerevisiae* (yeast). As conclusion, cell death was observed (Lv et al. 2011).

In another study, Xiao Shen et al. investigated the antibacterial effect of extract of blueberry (Vaccinium corymbosum L.) on *L. monocytogenes* and *S. Enteritidis*. In consequence, they showed blueberries have inhibitory activity on them. Also, chlorogenic acid, ellagic acid, quercetin, and quercetin-3-galactoside that are blueberry phenolic compounds displayed an inhibitory effect as linked to the dose (Shen et al. 2014).

Although the inhibitory, antimicrobial and bactericidal activities of phenolic acids have been shown, the molecular mechanisms of action of these substances have not been clearly elucidated. Proteomic approaches have become indispensable in the elucidation of the mechanisms of microorganisms. Nowadays, these types of studies can understand how bacteria react to antibiotics by examining proteins and may provide extra and information about the mechanisms of disease-making (Pérez-Llarena and Bou 2016).

Lanciotti et al. used the ethanol, Citral, carvacrol, E-2-hexenal, and thyme essential oil as a natural product to inhibit *Listeria monocytogenes* Scott A cells and analyzed the proteome changes of cells by two-dimensional gel electrophoresis.

As a result, totally of 223 proteins were found and they were identified with MALDI-TOF-TOF. The database search was done by the Mascot program. Proteins mainly were related to cell morphology and mobility, ribosomal proteins, general stress response, and nitrogen metabolism. Cells adapted to these products and showed improved survival (Lanciotti et al. 2019).

Miyamoto et al. were investigated the effect of nisin that is an antibacterial peptide on *Listeria monocytogenes* ATCC 7644 cells. Proteins were by LC-MS/MS as control versus nisin treated ones. Consequently, 179 proteins were identified. In nisin treated cells, antioxidant proteins were identified such as catalase and Dps. Actually, this indicates the activation of defense mechanisms. Besides, there was a higher abundance of flagellin. This situation allows the bacterium to evade from a nisin-containing environment (Miyamoto et al. 2015)

## 2.4. Aim of the Thesis

The purpose of the thesis is to perform the proteome analysis of *Listeria monocytogenes* 4b cells in the presence of phenolic acids that are 3-hydroxyphenyl acetic acid and 4-hydroxybenzoic acid against the control group by the shotgun proteomics approach. Achieved results may elucidate to understanding mechanisms of molecular action by explaining differential proteins that cause a change. In addition, results can evaluate in order to develop novel and natural antibacterial-candidate therapy methods as.

In the study, 3 hypotheses were tested:

I. If 3-hydroxyphenyl acetic acid and 4-hydroxybenzoic acid have antibacterial effects, then the treatment of *L. monocytogenes* with subinhibitory concentrations of phenolic acids would inhibit the growth of bacteria. On the contrary, if *L. monocytogenes* develop resistance against these phenolic acids, it would create a phenolic acid-resistant bacterial population.

II. If the treatment of bacteria with phenolic acids affects the growth pattern, it would generate changing with subinhibitory concentrations of phenolic acids in the protein profiles.

III. If more extensive data achieves with the shotgun proteomics, results would ascertain mechanisms of molecular action.

# CHAPTER 3

# EXPERIMENTAL PROCEDURES

## 3.1 Activation and Single Colony Production of Bacteria

The long-term stock cultures of the bacteria are kept in a medium containing 20% glycerol at -80$^{o}$C. Here, glycerol stabilizes bacteria, so it prevents damage to cell membranes and keeps the cell living being. Although bacteria can be preserved in long-term media for several years, for short-term maintenance, pure cultures are sub-cultured on plates every week and then plates are kept on at 4 oC of the purpose of protecting the viability. At the end of one week, cultures are transferred to a new fresh solid-medium.
In this study, Brain heart infusion broth (BHI, Fluka) and BHI agar (Fluka) were prepared for the growth of *L. monocytogenes* as a medium. After stock culture completely melted, for the activation of bacteria, two times inoculation was done (%4 and %2, respectively), it incubated at 37 $^{o}$C for 18 hours without shaking. The increase of bacteria appeared as turbidity in the broth. The streak plate method was applied in order to have a single colony of *L. monocytogenes*, and then incubated along 18 hours at 37 $^{o}$C.

## 3.2 Antibacterial Activity of Phenolic Acids

### 3.2.1. Preparation of Bacterial Culture

After single colony production, a colony of *L. monocytogenes* was inoculated into 4 ml of BHI media, incubated at 37$^{o}$C for 18 hours. Base on the previous studies, the optical density (OD) of *L. monocytogenes* was regulated to 0.18 which corresponds to the bacterial load of 1x10$^{8}$ colony-forming unit/ milliliter (cfu/ml) with a spectrophotometer at a wavelength of 600 nm. Then, this value was set to 10$^{6}$ cfu/ml in series dilution with the BHI broth medium as a starter culture. Dilution 1x10$^{4}$ cfu/ml was chosen as working solutions to designate the antimicrobial effect because of the sufficient amount for foodborne pathogens. Lastly, the spread plate of 10$^{5}$ cfu/ml and 10$^{6}$ cfu/ml dilutions were used to colony count and validation.

### 3.2.2. Preparation of Phenolic Acid Solutions

In this study, 3-Hydroxyphenylacetic acid (3-HPAA, Sigma Aldrich) and 4-Hydroxybenzoic acid (4-HBAA, Sigma Aldrich) were used as the phenolic acid. In order to see desired concentrations of phenolic acids as final concentration, they were prepared by using two-fold in the medium in the dark. (Table 3.1). 100µl of each concentration was mixed with 100µl of working solution ($10^4$ cfu/ml) in 96-well microtiter plate (Bio-Grainer, Germany), dilutions concentration was halved, and then optical density (OD) was measured at 600 nm for 0h via Thermo Multiscan Spectro Reader (Finland), after incubation at 37$^o$C, it measured for 24h. In outcome, the lowest substance concentration without bacterial growth was determined as minimum inhibitory concentration (MIC). Then, the spread plate technique was performed from $10^5$ cfu/ml and $10^6$ cfu / ml dilutions for counting and confirmation. The desired value was calculated for $10^5$ cfu / ml dilution because less than 30 single colonies were obtained that statistically unessential for $10^6$ cfu / ml dilution.

Table 3.1. Concentration of Phenolic Acids

| **Phenolic Acids** | **Concentration of plate** | **Concentration of tubes** |
|---|---|---|
| **3-HPAA and 4-HBA** | 1 mg/ml<br>1.5 mg/ml<br>2 mg/ml<br>2.5 mg/ml<br>3 mg/ml<br>3.5 mg/ml<br>4 mg/ml<br>5 mg/ml | 2 mg/ml<br>3 mg/ml<br>4 mg/ml<br>5 mg/ml<br>6 mg/ml<br>7 mg/ml<br>8 mg/ml<br>10 mg/ml |

## 3.3 Total Protein Extraction of Treated Bacteria

### 3.3.1. Preparation of Bacterial Culture

The starter culture was obtained by incubating a single colony of Listeria monocytogenes with 4 ml of BHI broth medium at 37 ° C for 18 hours, as in the previous was explain the method. Then, optical density (OD) was set necessary level (0.18) at 600 nm via Thermo multi-scan spectra reader and approved in terms of bacterial load, viable colony plate count.

### 3.3.2. Phenolic Acids

Inhibition value that retarded the growth at 18th hour of concentrations of phenolic acids was determined which corresponds to approximately 50 % (MIC50) inhibition value for the protein isolation. These values were given in Table 3.2.

Table 3.2 Minimum inhibitory concentrations of phenolics

| **Phenolics** | **Prepared concentration** | **~ MIC $_{50}$ values (Final concentration)** |
|---|---|---|
| **3-HPAA** | 5 mg/ml | 2.5 mg/ml |
| **4-HBA** | 3 mg/ml | 1.5 mg/ml |

### 3.3.3. Treatment with Phenolic acids

1 ml bacterial culture which has load of $10^8$cfu/ml were transferred 99 ml BHI broth medium thus, starter culture that has a load of $10^6$cfu/ml was achieved. Then, 200 ml BHI broth that has 4 ml of bacteria from the starter culture was prepared for 3 different flasks, (Phenolics groups, and control). Phenolic acids that are 3-HPAA, 4-HBA was weighed in different flasks as 1 g and 0.6 g, respectively and then added 20 ml distilled water, 180 ml BHI broth to every flask and they mixed with flasks that involve bacteria. As a result, at 400 ml final volume, 2.5 mg/ml of 3- HPAA and 1.5 mg/ml of 4-HBA was achieved. Next, the optical density (OD) of stressed bacteria was gauged at 600 nm

via spectrophotometer (Thermo, Multiscan Spectro Reader) for 0h and 18 hours. Later, in small volume serially dilution (1: 9, v / v) was done with 0.5 ml of four tubes.
The spread plate method was applied for 102 and 101 diluted tubes, calculation of cfu/ml done in the same way as section also 2.3.

### 3.3.4. Protein Isolation

After the samples were transferred into the centrifuge tube (Beckman), centrifuged with 10.00 g, at 4 $^{o}$ C for 20 minutes via a large bench centrifuge (Sigma, 6K15). Observed the pellet was washed in 20 ml of 0.85 % NaCl of a solution, again centrifuged with 20.000 g, at 4 $^{o}$ C for 20 minutes. Pellet was resuspended in 2 ml phosphate buffer saline (PBS) solution and then, in dry-ice, sonication was performed as for 9 seconds sonic, 9 seconds of an interval during to break up cell walls and membranes of bacteria. Next, cells were centrifuged with 20.000 g, at 4 $^{o}$ C along 20 minutes to remove the residual of cells, this time supernatants which include proteins were transferred into the clean 50 ml of falcons.

## 3.4. Make Sample Suitable for Mass Spectroscopy

### 3.4.1. Purification, Pre-Concentration of Proteins

The purpose of with remove undesirable substances that slow down the flow in protein samples was stirred with cold acetone, which causes precipitation of the protein. Besides, when the sample was purified, the pre-concentration of the samples were also adjusted. In order to have a pellet from protein, a supernatant was removed after thecentrifuge and the pellet was dissolved in resuspension buffer which suitable with the flow. In this study, the solution was prepared as 1 volume of sample (2ml), 4 volumes of acetone (8ml), and incubated at -20 $^{o}$C through overnight. Then this solution was centrifuged with 14.000 g, at 4 o C through 15 minutes. After centrifuge resuspension buffer was prepared which contains 7 M Urea, 2 M Thiourea, 0.1 M Tris-HCl (pH: 7-8) that known as rehydration buffer (2.1 gram urea and 0.76 gram thiourea were dissolved in 2ml Tris-HCl). Then it was complete to 5 ml. Next, the supernatant was detracted from tubes and at the bottom the pellet was mixed with resuspension buffer. Thus the pellet was re-dissolved.

### 3.4.2. Bradford Assay for Quantitative Analysis

To have the quantification of total protein, Bradford assay protocol also known as Coomassie brilliant blue G-250 dye was carried out. Here, according to the change of color in the presence of a standard protein was measured the desired concentration of proteins.

