CHARACTERIZATION OF HEAVY METAL RESISTANT BACTERIA USING INFRARED SPECTROSCOPY TOGETHER WITH CHEMICAL PATTERN RECOGNITION TECHNIQUES

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ABSTRACT

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Approximately 50 elements with the density larger than five are considered as heavy metals. Some of them are essential for growth, although they also form strong detrimental complexes in living organisms. Moreover, microorganisms can develop resistance toward metal burden. This exceptional microbial feature may have remarkable applications in industry, such as bioremediation of metal-contaminated waters and recovery of important metals from industrial wastes. Considering the above-mentioned potentials, the purposes of this work are: 1) To screen, identify and classify metal resistant bacterial strains 2) To characterize the heavy metal resistance related molecular alterations happening in these bacterial isolates 3) To develop and optimize robust detection techniques for the elucidation of metal resistance in bacteria.

In the first part of the study, it was aimed to develop a reliable method for identification of key molecular profile changes and thus for discrimination of Cd and Pb-resistant bacteria using ATR-FTIR spectroscopy coupled with unsupervised multivariate analysis techniques such as Hierarchical Cluster Analysis (HCA) and Principal Component Analysis (PCA).

In the second part, it was intended to shed light on the possible mechanisms of Ag resistance and on how these changes are reflected in the cell's structure and molecular

composition, especially in *E. coli* and *S. aureus*. Furthermore, in the current study, unsupervised chemometric methods, namely PCA and HCA were used, since they are broadly applied methods for the differentiation of microbial communities with impressive accuracy.

The third part of the study, originating from the above-mentioned investigations, demonstrates the prompt and successful discrimination and most importantly classification of Ag, Cd and Pb-resistant laboratory and environmental (freshwater) bacteria from their non-resistant counterparts. In this context, a supervised classification pattern-recognition tool, Soft Independent Modeling of Class Analogy (SIMCA), was applied in light of FTIR spectra of these bacterial isolates.

Information obtained from the ATR-FTIR spectroscopy coupled with chemometrics could serve as a guide for further studies on heavy metal resistance mechanisms, the detailed elucidation of which could in turn lead to the selection of the bacterial species best suited for bioremediation. The identification and classification of heavy metal resistant life forms in a fast and productive manner would also contribute to the establishment of sustainable, green and worthwhile biogeotechnological operations to rehabilitate the soil and water.

Keywords: Heavy metal resistant bacteria, Infrared, ATR-FTIR spectroscopy, Chemometrics, PCA, HCA, SIMCA

ÖZ

AĞIR METAL DİRENÇLİ BAKTERİLERİN KIZILÖTESİ SPEKTROSKOPİSİ VE KİMYASAL ÖRÜNTÜ TANIMA TEKNİKLERİ İLE BİRLİKTE NİTELENDİRİLMESİ

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Özkütlesi 5'ten büyük olan yaklaşık 50 metal element, ağır metal olarak bilinmektedir. Bazıları canlıların büyümesi için gerekli olmasına rağmen, canlı organizmalarda zararlı karmaşık formlar oluştururlar. Bununla birlikte, mikroorganizmalar metal yüküne karşı direnç geliştirirler. Bu kuraldışı mikrobik özelliğin, sulardaki kirliliğin biyolojik olarak giderilmesi ve sanayi atıklarından metallerin geri dönüşümünde kullanılması gibi, sanayide kayda değer önemli uygulamaları olabilir. Yukarıda belirttiğimiz potansiyeller göz önüne alındığında, bu çalışmanın amaçlarını,

1) Metal dirençli bakterilerin taranması, tanımlanması ve sınıflandırılması,

2) Bakterilerde ağır metal direnç mekanizmaları ile ilişkili moleküler değişimlerin karakterize edilmesi,

3) Bakterilerdeki metal direncini ortaya çıkarmak için hızlı tespit yöntemlerinin geliştirilmesi ve optimize edilmesi oluşturmaktadır.

Çalışmamızın ilk bölümünde, ATR-FTIR spektroskopisi ile birlikte gözetimsiz çoklu değişken analiz tekniklerinden hiyerarşik kümeleme (HCA) ve temel bileşen analizleri (PCA) kullanılarak, Cd ve Pb dirençli bakterilerde anahtar moleküler değişimlerin

tanımlanması ve bu bakterilerin ayrılmasında kullanılacak güvenilir bir metot geliştirilmesi amaçlandı.