In the protocol, firstly stock of bovine serum albumin (BSA) solution and then 8 dilutions of them were prepared in order to have achieved standard curve. Then, 240 µl of Bradford reagent, 5 µl of resuspension buffer, and 5 µl of all concentrations of standards were put into the hole of a 96-well microplate. At last, a calibration curve was done in Microsoft Office Excel. Another hole of the plate was loaded with 240 µl Bradford reagent, 5 µl of ultrapure water, and 5 µl of protein samples. The change of color was evaluated by the spectrophotometer. Then, unknown concentrations were specified according to the previous calibration curve.

### 3.4.3. In solution digestion

Proteins were digested for mass spectrophotometry. Hydrolysis of the peptide bonds was performed with the commonly used protease trypsin. Then, Dithiothreitol (DDT) and 2-Iodaacetamide (IAA) were used as reduction and alkylation buffer, respectively. Thereby, the disulfide bonds were cleaved.

Moreover, the molecular weight cut-off (MWCO, Merck Millipore) filter was utilized so that removing excess urea, some impurity. In digestion procedure of this study, the following steps were applied.

- Firstly, at the end of the quantification analysis, the sample concentration was determined as a 400 µg of total protein every sample.
- Each sample and enough of amount resuspention buffer together was put to into10 kDa molecular weight cut-off filter tube.
- 5 µl of 0.2 M DDT was prepared in 50 mMTris-HCl, pH: 8,5. Then this reagent was added the tube and waited through one hours.
- 20 µl of 0,2 M IAA was prepared same way of DDT in the dark, waited along one hours, after combined in the tube.
- 20 µl of 0,2 M DDT again was added for non-reacting IAA and waited for one hours.

- Then, samples were centrifuged during ten minutes with 14.000 rpm of speed due to decontamination from urea or thrioure.
- As washing solution 200 µl of 50 mMTris-HCl was used and samples was washed two times, again centrifuged.
- Trypsin solution was prepared with on rate of 1: 50 (enzyme: sample) in 50 mM of Tris-HCl.
- The final concentration for trypsin was 48 µg/ml. 200 µl of trypsin solution was annexed to each sample and held at 37 $^{o}$C in tubes (Protein Lo-Bind, Eppendorf) during overnight.
- Lastly, after incubation, the volume of samples was evaporated in a rotary till nearly 120 µl. (Actually, the volume of the loop was 100 µl but in the purpose of taking a precaution 120 µl of the volume was evaporated.)

### 3.4.4. Fraction Concatenation and LC-MS/MS

Complicated protein mixture has achieved after in-solution digestion section. In order to reduce complexity, a pre-separation step was done thanks to off-line HPLC fractionation. Fractionation was done led by SHIMADZU Prominence Ultra-Fast Liquid Chromatography with LC Solution version 1.25 SP3 software.A high pH reverse phase (RP) $C_{18}$ column that has sizes of 25 cm x 0, 46 cm x 5 um was utilized.

Then mobile phases were prepared. When mobile phase A was prepared ultrapure water in 10mM ammonium formate while mobile phase B was prepared 90% acetonitrile in 10mM ammonium formate and pH was set to 10 for both them.Also, injection volume was 100 µ,temperature of column and flow rate were set as 40$^{o}$C and 0.5 ml/minute, respectively.According to binary gradient LC time programme as given table in 3.3, samples were fractionated via a collector (Sunchrom Micro Fraction Collector) into 96 well plates for better separation orthogonality and they were collect with a certain an order in 2 ml of tubes.This order displayed in figure 3.1. After collecting, 12 fractions have achieved for each sample. Following, whole fractions of every sample exactly evaporated in a rotational vacuum concentrator.

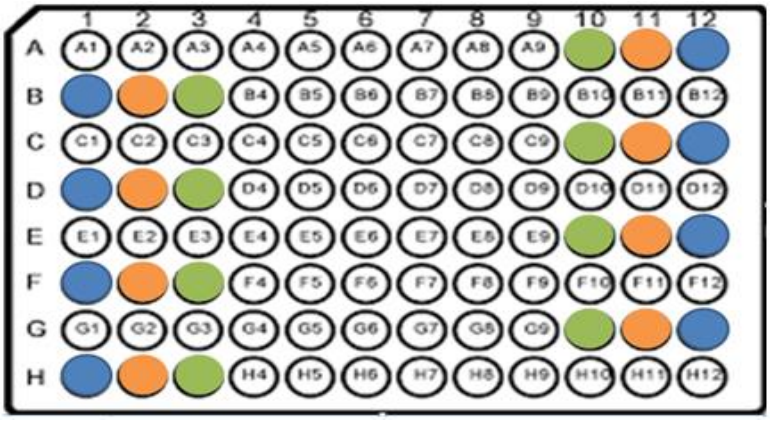


Figure 3.1 Collection order of fraction

Firstly, 20 ul buffer that include 2% acetonitrile, 0.1% formic acid was added every fractions. Before centrifugation of 10 minutes, fractions were holded in ultrasonic bath and on vortex during 15 and 5 seconds, respectively.

Then, in order to wet resin that found in ZipTip pipette tips 10 ul of %100 acetonitrile was dragged and droped for minimum three times, in the similar way, for condition of the tip 10 µl of %1 formic acid was done. Secondly, tips were treated with 15 cycle of sample for maximum binding. Then, tips were washed with 10 µl of %1 formic acid. For elution, 50% and 75% acetonitrile were used 2 times and one time, respectively. So, nearly total volume of 5 ul has achieved in lobindig tubes. Finally, 25 ul of 0.1% formic acid was added in this tube for the purpose of obtaining volume of 30 ul. All fraction of every sample was analysed two times to get technical repeat by reversed phase LC-ESI-MS/MS using Ultimate 3000 HPLC system (Dionex).

In this analysis size of used column (Sigma Supelco Ascentis®) was 15 cm × 500 µm x 2.7µm. Also MS profiling spectra were obtained using LTQ XL ESI Ion trap Mass Spectrometer (Thermo Scientific).Mobile phases (A and B) was prepared for LC analysis. Phase A is 0.1% formic acid in ultrapure water and phase B include 0.1% formic acid in acetonitrile.

Tubing of HPLC was connected to ESI, throughout the analysis spray voltage was maintained at 3.00 kV and injection volume as 10 µl; flow was defined as 5 µl/minute. LC-MS/MS time programme and data dependent acquisition other parameters for mass spectrometry were choosen as in Table 3.4 and the runs was complated.

Table 3.3 LC gradient programme

| **Time (min)** | **Mobil Phase B (%)** |
|---|---|
| 10.00 | 0 |
| 10.00 | 5 |
| 50.00 | 25 |
| 57.00 | 30 |
| 64.00 | 40 |
| 71.00 | 80 |
| 80.00 | 80 |
| 90.00 | 0 |
| 95.00 | 0 |

Next, raw datas that have individual runs were obtained ve they were converted to mascot generic file format (mgf) by using Thermo Proteome Discoverer 1.4. and then are merged to get single data for Mascot server.

### 3.4.5. Protein Identification over Bioinformatics Analysis

After merged files were obtained, a database related to L.Monocytogenes 4b was found out from the UniProt website (www.uniprot.org) and it was downloaded with FASTA format. Next, all these files were scanned by using the Mascot server version 2.3 (www.matrixscience.com).Parameters used in Mascot for all data were given in Table 3.5. Apart from these parameters, monoisotopic mass is marked and the decoy database that provides random matches were selected.At the end of the scanning, a report was obtained.

There, the name of common and differential proteins between L.*monocytogenes* samples which are 3-HPAA, 4-HBA, and control was copied to the excel sheet. As the last step, a Venn diagram was drawn by VIB from Ghent University for each sample to in detail see an association between proteins.

Table 3.4 LC-MS/MS time programme and parameters

| **LC Gradient Elution Method** | | **MS Data-Dependent Setting Parameters** | |
|---|---|---|---|
| ***Time(min)*** | ***Mobile Phase B*** | **Scan events (SE)** | 15 |
| 0.00 | 2% | **MS Scan Range** | 400-800 |
| 4.00 | 2% | **Activation Tipe** | CID |
| 5.00 | 5% | **Default Charge State** | 2 |
| 6.00 | 8% | **Isolaton width** | 2.0 |
| 36.00 | 22% | **Activation Time Q (ms)** | 30.00 |
| 39.00 | 35% | **Repeat Count and Duration** | 1-30 |
| 42.00 | 60% | **Exclusion List and Duration** | 500-30 |
| 45.00 | 90% | **Signal Treshold (counts)** | 5000 |
| 50.00 | 90% | **Normalized Collison Energy** | 35 |
| 55.00 | 2% | **Data Type** | Centroid |

Table 3.5 Conditions for Mascot server

| | |
|---|---|
| **Database** | LM4b |
| **Enzyme used in digestion** | Tyrpsin |
| **Taxonomy** | All entries |
| **Fixed Modifications** | Carbamidomethyl |
| **Variable Modifications** | Oxidation (M) |
| **Peptide Tolerance** | 1.5 Da |
| **MS/MS Tolerance** | 0.6 Da |
| **Peptide Charge** | 2+ and 3+ |
| **Data Format** | Mgf |
| **Instrument** | ESI-TRAP |

# CHAPTER 4

# RESULTS AND DISCUSSIONS

In this study, two different phenolic acids (3-HPAA, 4-HBA) treated *L. monocytogenes* were subjected to mass-spectrometry based shotgun proteomic analysis to understand the antimicrobial action mechanisms of these phenolic acids. The bacteria develop an adaptation mechanism for its survival at the changing or under the stress conditions by regulating their (Katarína et al. 2019).

In this direction, firstly the to understand of the antimicrobial activity of phenolic acids that are 3-HPAA and 4-HBA against *L. monocytogenes*, microtiter plate assay was done. After exposure to the phenolic acids, according to the absorbance measurement that has at the end of 18 h incubation at 37$^{o}$C, growth curves of bacteria were drawn as time (hour) versus optical density (OD, 600nm). Standard deviations were extremely low. Then minimum inhibitory concentrations (MIC) values of each phenolic acid were determined.

As seen in Table 4.1, concentrations of 2.5 mg/ml of 3-HPAA and 3 mg/ml of 4-HBA were the lowest concentration that inhibited the growth of bacteria on *L. monocytogenes* 4b cells. These results showed that both phenolic acids have an antimicrobial effect on these bacteria. Therefore, these concentrations were used in protein isolation as sublethal concentrations.

For the isolation, proteins that belong to all strains (the control and phenolic acids with ones) were isolated, and then they digested with the trypsin. Achieved peptides were fractioned by high pH reverse-phase chromatography, the latest 12 fractions were obtained by concatenation, and each one was analyzed using LC-MS/MS.

At the end of these, to identifying proteins, the database search was performed against the UniProt database with the Mascot search engine. Values of 1.2 Da and 0.6 Da represent default peptide and MS/MS tolerance values of Mascot search engines, respectively, but in this study, these values that are 1.5 Da and 0.6 Da were determined as more suitable tolerances. Also, the target sequence was searched against the decoy database. Purposes of this, as random, to introduce reliable peptide-spectrum match (PSM) and obtain a minimal false discovery rate.

Table 4.1 Percent inhibition values of phenolic acids

| Phenolic Compound | Final Concentration | Percent Inhibition |
|---|---|---|
| **3-HPAA** | 1mg/ml | 3,450712 |
| | 1,5mg/ml | 14,34895 |
| | 2mg/ml | 15,45064 |
| | **2,5mg/ml** | **38,7051** |
| | 3mg/ml | 103,0651 |
| | 3,5mg/ml | 105,939 |
| | 4mg/ml | 104,0794 |
| | 5mg/ml | 102,2039 |
| **4-HBA** | 1mg/ml | 21,91198 |
| | **1,5mg/ml** | **45,79743** |
| | 2mg/ml | 88,87892 |
| | 2,5mg/ml | 101,0939 |
| | 3mg/ml | 101,2015 |
| | 3,5mg/ml | 101,3337 |
| | 4mg/ml | 101,3099 |
| | 5mg/ml | 101,1021 |

After the phenolic acid treatments, changes were detected in the protein profile of *L. monocytogenes*. The Venn diagram shown in Figure 4.1 demonstrates the identified total protein numbers: 99 and 106 proteins were identified in 3-HPAA and 4-HBA

treated *L. monocytogenes,* respectively. On the other hand, 170 proteins were identified in the control group.

## 4.1 Identified Proteins after 3-HPAA Treatment

As seen in the Venn diagram (Figure 4.1), 8 proteins of the identified 99 proteins were overlapped with 4-HBA treated group, 12 proteins with control groups. On the other hand, 79 proteins were only obtained in 3-HPAA treated group.

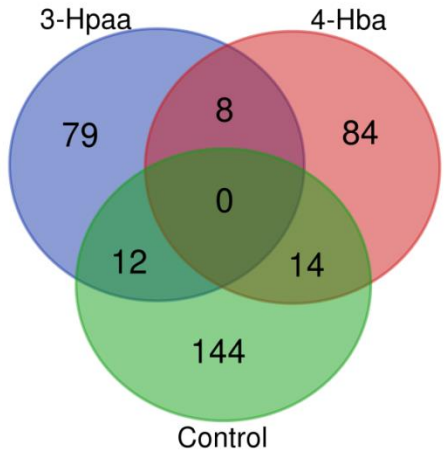



Figure 4.1 Venn diagram showing the numbers of unique and overlapping proteins identified in the presence of 3-HPAA or 4-HBA.

Each protein was functionally categorized based on the UniProt database. Mainly, these groups were relevant as DNA, RNA, ribosomal and protein synthesis, cell wall/ membrane/ flagella, metabolism, redox related, and virulence. Percentages of groups were calculated: 5.6%, 10.1 %, 14.6 %, 14.6 %, 51.7 %, 1.1 %, and 2.2 % for DNA, RNA, ribosomal and protein synthesis, cell wall and membrane, metabolism, redox, and virulence, respectively. (Figure 4.2). As a result, at the end of 3-HPAA treatment, the proteins in which metabolism-related had the highest percentage. These results were not surprising, because the great number of proteins generally participate in the metabolic pathways.

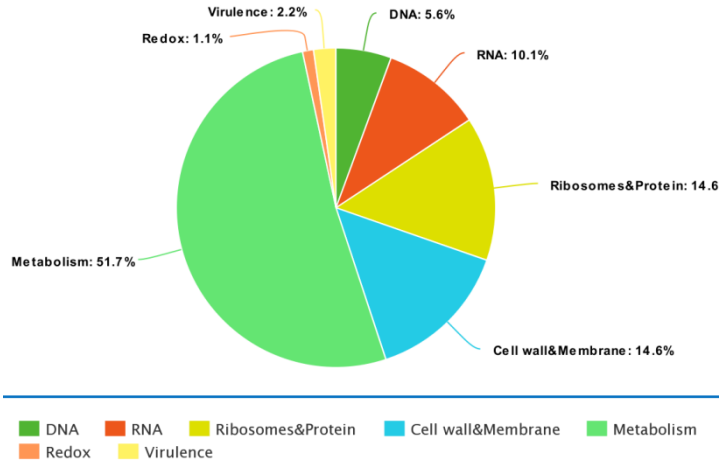



Figure 4.2 The percentages of proteins for each group of function in the presence of 3-HPAA detected proteins

### 4.1.1. DNA Related Proteins after 3-HPAA Treatment

Proteins related to DNA of *L. monocytogenes* that identified in the presence of 3-HPAA were listed as DNA binding (DNA-binding protein, uncharacterized HTH-type transcriptional regulator yulB, and uncharacterized protein ytoI) and DNA repair (UvrABC system protein A uvrA, and DNA polymerase/3'-5' exonuclease PolX) in table 3.1.

DNA-related proteins and their interactions suggest that the bacteria maintain their vital activities to overcome the stress and prevent permanent changes in the DNA sequence. Organisms have three fundamental DNA repair mechanisms to maintain genomic integrity throughout life, including base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). DNA lesions that deterioration on the helix structure of DNA can be repaired via the NER mechanism. Here, known as exonuclease ABC (UvrABC) protein have an essential role. This protein has homologs of the Uvr system that including UvrA, UvrB, and UvrC.

NER pathway starts with the binding of UvrA to disruption of uvrA sensitizes the cells to agents that cause DNA damage because of the non-activation of this pathway (Lenhart et al. 2012). UvrABC system protein A (UvrA) was detected in the protein profile of *L. monocytogenes* after with treatment of 3-HPAA.

In this state, we can say that the DNA of *L. monocytogenes* was damaged, and the bacteria might overcome this problem by using the UvrABC system to repair its DNA(Cappa, Cattivelli, and Cocconcelli 2005).

On the other hand, a DNA polymerase, PolX is, required for the replication of DNA. It can provide the continuation of replication via the 3'–5' exonuclease activity of which the enzyme recognizes the wrong base pair and excises these mismatched bases.These proteins could demonstrate that *L. monocytogenes* performed DNA repair and replication during the treatment with 3-HPAA to hold harmless its DNA.

Table 4.2 DNA-Related Proteins

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R3Z6** | DNA-binding protein | BN389_08330 | DNA binding |
| **A0A0E1R3Z7** | HTH-type transcriptional regulator | yulB_2 | DNA-binding |
| **A0A0E1RAP5** | UvrABC system protein A | uvrA_2 uvrA BN389_24510 | DNA repair |
| **A0A0E1R6A2** | DNA polymerase/3'-5' exonuclease PolX | polX BN389_12490 | DNA repair; DNA-directed DNA polymerase activity; exonuclease activity |
| **A0A0E1R8V3** | HTH-type transcriptional regulator CysL | cysL BN389_21790 | DNA-binding transcription factor activity |

## 4.1.2. RNA Related Proteins after 3-HPAA Treatment

The proteins taking roles in the RNA-related processes of the bacteria were listed in Table 3.2; generally, these proteins function in transcription and transcriptional regulation.

GatA is one of the genes of the transcriptional unit of glutamyl-tRNA Gln amidotransferase (others are gatB, and gatC). It is an enzyme and necessary in order to generate the correctly charged Gln-tRNA(Gln) in the glutaminyl-tRNA synthetase deficiency organisms during translation.

RNA polymerase, also called RNAP, is a molecule that is employed for RNA synthesis from DNA. In eukaryotes and prokaryotes, DNA-bound RNA polymerases have an essential role due to transferring the message for protein synthesis, and interaction between DNA linked proteins and transcriptional machinery might cause activation of

transcription (Chatterji et al. 2007).Control of the process of gene transcription permits the adaptation of cells to changing fields because of the effect on patterns of gene expression. Thus, the cells continue metabolic processes. This enzyme has five subunits, subunit omega (ω) is the smallest and encoded as rpoZ. Although the function of rpoZ is unclear, it is known that it has a role in the operating of denaturated RNA polymerase and the preservation of the biggest subunit(Mathew and Chatterji 2006). In addition to this, the result of a study was the necessity of RNAP subunit ω (rpoZ) for some biological processes (e.g.the development of the shape of organisms) and the production of the antibiotic (Kojima et al. 2002)**.**These proteins might be the result of an indicator of fast adaptation to the existence of 3-HPAA, and in this way, bacteria try to sustain the survival and operate their processes.

The ypsC gene is one of the RNA methyltransferases and responsible for rRNA variation. Methylation is a kind of chemical modification and it has a part in the adjustment of structure and function of ribosomes due to happening in the functional fields of the ribosome(Sergeeva, Bogdanov, and Sergiev 2015).The RNA methylations play a role in ribosome biogenesis(Xu et al. 2008)**,** and correct translational initiation (Burakovsky DE et al. 2012).

Antibiotics can block regulations for ribosomal activity in bacteria. Enzymes of RNA methyltransferases affect antibiotic resistance. The expression of many rRNA methyltransferases can change under different stress conditions (Vester & Long, 2009). After this information can comment that RNA methyltransferase is significant at the protein profile of the bacteria, which is phenolic acid treatment.

Table 4.3 RNA-Related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R993** | Transcriptional regulator | BN389_27030 | DNA binding |
| **A0A0E1RCN9** | Probable inactive metalloprotease | ymfF BN389_14170 | Metal ion binding |
| **A0A0E1RDQ0** | Glutamyl-tRNA(Gln) amidotransferase subunit A | gatA BN389_17820 | Amidase activity |
| **A0A0E1R7L3** | CA-adding enzyme | cca BN389_19300 | tRNA modification |

(cont.).