Çalışmanın ikinci kısmımda ise, özellikle *E. coli* ve *S.aureus* bakterilerinde, Ag dirençlilik mekanizmaları ve bu mekanizmanın hücre yapısı ve moleküler kompozisyonunda ne tür değişiklere yol açtığının belirlenmesine yönelik ışık tutma amaçlanmıştır. Bununla birlikte, mikrobiyal toplulukların ayrılmasında kullanılan ve yüksek doğruluk verebilen metotlar olması nedeniyle, gözetimsiz kemometrik metotlardan PCA ve HCA kullanıldı.

Çalışmanın üçüncü kısmı, yukarıda bahsedilen araştırmalara dayanarak, Ag, Cd ve Pb dirençli laboratuvar ve çevresel bakteri suşlarının dirençli olmayan suşlardan çabuk ve başarılı bir şekilde ayrımı, daha da önemlisi sınıflandırılmasını içermektedir. Bu kapsamda, bakteri izolatlarına ait FTIR spektrumlarına, sınıflandırma amaçlı olarak gözetimli örüntü tanımlama metotlarından Sınıf Analojisinin Hafif Bağımsız Modellemesi (SIMCA) uygulandı.

ATR-FTIR spektroskopisi ile kemometrik analiz yöntemlerinin birlikte kullanılmasından elde edilen bilgiler, ağır metal direnç mekanizmalarıyla ilgili ileriki çalışmalara rehber olabilir, diğer bir deyişle bu mekanizmaların açıklanması, biyoremediasyon için en uygun bakteri türlerinin doğru olarak seçimini sağlayacaktır. Hızlı ve etkin bir şekilde ağır metal dirençli yaşam formlarının tanımlanması ve sınıflandırılması, suyun ve toprağın iyileştirilmesinde kullanılacak sürdürülebilir, faydalı ve yeşil biyojeoteknolojik uygulamaların kurulmasına da katkı sağlayacaktır.

Anahtar Kelimeler: Ağır metal dirençli bakteriler, Kızılötesi, ATR-FTIR spektroskopisi, Kemometrik, PCA, HCA, SIMCA

Dedicated to the memory of the ancestors those who devoted their lives for the well-being of our nation

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Ag	Silver
ATR	Attenuated Total Reflectance
Cd	Cadmium
EPS	Extracellular Polysaccharides
FTIR	Fourier Transform-Infrared Spectroscopy
HCA	Hierarchical Cluster Analysis
IR	Infrared Spectroscopy
LPS	Lipopolysaccharide
MIC	Minimal Inhibitory Concentration
NA	Nutrient Agar
NB	Nutrient Broth
OD	Optical Density
Pb	Lead
PCA	Principal Component Analysis
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
SEM	Standard Error of Mean
SIMCA	Soft Independent Modeling of Class Analogy Analysis

CHAPTER 1

INTRODUCTION

1.1 Heavy metals and pollution of the environment

Heavy metals are commonly found elements in the universe. In chemistry, around 50 elements are identified as heavy metals based on their atomic density. The density should be larger than 5 g/cm^3 in order to be considered as heavy metals (Kumar et al., 2015). From the environmental point of view, the elements generating toxicity at small quantities and harm to the environment with no essential role for living organisms are recognized as heavy metals (Kumar et al., 2015). Metals such as cadmium, chromium, copper, lead, silver and zinc are commonly found pollutants in the ecosystem (Hong et al., 2011). Anthropogenic input like urbanization, persistent agricultural activities and various integrated industrial facilities are major causes for planetary heavy metal contamination (Herrera-Estrella and Guevara-Garcia, 2009). Another ecological disaster induced by humans is the contamination of aquatic sources by heavy metals (Hong et al., 2011). In this context, metal refining from ores, chemical, metallic, plastic, paint/coating, battery and tire productions and natural oil/gas extraction processes have detrimental outcomes. In addition, the energy-producing facilities, electroplating, concrete, and steel and porcelain industries release their heavy metal wastes directly to aquatic environments (Freitas et al., 2008; Kumar et al., 2015).

Cadmium (Cd) as an important environmental contaminant has been utilized in various industrial processes. It is also abundantly found in the surface of Earth (approximately 0.1 mg/kg). The highest natural concentrations of Cd are measured in complex rocks.

These complex structures are formed by the accumulation and precipitation of mineral compounds with rock particles (Tchounwou et al., 2012).