Table 4.3 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R901** | DNA-directed RNA polymerase subunit omega | rpoZ BN389_18510 | DNA-directed 5'-3' RNA polymerase activity |
| **A0A0E1RFM4** | Ribonuclease R (RNase R) | rnr BN389_24120 | Nonspecific degradation of rRNA) |
| **A0A0E1RE54** | Putative RNA methyltransferase YpsC | ypsC BN389_19120 | rRNA modification |
| **A0A0E1R5B6** | Uncharacterized glycosyltransferase ydaM | ydaM BN389_05660 | Synthesis of extracellular polysaccharide, controlled by SigB |
| **A0A0E1R6X8** | tRNA (Adenine(22)-N(1))-methyltransferase | trmK BN389_14790 | tRNA (adenine-N1-)-methyltransferase activity |

### 4.1.3. Ribosome and Protein Synthesis Related Proteins after 3-HPAA Treatment

Methionine-tRNA ligase, protein translocase subunit SecE, condensation domain-containing protein, pyrroline-5-carboxylate reductase, DEAD-box ATP-dependent RNA helicase CshA, 30S ribosomal protein S2, histidine--tRNA ligase, 30S ribosomal protein S16, ribosome-binding factor A, translation initiation factor IF-2, a negative regulator of genetic competence ClpC/MecB, elongation factor 4 (EF-4), and bifunctional protein FolD were ribosome and protein synthesis related proteins that obtained after exposure to 3-HPAA (Table 4.4).t-RNA ligases are essential components that have an important role in protein translation in whole organisms. These proteins transport the amino acid to the ribosome and by this means, it persistences growth of peptide chains (Ferro ve ark., 2018). Apart from this, recently, inhibition of processes of these proteins was approached in terms of drug development. (Ho et al., 2018; Lee et al., 2018). Methionine-tRNA ligase that codes with metG was obtained for adaptation after the treatment of 3-HPAA, it could be concluded that this phenolic acid caused the influencing of the protein profile of *L. monocytogenes.*

Translocon is a protein complex that relates to translocation between membranes. Pathogen bacteriacollect the translocons from the host membranes at the same time so that virulence factors are sent to target cells. This protein complex consists of Sec proteins. Proteins that belong Sec family transports the polypeptides throughout the plasma membrane, and they are a chaperone protein (SecB), an ATPase (SecA), an integral membrane complex (SecCY, SecE, and SecG), two additional membrane proteins (SecD and SecF) (Bieker, Phillips, and Silhavy 1990). Sec E plays a role as an export apparatus, and it is crucial for base cellular functions.However, with the presence of Sec E protein after 3-HPAA treatment we can comment that inhibition of bacterial virulence of *L. monocytogenes*.

RNA helicases catalyze double-stranded RNA molecules as energy-dependent. These enzymes found in DEAD-box have a part in RNA metabolisms, such as synthesis of RNA, disruption of RNA, and RNA-RNA interactions. CshA is a cold shock-linked helicasewhich involved inRNA degradation.It has beenshownthat in Bacillus subtilis, CshA and CshB disrupt structures in RNA so thatit prevents the initiation of translation (Hunger et al. 2006). Besides, S. Oun al demonstrated that biofilm formation and irregular hemolysis are increased after mutation of the CshA gene in *Staphylococcus aureus* (Oun et al. 2013)**.** As a result, the increase of the CshA gene in the presence of 3-HPAA can affect the translation.

Ribosomal protein S2 is the main component of the translation machinery, and it has a role in decoding of mRNA according to base matching between codon and anticodon. One of the mutants, rpsB, has thermo-tenderness. It has been shown by A. Okuyama et all that the absence of 30S ribosomal subunit protein S2 in kasugamycin-resistant mutant via 2D-electrophoresis (Okuyama, Yoshikawa, and Tanaka 1974).Besides, although protein S16 is not directly participating in translation, its role in theassemblyof the 30S ribosomal proteins is crucial(Blokhin et al. 2019)**.** The outcomes might demonstrate that these proteins promote thechanges in translation and resistance mechanisms.

Ribosome-binding factor A (RbfA) is a shock protein that helps reproduction for bacteria at the low temperatures. Moreover, this protein shows attraction to 30 S ribosomal subunits, but not to 70S**.** This protein takes part also in the initiation of translation, and thereby, considered to be influenced the translation.

Genetic competence is a physiological statewhich takes a limited time ofthe bacterial life cyclethat might change based on bacteria or environmental conditions. This state allows bacteria to bind and take up the free DNA segment from its surrounding.

It is controlled by many regulatory genes such as mecA, clpC/mecB, comK, and the ability to getting genetic material is a prerequisite for transformation (Kruger, Volker, and Hecker 1994).

The MecB protein controls degradative enzymes (Msadek, Kunst, and Rapoport 1994). After the 3-HPAA treatment, it can be considered that the negative regulator of genetic competence, ClpC/MecB mightestablish a connection between the competence pathway and the stress response.

Protein synthesis is an essential event for living cells, and several protein factors participate in here. There are also additional factors that take a role in protein synthesis by recovering ribosomes in a stressful environment such as alternative ribosome-rescue factor A, elongation factor 4 (EF4), transfer mRNA (tmRNA). The main functions of EF4 are to promote back-translocation, to separate elongate ribosomes, and help in 30S biogenesis. Depending on the severity of stress, EF4 supports cell survival or death. EF4 was observed in the ribosome profiling analysis of cells when cells had been exposed to 3-HPAA. This might indicate that EF4 functions under phenolic acid conditions and thanks to this, may provide liveliness for cells by continuing protein synthesis (B. Liu and Chena 2018).

Table 4.4 Ribomal and Protein Synthesis Related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R2M7** | Methionine--tRNA ligase | metG BN389_01900 | Methionine-tRNA ligase activity, tRNA binding |
| **A0A0E1R2V1** | Protein translocase subunit SecE | secE BN389_02600 | Protein secretion |
| **A0A0E1R2S2** | Pyrroline-5-carboxylate reductase | proC BN389_04130 | Pyrroline-5-carboxylate reductase activity |
| **A0A0E1R5C0** | DEAD-box RNA helicase | shA BN389_08940 | RNA helicase activity |

(cont.).

Table 4.4 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R5C0** | DEAD-box RNA helicase | shA BN389_08940 | RNA helicase activity |
| **A0A0E1R6G8** | Histidine--tRNA ligase | hisS BN389_15450 | Histidine-tRNA ligase activity |
| **A0A0E1R7X8** | 30S ribosomal protein S16 | rpsP BN389_18240 | Structural constituent of ribosome |
| **A0A0E1R6L6** | Translation initiation factor IF-2 | infB BN389_13490 | GTPase activity; translation initiation factor activity |
| **A0A0E1R983** | Negative regulator of genetic competence ClpC(MecB) | clpC BN389_02470 | Protein degradation, positive regulator of autolysin |
| **A0A0E1R702** | Elongation factor 4 | lepA BN389_15040 | GTPase activity; ribosome binding; translation elongation factor activity |
| **A0A0E1R6P8** | Bifunctional protein FolD | folD BN389_13840 | Methenyltetrahydrofolate cyclohydrolase activity |
| **A0A0E1R301** | Condensation domain-containing protein | BN389_04830 | Condensation domain is a protein domain found in many multi-domain enzymes that synthesize peptide antibiotics. |
| **A0A0E1R6D1** | 30S ribosomal protein S2 | rpsB BN389_16830 | Structural constituent of ribosome |

### 4.1.4. Cell wall, Membrane, and Flagella RelatedProteins after3-HPAA Treatment

Treatment of *L. monocytogenes* with 3-HPAA resulted in a differencein tenproteins associated with the cell wall, membrane, and flagella (Table 4.5). One of them that are bifunctional protein GlmU was the most remarkable protein.

Bifunctional protein GlmU has Udp-n-acetylglucosamine phosphorylase activityand alsoinvolved in the cell wall organization. GlmU is a target protein for the development of antimicrobial compounds againstboth gram-negative and positive bacteria(Olsen, Vetting, and Roderick 2007). Lack of this protein might generate disruptions and alterations on the peptidoglycan structure. On the other hand, in the protein profile of 3-HPAAtreated *L. monocytogenes*, bifunctional protein GlmU was observed. Bacteria may have synthesized this protein to overcome phenolic acid stress and prevent potentialmembrane loss. Furthermore, some studies have shown that bacteria reveal physical defense mechanisms to counteract adverse environmental conditions. For example, Changing Chen et al. presented that proteins such as GlmU that participated in biofilm formation and cell wall biosynthesis were up-regulated in response to alkylation stress (Chen et al. 2019).

Table 4.5 Cell wall, Membrane and Flagella Related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R7H0** | Cell division protein FtsL | ftsL BN389_20680 | Septum formation |
| **A0A0E1R9S5** | D-alanyl-D-alanine carboxypeptidase dacA | dacA BN389_27300 | Serine-type D-Ala-D-Ala carboxypeptidase activity |
| **A0A0E1R7W3** | Septum site-determining protein DivIVA | divIVA BN389_20400 | Division site selection, chromosome segregation |
| **A0A0E1R948** | Bifunctional protein GlmU | glmU BN389_02120 | Cell wall organization |
| **A0A0E1R292** | Copper homeostasis protein | cutC BN389_00300 | Copper ion binding |
| **A0A0E1R471** | Flagellar motor switch protein FliG | fliG BN389_07600 | Motor activity |
| **A0A0E1R2Z0** | Gfo/Idh/MocA family oxidoreductase | BN389_03000 | Oxidoreductase activity |
| **A0A0E1REZ5** | Peptidoglycan bound protein | BN389_22120 | Collagen binding |

### 4.1.5. MetabolismRelatedProteins after3-HPAA Treatment

When the effect of 3-HPAA on metabolism was examined, differences in the expression of proteins related to the purine and pyrimidine metabolism, amino acid metabolism, energy metabolism, fatty acid carbohydrate metabolism, and phospholipid metabolism were seen (Table 4.6).

Adenylosuccinate synthetase (AdSS) and adenylosuccinate lyase (ASL), enzymes that were encodedby purA and purB genes, respectively, play a role in the purine biosynthesis. According to the studiesin literature, both AdSS and ASL proteins hadbeen defined as vital components for purine recovery in bacteria. Apart from this, the necessity of purB was documented as a potential therapeutic target in the *L. donovani* and *S. aureus*(Boitz et al. 2013; Fyfe et al. 2010).

Glutamine transport ATP-binding protein, GlnQ, is an identified protein in the presence of 3-HPAA stress in *L. monocytogenes*, is responsible for the amino acid metabolism and glutamine transport. Glutamine synthetase (GlnA) that converts glutamate and ammonia to glutamine is one of the main enzymes in nitrogen metabolism. It functions as a nitrogen donor, thereby the level of this protein has vital importance. Additionally, several studies demonstrated that glutamine metabolism was important for the virulence of bacteria. Gee W. Lau et al. showed that glnQ and glnA genes participate in the virulence pathway of *S. pneumoniae*. Consequently, these indicate that *L. monocytogenes* cells with 3-HPAA might increase the nitrogen metabolism for survival(Lau et al. 2001).

Most of the time, sugars are primarycarbonsources that have been utilized by bacteria for the production of ATP. Also, it was reported that amino sugars are employed in bacterial components such as peptidoglycan or lipoteichoic acid synthesis (Kawada-Matsuo et al. 2012). If the disturbance of this regulation, the virulence of bacteria will be change. Therefore, sugar metabolism is essential for both bacterial physiology and the virulence of bacteria(Kawada-Matsuo, Oogai, and Komatsuzawa 2016). Glucosamine-6-phosphate deaminase (NagB) has a central role in sugar distribution to the glycolysis pathway and to cell wall biosynthesis. This protein observed after exposure of *L. monocytogenes* to 3-HPAA; it might indicate the cells' increased demands for amino sugar to be used in carbohydrate pathways.

ATP synthase subunit b, encoded by atpF that is found ubiquitously in energy-transducing produces ATP in the presence of a proton gradient within the cells.

Insufficiency of this protein can limit metabolic processes due to energy depletion. On the other hand, ATP synthase subunit b was identified in 3-HPAA treated *L. monocytogenes* (Table 4.6). Therefore it mightindicatethat 3-HPAA presence did not result in the complete disruption of this membrane-bound enzyme complex (Guo, Suzuki, and Rubinstein 2019). ABC transporters involve two domains that are ATP cassette and transmembrane, it is an importer that pumps nutrient to bacteria, also, is an important virulence factor in bacteria because of having a role in the secretion of antimicrobial agent or toxins (Davidson and Chen 2004; Rees, Johnson, and Lewinson 2009). Identification of putative ABC transporter ATP-binding protein exp8 in 3-HPAA treated *L. monocytogenes* (Table 4.6) might demonstrate the less access of bacteria to the nutrients or inability of bacteria to utilize the present nutrients within the media.

Table 4.6 Metabolism-Related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R3Z8** | Adenylosuccinate synthetase (AMPSase) (AdSS) | purA BN389_00660 | GTP binding; magnesium ion binding |
| **A0A0E1R4J7** | Histidinol dehydrogenase (HDH) | hisD BN389_06040 | Zinc ion binding |
| **A0A0E1R677** | Acetyl-CoA acetyltransferase | thlA BN389_14400 | Acetyl-CoA C-acetyltransferase activity |
| **A0A0E1R4V5** | Alpha-ribazole-5'-phosphate phosphatase | cobC BN389_11680 | Alpha-ribazole phosphatase activity |
| **A0A0E1R5G5** | Ethanolamine ammonia-lyase light chain | eutC BN389_11950 | Cobalamin binding |
| **A0A0E1R770** | Adenylosuccinate lyase | purB BN389_18000 | Succinate AMP-lyase activity |
| **A0A0E1R5D9** | Glutamine synthetase | glnA BN389_13230 | ATP binding; glutamate-ammonia ligase activity |

(cont.).

Table 4.6 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R9V2** | Putative mannose-6-phosphate isomerase | BN389_21760 | Isomerase activity |
| **A0A0E1R9F3** | Uncharacterized sugar epimerase | yhfK BN389_23590 | Lyase activity |
| **A0A0E1R9K2** | Cellobiose-specific phosphotransferase enzyme IIB component | celA_4 BN389_26500 | Protein-N(PI)-phosphohistidine-sugar phosphotransferase activity |
| **A0A0E1R6H8** | Glucosamine-6-phosphate deaminase | nagB_2 nagB BN389_09860 | N-acetylglucosamine utilization |
| **A0A0E1RAE6** | Inosine5'-monophosphate dehydrogenase | guaB BN389_27340 | Metal ion binding; nucleotide binding |
| **A0A0E1R1Q6** | Arginine deiminase | arcA BN389_00530 | deiminase activity |
| **A0A0E1RBI7** | Periplasmic beta-glucosidase | bglX_2 BN389_27610 | Beta-glucosidase activity |
| **A0A0E1R5K5** | Putative aminopeptidase | ysdC BN389_12350 | Aminopeptidase activity |
| **A0A0E1R8I5** | Guanine/hypoxanthine permease | pbuO BN389_22850 | Nucleobase transmembrane transporter activity |
| **A0A0E1R4L2** | Probable endopeptidase p60 | iap BN389_06190 | Uptake of niacin |
| **A0A0E1R7V0** | Acetolactate synthase | alsS BN389_20250 | Magnesium ion binding; thiamine pyrophosphate binding |
| **A0A0E1R5M1** | Isopentenyl-diphosphate delta-isomerase | fni BN389_14080 | FMN binding; isopentenyl-diphosphate delta-isomerase activity; magnesium ion binding |

(cont.).

Table 4.6 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R905** | 3-oxoacyl-[acyl-carrier-protein] synthase 2 | fabF BN389_22340 | Biosynthesis of fatty acids |
| **A0A0E1RDR0** | Bifunctional purine biosynthesis protein PurH | purH BN389_17920 | Phosphoribosylaminoimid azolecarboxamide formyltransferase activity |
| **A0A0E1R7F3** | Putative ABC transporter ATP-binding protein exp8 | exp8 BN389_16740 | ATPase activity; ATPase-coupled transmembrane transporter activity |
| **A0A0E1R8P9** | ATP synthase subunit alpha | atpA2 atpA BN389_24930 | ATP binding; hydrolase activity; proton-transporting ATP synthase activity, rotational mechanism |
| **A0A0E1R4R8** | GMP synthase [glutamine-hydrolyzing] | guaA BN389_11230 | GMP synthase activity; pyrophosphatase activity |
| **A0A0E1RAN7** | Thioredoxin reductase | trxB_2 BN389_24410 | Keeps thioredoxin |
| **A0A0E1R414** | Pyridoxine kinase | pdxK BN389_07050 | Phosphomethylpyrimidine kinase activity; pyridoxal kinase activity |
| **A0A0E1R8B4** | Phosphopentomutase | deoB BN389_19790 | Magnesium manganese an ion binding; phosphopentomutase activity |
| **A0A0E1R5U9** | Pyruvate dehydrogenase E1 component subunit beta | pdhB BN389_10840 | Pyruvate dehydrogenase (acetyl-transferring) activity |
| **A0A0E1R7T1** | Uncharacterized protein | ypgQ BN389_18040 | Degradation of excessive or abnormal nucleotides |
| **A0A0E1RC30** | Corrinoid adenosyltransferase | yvqK BN389_12270 | Cob(I)yrinic acid a,c-diamide adenosyltransferase activity |

(cont.).

Table 4.6 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R6X2** | Probable manganese-dependent inorganic pyrophosphatase | ppaC BN389_14740 | Inorganic diphosphatase activity; manganese ion binding |
| **A0A0E1R3R7** | Uncharacterized protein | ykgB BN389_05950 | Pentose phosphate pathway |
| **A0A0E1REZ1** | Uncharacterized protein yhcK | yhcK BN389_22070 | Phosphorelay sensor kinase activity |
| **A0A0E1R8N6** | Glutamine transport ATP-binding protein | glnQ BN389_17660 | Uptake of amino acids |
| **A0A0E1R8S0** | Putative heme-dependent peroxidase | BN389_21440 | Heme binding; metal ion binding; |
| **A0A0E1R608** | Uncharacterized ABC transporter ATP-binding protein HI_0663 | BN389_11490 | ATPase-coupled transmembrane transporter activity |

### 4.1.6. Redox Related Proteins after 3-HPAA Treatment

NADPH dehydrogenase and thioredoxin-like protein, which are the remarkable cell hemostasis and redox related proteins were detected in the protein profile of *L. monocytogenes* after phenolic acid application (Table 4.7).

Nicotinamide adenine dinucleotide phosphate (NADPH) has a role as an electron donor in whole organisms. However, NADPH dehydrogenase is a response to a toxic substance in biological proses of bacteria. Detection of this protein might display that *L. monocytogenes* manages to respond to the oxidative stress caused by 3-HPAA.

Thioredoxin is a class of redox proteins that participate in biological processes and known to be present in entire organisms, including bacteria and fungi. Thioredoxins act as electron donors for some enzymes and it is helpful for decrease oxidative stress. Thioredoxin-like protein that encoded by ykuU gene was detected after 3-HPAA treatment. Since functioned in oxidative stress, the presence of this protein could be speculated that the redox and homeostasis might be damaged with 3-HPAA application and protect the cell from oxidative stress during cell survival.

Table 4.7 Cell homeostasis and Redox-Related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R9A3** | Putative monooxygenase | ycnE_2 BN389_25400 | Monooxygenase activity |
| **A0A0E1R9L1** | NADPH dehydrogenase | namA BN389_24340 | FMN binding |
| **A0A0E1R678** | Thioredoxin-like protein | ykuU BN389_16280 | Protection against peroxide stress |

## 4.1.7. Virulence Related Proteins after 3-HPAA Treatment

After treatment, when the effect of 3-HPAA on *L. monocytogenes* pathogenicity was examined, 4 differently identified proteins were found (Table 4.8). Two of them were remarked, aminopeptidase, and alcohol dehydrogenase.

Aminopeptidases function the cleavage of amino acids from the N-terminal of proteins or peptides. Bacteria can exhibit some aminopeptidasic events related to cell envelope or extracellular. Moreover, it might accept as a promising antimicrobial target due to having a role in the virulence of the bacteria. In the protein profile of *L. monocytogenes* aminopeptidase (ampS) protein was observed. This might present that 3-HPAA seriously affected the amino acid metabolism of bacteria.

The alcohol dehydrogenase has a role in the conversion of acetaldehyde to ethanol and also, the expression of this protein can change during biofilm formation. After phenolic acid treatment, the presence of this protein can consider that 3-HPAA did not adversely affect the growth and viability of *L.monocytogenes* cells.

Table 4.8 Virulence related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R8K2** | Phosphoglucomutase | pgcA_2 BN389_24380 | Biosynthesis of peptidoglycan |

(cont.).

Table 4.8 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1RBR4** | Putative CDP-glycerol | tarB BN389_11170 | Glycero-phosphotransferase |
| **A0A0E1R7L5** | Aminopeptidase | ampS BN389_17390 | Biofilm formation |
| **A0A0E1R5E0** | Alcohol dehydrogenase | yqhD BN389_05910 | Biofilm formation |

## 4.2. Identified Proteins after 4-HBA Treatment

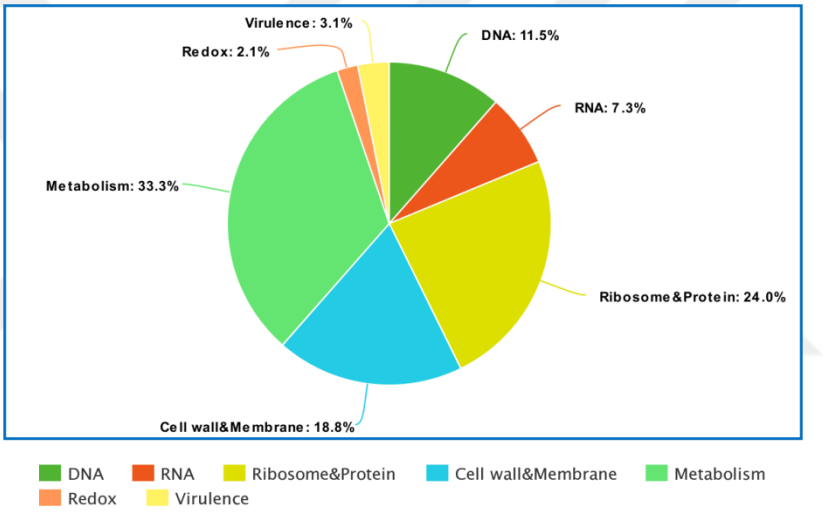



Figure 4.3The percentages of proteins for each group of function in the presence of 4-HBA detected proteins

The results showed that (Venn diagram, Figure 4.1), 8 of the identified 106 proteins overlapped with 3-HPAA treated group,14 with control groups. However, 84 proteins were only obtained in 4-HBA treated group. Based on their functions these proteins were classified related with DNA; RNA; ribosomes and protein; cell wall and membrane; metabolism; redox and cell homeostasis; and virulence and their percentages calculated as 11,5 %, 7,3 %, 24 %, 18,8 %, 33,3 %, 2,1%, 3,1%, respectively (Figure 4.3). According to these, the metabolism-related proteins showed the highest percentage, since the metabolism pathways involve many proteins. The virulence- related proteins displayed the lowest percentages, and it could be displayed that the virulence of the bacteria was rarely affected after exposure of 4- HBA.

### 4.2.1. DNA Related Proteins after4-HBA Treatment

The 4-HBA treatment showed the DNA protein profile listed in table 4.9.According to these proteinsand their interactions, remarkable proteins which areATP-dependent helicase/deoxyribonuclease subunit B, ATP-dependent RecD-like DNA helicase, sporulation initiation inhibitor protein, DNA polymerase/3'-5' exonuclease PolX, DNA ligase, DNA gyrase subunit B functioned in DNA replication and repair.

ATP-dependent helicase/deoxyribonuclease subunit B(AddB) recognizes the cross-over Hotspot Instigator (Chi) site that generates suitably a DNA molecule; thus, the DNA repair process is instigated via recombination. AddB protein was observed in the protein profile of *L. moncytogenes* in the media containing 4-HBA. This might indicate that bacteria manage to repair their own DNA, in this way cells maintenances efficient survival and replication(M. Wilkinson and Wigley 2014). Additionally, sporulation initiation inhibitor protein (soj) provides the survival of bacteria in extremely unsuited situations such as acidic, cold, dry conditions, detection of soj may show that *L. monocytogenes* cells may continue the life activities by overcoming 4 HBA media.

The polX gene is one of the DNA polymerases and essential for replication of DNA, it identifies a mismatched base pair with the 3'–5' exonuclease activity and excises these wrong base pairs, thus, it may continue the replication continuity.

DNA ligase (LigA) is an important step for many fundamental reactions within the cell such as DNA replication, repair, recombinations, joining of suitable DNA ends, and in breaks of phosphodiester linkages in double-stranded DNA. Detection of LigA can show that the *L. monocytogenes* cell repaired its damaged DNA (A. Wilkinson, Day, and Bowater 2001)

DNA gyrase is one of the required proteins for influent cell division of the supercoiling of chromosomal DNA.

DNA gyrase subunit B, GyrB, negatively mediates the supercoiling of double-stranded DNA and supports DNA replication, transcription, recombination, and repair via DNA topological change.

İdentify of topological change-related protein such as GyrB in the presence of 4-HBA may show the need for more eased progress of replication (KAYIŞ 2019).

Table 4.9 DNA Related

| PROTEIN ID | PROTEIN NAMESE | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R7T6** | Ribonucleoside-diphosphate reductase | nrdA BN389_21880 | ATP binding; thioredoxin disulfide as acceptor |
| **A0A0E1R838** | ATP-dependent helicase/deoxyribonuclease subunit B | addB BN389_22980 | DNA repair/ recombination |
| **A0A0E1R3J8** | HTH-type dhaKLM operon transcriptional activator | dhaS BN389_05150 | DNA binding |
| **A0A0E1R476** | Putative DNA-binding protein | ywzG BN389_07650 | DNA binding |
| **A0A0E1R729** | ATP-dependent RecD-like DNA helicase | recD2 BN389_15340 | Replication fork progression |
| **A0A0E1R9F0** | Sporulation initiation inhibitor protein | soj BN389_27680 | Forespore chromosome Sporulation initiation |
| **A0A0E1R6A2** | DNA polymerase/3'-5' exonuclease PolX | polX BN389_12490 | DNA repair |
| **A0A0E1R2B5** | Single-stranded DNA-binding protein (SSB) | ssb1 BN389_00550 | DNA replication, repair, and recombination |
| **A0A0E1R754** | DNA ligase | ligA BN389_17850 | DNA replication |
| **A0A0E1R3U5** | DNA gyrase subunit B | gyrB BN389_00060 | DNA supercoiling, control of replication initiation |

## 4.2.2. RNA Related Proteins after 4-HBA Treatment

The RNA related proteins listed in table 4.10 were only identified in the 4-HBA treated group.

These proteins were transcription termination/antitermination protein, DNA-directed RNA polymerase subunit beta', 5'-3' exonuclease Probable DNA-directed RNA polymerase subunit delta, transcription termination/antitermination protein. They generally took roles in transcription and transcriptional regulation process of the

bacteria. NusA gene is a termination protein that affects the transcription. This gene cause changes in RNA polymerase, prevent interaction between the RpoA and RNA.
In addition, the NusG gene that belongs to the same famıly was observed. This gene enhances the transcription elongation rate by RNA polymerase. İdentification of these proteins required for initiation, elongation, and transcription of genes might indicate that *L. monocytogenes* cells overcome the 4-HBA stress and continue the transcription.
DNA-directed RNA polymerase enzyme (RNAP) found in the whole organism synthesis the RNA from the DNA template. The subunit β' (RpoC gene) is the largest subunit, catalyzed the transcription of DNA to RNA. Aditionally, an additional subunit that termed subunit delta or RpoE gene that belongs to the RNAP family was observed in the protein profile of L. *monocytogenes.* In the literature, Andy Weiss et al. displayed that this gene is an important component in the S. aureus transcription and has a critical role during infection (Weiss et al. 2014). After 4-HBA treatment, with the observation of RpoC and RpoE genes, *L. monocytogenes* cells can be efficiently adapt to the changing media and sustain the metabolic process for survival.

Table 4.10 RNA related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R7J1** | Transcription termination/antitermination protein NusA | nusA BN389_13460 | DNA-binding transcription factor activity |
| **A0A0E1R7L3** | CCA-adding enzyme | cca BN389_19300 | ATP:3'-cytidine-cytidine-tRNA adenylyltransferase |
| **A0A0E1R4I8** | Transcription termination/antitermination protein NusG | nusG BN389_02610 | Interacts with the transcription termination factor Rho |
| **A0A0E1RAV9** | Probable DNA-directed RNA polymerase subunit delta | rpoE BN389_25210 | DNA-directed 5'-3' RNA polymerase activity |
| **A0A0E1R4K2** | DNA-directed RNA polymerase subunit beta' | rpoC BN389_02760 | DNA-directed 5'-3' RNA polymerase activity |
| **A0A0E1R961** | 5'-3' exonuclease | ypcP BN389_19060 | Removal of RNA from Okazaki fragments |

### 4.2.3. Ribosomal and Protein synthesis Related Proteins after 4-HBA Treatment

Treatment of L. *monocytogenes* with 4-HBA resulted in the differential 23 proteins in terms of ribosomal and protein synthesis, 15 of them were remarkable proteins (Table 4.11).

The shock-linked CshA gene is an RNA helicase found in DEAD-box, participate in RNA metabolism. After exposure to both of phenolic acids that are 3-HPAA and 4-HBA, CshA gene was observed in the protein profile of *L. monocytogenes*. When the studies in the literature are examined, results showed that the presence of the CshA gene affects the initiation of translation in the bacteria due to the disruption of RNA (Hunger et al. 2006).

t-RNAs are the main component that transfers the aminoacids to ribosomes, thus it allows the growth of the peptide chain in the translation. The t-RNA ligases such as gltX and valS were detected after 4-HBA treatment, which may present that after the phenolic acid treatment, an increase for some tRNAs via the metabolism changes for adaptation. The identification of these t-RNA ligases could be concluded that the 4-HBA affected the protein synthesis profile of *L. monocytogenes* ("Antibiotic Resistance in Bacteria Caused by Modified Nucleosides in 23S Ribosomal RNA | Request PDF" n.d.).

30S ribosomal protein S21 that encoded by the rpsU gene plays a role in the polypeptide synthesis. There is not much a known about function and role in stress resistance of ribosomal protein S21 but in a study, the reduced growth rate, unaffected motility resulted in*L. monocytogenes*. In this study, about the identification of the rpsU gene it could be concluded that 4-HBA affects the protein synthesis thereby the growth ratio of cells can decrease(Metselaar et al. 2015).

30S ribosomal protein S15 (rpsO) is a primary binder protein functioned in the assembly of the small subunit of the ribosome. The protein S15 is sensitive to cold and critical for assembly in the non-ideal conditions. Identification of the *rpsO* gene may show that cells overcome the 4-HBA, so protein synthesis continues(Bubunenko et al. 2006). 30S ribosomal protein S17 (rpsQ) is one of the main ribosomal proteins functioned in the assembly of the ribosomal small subunit and it biologically participates in the resistance of antibiotics. The existence of this protein might indicate that 4-HBA blocks the proper ribosomal structure and assembly process.

The L36 is a protein that belongs to the family of the 50S subunit and highly protected in bacteria. Ribosomes involving L36 are untruly assembled. The detection of protein L36 in the presence of 4-HBA which caused an unideal condition might result in the detrimental effects on *L.monocytogenes* by damaging the ribosomal assembly.

50S ribosomal protein L14 (rplN) is the primary RNA-binding protein, which ability to bind directly to 23S rRNA. This protein constitues part of B5 and B8 bridges, which is required for proper ribosome assembly and translation in the 70S ribosome. This might indicate that 4-HBA seriously affected the ribosomal assembly pathways of *L. monocytogenes* cells.

50S ribosomal protein L5 (rplE) can avoid the formation of the ribosome assembled in bacteria cells. The identification of this protein suggested that 4-HBA eventually damage the ribosome structure and assembly.

The ribosomal protein L19 (rplS), which belongs to the 50S subunit has a role in the structure and function of the aminoacyl-tRNA binding site. As known, tRNA ligases are crucial for protein translation in bacteria. Therefore, the detection of this protein is presented that phenolic acid affected protein translation.

Table 4.11 Ribosomal and Protein synthesis related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R5C0** | DEAD-box ATP-dependent RNA helicase CshA | cshA BN389_08940 | Important for adaptation to low temperatures |
| **A0A0E1RD43** | Valine--tRNA ligase | valS BN389_15770 | Aminoacyl-tRNA editing activity |
| **A0A0E1R862** | 3'-5' exonuclease DinG | dinG BN389_19240 | Response to DNA damage |
| **A0A0E1R987** | Glutamate--tRNA ligase | gltX BN389_02520 | Glutamate-tRNA ligase activity; zinc ion binding |
| **A0A0E1R6Z2** | 30S ribosomal protein S21 | rpsU BN389_14940 | Structural constituent of ribosome |
| **A0A0E1R6M1** | 30S ribosomal protein S15 | rpsO BN389_13540 | Structural constituent of ribosome |
| **A0A0E1R927** | Ribose-5-phosphate isomerase B | rpiB_3 BN389_26280 | Ribose-5-phosphate isomerase activity |

(cont.).

Table 4.11 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R7X8** | 30S ribosomal protein S16 | rpsP BN389_18240 | Structural constituent of ribosome |
| **A0A0E1R9Y9** | 50S ribosomal protein L36 | rpmJ BN389_25690 | Structural constituent of ribosome |
| **A0A0E1R4V3** | Chemotaxis protein methyltransferase | cheR BN389_07290 | Motility, chemotaxis |
| **A0A0E1R8Z1** | 50S ribosomal protein L28 | rpmB BN389_18410 | Structural constituent of ribosome |
| **A0A0E1RG36** | 50S ribosomal protein L14 | rplN BN389_25820 | Structural constituent of ribosome |
| **A0A0E1R9E2** | 50S ribosomal protein L5 | rplE BN389_25800 | Structural constituent of ribosome; tRNA binding |
| **A0A0E1R8B0** | Pseudouridine synthase | rluB BN389_19740 | 23S rRNA modification |
| **A0A0E1R7W5** | 50S ribosomal protein L19 | rplS BN389_18140 | Structural constituent of ribosome |
| **A0A0E1R2R0** | 50S ribosomal protein L25 | rplY ctc BN389_02250 | 5S rRNA binding; structural constituent of ribosome |
| **A0A0E1RB21** | 50S ribosomal protein L4 | rplD BN389_25910 | rRNA binding; structural constituent of ribosome |
| **A0A0E1RBL9** | tRNA uridine 5-carboxymethylaminomethyl modification | mnmG gidA BN389_27910 | Flavin adenine dinucleotide binding |
| **A0A0E1R822** | Aminotransferase | aspC BN389_22830 | They are important in the synthesis of amino acids, which form proteins |
| **A0A0E1R1S5** | 50S ribosomal protein L9 | rplI BN389_00630 | rRNA binding; structural constituent of ribosome |
| **A0A0E1REA5** | GTPase Der (GTP-binding protein EngA) | der BN389_19620 | Ribosome assembly |
| **A0A0E1R930** | Carboxy-terminal processing protease CtpA | ctpA BN389_18760 | Serine-type peptidase activity |
| **A0A0E1R8Y4** | 30S ribosomal protein S17 | rpsQ BN389_25830 | rRNA binding; structural constituent of ribosome |

### 4.2.4. Cell wall, Membrane and Flagella Related Proteins after 4-HBA Treatment

After the application of 4-HBA treatment of *L. monocytogenes*, proteins were listed in terms of the cell wall, membrane, and flagella in table4.12. Glutamine--fructose-6-phosphate aminotransferase, Internalin-I,lipoteichoicacid synthase, probable undecaprenyl-phosphate N-acetylglucosaminyl 1-phosphate transferase, N-acetylmuramic acid 6-phosphate etherase, putative penicillin-binding protein pbpX, UDP-N-acetylmuramate--L-alanine ligase, D-alanine--D-alanine ligase are remarkable proteins from the detected proteins.

Glutamine--fructose-6-phosphate aminotransferase (GlmS) is a protein that participates in the metabolism of amino sugar which is necessary for cell wall synthesis. Observation of the glmS gene can be because of continuing the cell wall integrity and structure caused by 4-HBA.

Internalins are surface proteins of on *L. monocytogenes*, are used for the invasion of mammalian cells. In addition to the two well-known forms (InlA and InlB) of these proteins, Internalin-I (InlI) was identified as a new internalins protein. Results of literature works show that InlI could not be necessary for pathogenesis but may function in infection conditions. (Sabet et al. 2005). InlI was found in the protein profile of the bacteria after 4-HBA treatment. This may indicate *L. monocytonegenes* cells continued to invade in the presence of 4-HBA.

Lipoteichoic acid (LTA) is a vital cell envelope component, in gram-positive bacteria, LTA synthesis is required for the growth and cell division process. The cell envelope is a natural protective barrier against many adverse conditions such as antimicrobial agents. Though, it could be speculated that the detection of ltaS1 resulted in the cell division changes in *L. monocytogenes*. Addition, the identification of some of the cell envelope proteins might demonstrate that 4-HBA affected the bacterial cell wall integrity and cell division as an antimicrobial agent (Garufi et al. 2012).

Teichoic acids are glycopolymers found on the surface of Gram-positive bacteria. It plays a role in cell shape determination, impacts everything from antibiotic susceptibility to survival in a host. TagO gene is necessary for the biosynthesis of teichoic acid. The teichoic acid biosynthetic pathway has been guessed to be two different types of possible antibiotic targets; antivirulence targets and antibiotic targets. Peschel et al. suggested that teichoic acid biosynthesis is an antivirulence target in

*S. aureus.* This situation has attracted attention as the resistance in pathogenicity of the organism cannot easily develop as in traditional antibiotic targets (Peschel et al. 2000). TagO gene was identified in the presence of phenolic stress. This might signal that 4-HBA could attenuate the virulence of *L. monocytogenes.*

The peptidoglycan layer provides the strength to the cell wall, helps the regulation of cell shape and acts as a selective mesh for molecules from the external environment. Peptidoglycan synthesis is one of the biogenesis pathways in bacteria. There are several proteins involved in this pathway: Mur ligases (MurA, MurB, MurC, MurD, MurE and MurF) and penicillin-binding proteins (PBPs). These proteins were accepted as attractive candidate antibacterial agents for bacteria at the results of the investigations (Y. Liu and Breukink 2016).

The MurC gene is responsible for the addition of L-alanine on UDP-MurNAc in the peptidoglycan synthesis stage. The identification of the MurC might be due to continuing the cell wall integrity and structure caused by 4-HBA. Thus, cell division and membrane integrity of *L. monocytogenes* were severely affected by 4-HBA exposure.

Beta-lactam antibiotics bind to penicillin-binding proteins (PBPs),then it leads to peptidoglycan layer disruption. Putative penicillin-binding protein pbpX that belongs to the PBPs family was observed in the presence of 4-HBA. This might show the decreased susceptibility of *L. monocytogenes* to the host defense and the antibiotics.

D-alanine--D-alanine ligase (DDl), is one of the differentprotein that has a role in peptidoglycan synthesis.

One study has shown that obtaining DDl can be used as a substrate that provides resistance to antibiotics used against life-threatening infections(S. Liu et al. 2006).

In this study, the detection of DDI may indicate that *L. monocytogenes* cells could not escape the effect of phenolic acid. Therefore, 4-HBA can use as an antibacterial agent against *L. monocytogenes* infection.

Protein murNAc-6P etherase (MurQ) is required the recycling of the peptidoglycan layer subunits during cell growth in this way bacteria save energy.

After the application of 4-HBA, murQ was observed, this could indicate that the cell wall was damaged and *L. monocytogenes* attempted to fix it (Borisova et al. 2016).

Table 4.12 Cell wall, membrane, flagella related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1RAR0** | Glutamine--fructose-6-phosphate aminotransferase | glmS BN389_07720 | Cell wall synthesis |
| **A0A0E1R4A6** | Zinc transport system ATP-binding protein | adcC BN389_01710 | ATPase activity; ATP binding zinc uptake |
| **A0A0E1RGD1** | GW domain-containing protein | BN389_26820 | GW domains are unlikely to act as functional mimics of SH3 domains since their potential proline-binding sites are blocked or destroyed |
| **A0A0E1R7G9** | Internalin-I | inlI_3 BN389_16940 | Invasion |
| **A0A0E1R5S6** | Flagellar biosynthesis protein flhA | flhA BN389_07260 | Flagellum and nanotube assembly |
| **A0A0E1RBB6** | Lipoteichoic acid synthase | ltaS1 BN389_09570 | Sulfuric ester hydrolase activity |
| **A0A0E1R5L3** | Probable undecaprenyl-phosphate N-acetylglucosaminyl 1-phosphate transferase | tagO BN389_09890 | Biosynthesis of teichoic acid |
| **A0A0E1RBB6** | Lipoteichoic acid synthase | ltaS1 BN389_09570 | Sulfuric ester hydrolase activity |
| **A0A0E1R7B4** | UDP-N-acetylmuramate--L-alanine ligase | murC BN389_16290 | Peptidoglycan precursor biosynthesis |
| **A0A0E1RA78** | Putative penicillin-binding protein pbpX | pbpX BN389_05770 | Peptidoglycan biosynthesis |
| **A0A0E1R443** | D-alanine--D-alanine ligase | ddl BN389_08830 | Peptidoglycan precursor biosynthesis |
| **A0A0E1R8F3** | MurNAc-6-P etherase | murQ BN389_16860 | Cell wall turnover |
| **A0A0E1RAC0** | Uncharacterized protein SSP1546 | BN389_06270 | Glycerone kinase activity; lipid binding |
| **A0A0E1R5U7** | Flagellar hook-associated protein 1 (HAP1) | flgK BN389_07510 | Motility and chemotaxis |
| **A0A0E1R424** | Cell wall surface anchor family protein | BN389_08630 | Cell wall surface anchor |

(cont.).

Table 4.12 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R7Z6** | Phosphopantetheine adenylyltransferase | coaD BN389_20800 | Lipopolysaccharide biosynthesis |
| **A0A0E1REN7** | Uncharacterized ABC transporter ATP-binding protein YdiF | ydiF BN389_21020 | ATPase activity; ATP binding; DNA binding |

## 4.2.5. Metabolism Related Proteins after 4-HBA Treatment

Bifunctional adenosylcobalamin biosynthesis protein CobU, NAD(P)H dehydrogenase [quinone] 1, L-lactate dehydrogenase (L-LDH), 3-oxoacyl-[acyl-carrier-protein] reductase FabG, GTP-sensing transcriptional pleiotropic repressor, HTH-type transcriptional regulatorare notable metabolism-related proteins (Table 4.13). Bifunctional adenosylcobalamin biosynthesis protein CobU catalyzes ATP-dependent phosphorylation of adenosylcobinamide (coenzyme B12). After phenolic acid treatment, protein CobU was observed in the protein profile of bacteria. This might be indicated that *L. monocytogenes* cells tend to increase metabolic activities in the presence of 4-HBA.

In bacteria, 3-oxoacyl-[acyl-carrier-protein] reductase (fabG), and 3-oxoacyl-[acyl-carrier-protein] synthase 2 (fabF) are essential enzymes that participate in type II fatty acid biosynthesis. While the fabG catalyzes a reaction that necessary for the elongation cycle of fatty acid biosynthesis, the fabF gene catalyzes a condensation reaction. The detection of these genes might be because of the utilization of fatty acids in the repair process of disorders of the cell membrane resulting from 4-HBA as well as cell wall maintenance.

NAD(P)H: quinone oxidoreductase 1 (NQO1) is a flavoenzyme that responds to environmental stress. İt provides protection when electron transfer chains are affected or when in the environment of highly oxidative caused by a variety of metabolic events. NQO1 gene was identified only *L. monocytogenes* cells with 4-HBA treated. It might indicate the preference of bacteria to use non-oxidative metabolic pathways to protect cells from oxidative damage.

L-Lactate is a product that achieved from especially fermentation of glucose reactions. L-lactate dehydrogenase (L-LDH) catalyzes the oxidation of L-lactate to pyruvate. L-

LDH in *L. monocytogenes* was observed in the presence of 4-HBA. Inducement of this protein might have been required to generate more increasing equivalents to be used in metabolism. In addition, this protein can indicate that the energy needs of the cells also are provided. GTP-sensing transcriptional pleiotropic repressor (CodY) is a GTP binding protein that detects intracellular GTP concentration as an indicator of nutritional limitations. Identification of this protein after 4-HBA application might indicate the less access of bacteria to the nutrients or inability of *L. monocytogenes* to use the present nutrients within the media. Moreover, the literature showed that the CodY gene regulates virulence mechanisms in the pathogen gram-positive bacteria. Observed that the expression of the most important virulence genes often associated with the exhaustion of available nutrients. In a study, Nicholas R Waters et alshowed that in the starvation conditions, *S. aureus* used CodY to limit host damage (Waters et al. 2016).

Table 4.13 Metabolism related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1RC11** | Cobalt-precorrin-5B C(1)-methyltransferase | cbiD BN389_12120 | Methyltransferase activity |
| **A0A0E1R652** | Putative dipeptidase | ykvY BN389_16030 | degradation of peptides |
| **A0A0E1R8W3** | NAD(P)H dehydrogenase [quinone] 1 | NQO1 BN389_18160 | NAD(P)H dehydrogenase (quinone) activity |
| **A0A0E1R6W9** | Hydrolase family protein | BN389_11460 | Hydrolase activity |
| **A0A0E1R860** | Isocitrate dehydrogenase [NADP] | icd BN389_15910 | Isocitrate dehydrogenase (NADP+) activity; magnesium ion binding; NAD binding |
| **A0A0E1RB96** | Phosphosugar-binding transcriptional regulator, RpiR family | BN389_26660 | Carbohydrate derivative binding; DNA binding; DNA-binding transcription factor activity |
| **A0A0E1RDM4** | ABC transporter, substrate-binding protein | BN389_17570 | SBP-dependent ABC transporters recognize a broad range of ligands from metal ions |
| **A0A0E1R7Z9** | Fumarate hydratase class II | fumC BN389_22580 | Fumarate hydratase activity |

(cont.)

Table 4.13 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R6V7** | 3-oxoacyl-[acyl-carrier-protein] reductase FabG | fabG BN389_18330 | Fatty acid biosynthesis |
| **A0A0E1RA35** | Hydrolase, CocE/NonD family | BN389_05320 | Dipeptidyl-peptidase activity |
| **A0A0E1R848** | Folylpolyglutamate synthase | folC BN389_15760 | Biosynthesis of folate |
| **A0A0E1R470** | Protoporphyrinogen oxidase | hemY BN389_09130 | Oxygen-dependent protoporphyrinogen oxidase activity |
| **A0A0E1R3R7** | Uncharacterized protein | ykgB BN389_05950 | Pentose phosphate pathway |
| **A0A0E1R967** | Uracilphosphoribosyltransferase | upp BN389_25000 | Magnesium ion binding |
| **A0A0E1R558** | Carbon-nitrogen family | BN389_08190 | Hydrolase activity |
| **A0A0E1RCN4** | Pyrroline-5-carboxylate reductase 3 | proG BN389_14120 | Pyrroline-5-carboxylate reductase activity |
| **A0A0E1RF85** | PTS system fructose-specific EIIABC component | fruA_4 BN389_23020 | Fructose uptake and phosphorylation |
| **A0A0E1RBF5** | GTP pyrophosphokinase | yjbM BN389_09970 | GTP diphosphokinase activity; kinase activity |
| **A0A0E1R849** | Ferrous iron transport protein B | feoB BN389_21350 | Ferrous iron transmembrane transporter activity; GTP binding |
| **A0A0E1R3F6** | L-lactate dehydrogenase (L-LDH) | ldh1 ldh BN389_02240 | L-lactate dehydrogenase activity |
| **A0A0E1R646** | Ethanolamine ammonia-lyase heavy chain | eutB BN389_11940 | Ethanolamine ammonia-lyase activity |
| **A0A0E1R905** | 3-oxoacyl-[acyl-carrier-protein] synthase 2 | fabF BN389_22340 | Fattyacid biosynthesis |
| **A0A0E1R5V0** | Uncharacterized protein yqeY | yqeY BN389_14930 | Carbon-nitrogen ligase activity, with glutamine as amido-N-donor |
| **A0A0E1RCE6** | HTH-type transcriptional regulator | glnR BN389_13220 | DNA binding, regulation of glutamine synthesis |

(cont.).

Table 4.13 (cont.).

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R853** | Hydrolase, alpha/beta fold family | BN389_21400 | Hydrolase activity |
| **A0A0E1R6S3** | GTP-binding protein TypA/BipA homolog | typA BN389_10960 | GTPase activity; GTP binding |
| **A0A0E1R565** | Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex | pdhC BN389_10850 | Transferring acyl groups links glycolysis and TCA cycle |
| **A0A0E1R936** | Purine nucleoside phosphorylase DeoD-type | deoD BN389_18810 | Purine-nucleoside phosphorylase activity |
| **A0A0E1R6Y8** | Bifunctional adenosylcobalamin biosynthesis protein CobU | cobU BN389_11660 | Cobinamide phosphate guanylyltransferase activity; GTP binding |
| **A0A0E1R5S7** | GTP-sensing transcriptional pleiotropic repressor | codY BN389_13050 | Regulation of a large regulon in response to branched-chain amino acid limitation |

## 4.2.6. Redox Related Proteinsafter 4-HBA Treatment

Two proteins related with the redox and cell homeostasis identified in 4-HBA treated *L. monocytogenes* were listed in table 4.14. Alcohol dehydrogenases (ADH) simplifies the interactions between alcohols, aldehydes, and ketones. Lack of the Adh gene can affect some properties such as unbalance of redox in the cell or acetaldehyde accumulation. After 4-HBA exposure, NADPH-dependent butanol dehydrogenase (adh1) was observed in the protein profile of *L.monocytogenes*. This situation can show that cells overcame the phenolic stress, keeps growing.

Table 4.14 Redox related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R5F5** | NADPH-dependent butanol dehydrogenase | adh1 BN389_11850 | Metal ion binding; oxidoreductase activity |
| **A0A0E1RBP3** | Thioredoxin-like_fold domain-containing protein | BN389_10920 | Thioredoxins acts as antioxidants by facilitating the reduction of other proteins |

### 4.2.7. Virulence Related Proteins after 4-HBA Treatment

One of the functional categories was made for the proteins related to the pathogenicity of bacteria. When the effect of 4-HBA on *L. monocytogenes* pathogenicity was examined, 3 differently identified proteins were found (Table 4.15).Generally, these proteins had functions in continuation of infection, fighting against the host defense system, response to antibiotics and immune system. Antigen A (lmaA) is a surface protein that is intrinsic to *L. monocytogenes* and functioning in the attachment to the host cells. lmaA gene can stimulate an immune response due to acceptance as a threat. The appearance of this protein after 4-HBA application might cause highvirulence of *L. monocytogenes* by resulting in a type defensible to host immune cells and antibiotics. The efflux of chemically compounds such as antibiotics by membrane-bound multidrugtransporters is important in bacterial multidrug resistance (MDR). There is more thanone gene that participates in the regulation of transcription of the MDR transporter genes and the HTH-type transcriptional activator (mta) is one of them. The possible appearance of this protein with 4-HBA might matter in terms of increased resistance ability of *L. monocytogenes* against multiple drugs. Bacterial secretion system is a cellular device that utilizes in the secreting of virulence factors to manipulate the host or to fight with the immune system (Garufi, Butler, and Missiakas 2008). ESAT-6-like protein is a type of secretion system that had been identified in also different strains. Exported effector protein EsxA, and EsxB are members of the ESAT-6 like system. The identification of these proteins may represent a significant effect on the virulence of pathogenic bacteria. A study concluded that the presence of these genes increases abscess formation in *S. aureus Newman* (Burts et al. 2005). In this study, EsxA gene was observed in 4-HBA treated cells. It might indicate that significantly developed resistance, defense and increased susceptibility to antibiotics.

Table 4.15 Virulence related

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R1T1** | ESAT-6 protein | esxA BN389_00680 | Protein secretion |

(cont.).

Table 4.15 (cont.)

| PROTEIN ID | PROTEIN NAMES | GENE NAMES | FUNCTION |
|---|---|---|---|
| **A0A0E1R369** | HTH-type transcriptional activator | mta BN389_05630 | Regulation of multidrug-efflux transporter genes, Resistance against toxins/ antibiotics |
| **A0A0E1R8X4** | Antigen A | lmaA BN389_01370 | It is regarded as a threat by the immune system and is capable of stimulating an immune response |

# CHAPTER 5

# CONCLUSION

Proteomics researches are an invaluable methodfor the identification of stress-response in pathogenic bacteria. The proteomic approach allows the identification of the protein profiles of bacteria under the stress conditions and, it provides the identification of primary or secondary targets by remarkable protein changes.

In this study, mechanisms of molecular markers for the antimicrobial effects of water-soluble phenolics 3-hydroxyphenyl acetic acid (3-HPAA) and 4-hydroxybenzoic acid (4-HBA) on *Listeria monocytogenes*, which is a dangerous food-related pathogen, were elucidated via the using the MS-based proteomics approach.

According to the outcomes obtained from shotgun proteomics, various changes were observed in DNA, RNA, metabolism, cell wall, protein synthesis, redox, and pathogenicity related pathways of the bacteria due to the adaptation process to both phenolic acids-containing environment.

The most remarkable targets for tested phenolic acids could be presented as their effects on the protein synthesis mechanisms and the ribosome. After the phenolic acid application, and then the look at the protein profile of the *L. monocytogenes*, too much sub-unıt that participates in protein synthesis was observed. This might show that cell synthesizes proteins but not achieve assembly map in the presence of phenolic acids.

To the best of our knowledge, the studies of clarification of the antimicrobial effects of phenolic acids with proteomics studies are very limited. The significance of this study was the determination of the possible ways by shotgun proteomics approach to combat *L.monocytogenes* that able to develop resistance against neither 3-HPAA nor 4-HBA.

The result indicate that 4-HBA has a higher antimicrobial effect than that of 3- HPAA. The4-HBA showed higher inhibition percentage at the same concentration. This might be due to their carboxyl groups are attached to the benzene ring differently. In 3-HPAA, the carboxylic group doeslink to the benzene ring via an ethylene group, in contrast, in 4-HBA, the carboxylic group is directly attached. These structural differences might be the reason for the variations of the antimicrobial effects between 3-HPAA and 4-HBA. Besides that, 4-HBA are more polar than 3-HPAA because of the side chain. The chain

of 4-HBA can help the transfer of acid through the cell membrane, and this is important for inhibition.

These achieved results can be lighted to further studies for investigating ofphenolic acids as a novel and the natural antibacterial-candidate for therapy methods as.

The great progress has been made in mass spectrometry, which can provide faster protein sequencing, identification, quantification, localization, and protein-protein interactions. Future studies may be carried out with a better mass spectrometer, which in terms of advanced scanning speed and sensitivity to confirm the obtained results.

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