Although the concentration of lead (Pb) in nature is very low, human activities prominently pollute the environment by manufacturing lead-acid batteries, weaponry, instruments buffering X-rays and different types of metallic items (Tchounwou et al., 2012).

Silver (Ag) is an extremely toxic heavy metal coming after mercury (Savery et al., 2013). Disintegration and corrosion of land and rock are natural, whilst mining, electronic and cloud seeding processes are man-made sources of Ag pollution. Discharges of Ag from these industrial wastes have large impact in the contamination of the marine ecosystem (ATSDR, 1990; Savery et al., 2013).

1.2 A summary of microbial heavy metal resistance mechanisms

Even though some heavy metals are used as micronutrients, high concentrations of heavy metals are toxic. To prevent metal toxicity, cells have to eliminate metals rapidly and expeditiously. Generally, there are two main mechanisms to gain resistance to heavy metals: eukaryotes form complex structures from toxic metal cations and prokaryotes decrease heavy metal burden by actively discharging these ions out of the cell. Some binding factors and some enzymes that function in oxidation, reduction, methylation, and demethylation reactions work as defenders against heavy metal toxicity (Hynninen, 2010). In this part of the thesis, the resistance mechanism to Cd, Pb and Ag in bacteria are presented, since the interactions of these particular metals with several bacterial isolates were studied and discussed.

Cd, Pb and Ag have no biological importance for the organism. Just a small quantity of Cd is sufficient to cause lethality in terrestrial microbial life forms. Intake of Cd into cells occurs by ion uptake complexes, which have two valences (Zn^{2+}, Ca^{2+}, Mn^{2+}) (Prapagdee and Watcharamusik, 2009). The relationship of Cd with numerous microbes were previously analyzed (Banjerdkij et al., 2003) and it was reported that Cd might alter the constituents of these entities (Dobson, 1992; Prapagdee and

Watcharamusik, 2009). The genetic basis of Cd resistance systems in bacteria are well characterized compared to other metals (Saluja and Sharma, 2014). Previous studies showed that in the CadA resistance system, a plasmid-encoded Cd efflux system is used, which relies on CadA protein (P-type ATPase) (Tsai and Linet, 1993). Nevertheless, gram-negative bacteria employ a multi-protein chemiosmotic antiport system (Silver, 1996). The cation efflux system, called the *czc* system, removes cobalt, zinc, and cadmium from cells, which can get inside the cells quickly by a rapid and non-specific transport system for Mg^{2+}. The *czc* is made of three subunits: CzcC, CzcB, and CzcA (Saluja and Sharma, 2014). CzcA is known as RND protein (Tseng et al., 1999) and contains two hydrophilic domains which are found in the periplasm (Goldberg et al., 1999), similar to the other subunits of the *czc* complex, CzcB and CzcC. The constructive *czcCBA* genes are regulated by at least six known regulatory proteins (CzcD, CzcR, CzcS, CzcN, CzcI) and one unknown sigma factor (RpoX). CzcA and CzcB proteins pump zinc ions out of the cell, while CzcB protein is required for Cd resistance mechanism and cobalt transportation (Saluja and Sharma, 2014). *CzcA* mutation has described to eliminate zinc and cadmium resistance. Accordingly, CzcA is proposed as the actual efflux-transportation protein while the other structural proteins are assumed to regulate CzcA's specificity for exportation of the ions (Nies and Silver, 1995). Because of the existence of various anionic structures, bacteria are negatively charged and can easily bind positively charged metal ions. Therefore, isolation and characterization of heavy metal resistant bacteria can help to develop new applications for environmental protection studies (Saluja and Sharma, 2014).

Transporters such as P-type ATPases belong to the transport protein family that relocates ions against the concentration gradient using energy obtained by ATP hydrolysis. "P-type" means the generation of a phosphoenzyme intermediate during the reaction. The energy required for the translocation of an ion across biological membranes is gained from the energy released by the removal of the phosphate from ATP. The required substances for this reaction are positively charged ions like protons, cadmium, silver, lead and others (Hynninen, 2010). Transport proteins are also important in heavy metal equilibrium and resistance. These efflux transporters are members of P1B-type subfamily of ATPases (Axelsen and Palmgren, 1998) which are divided into two subgroups, Cu^{+}/Ag^{+}-translocators and $Zn^{2+}/Cd^{2+/}Pb^{2+}$-translocators,

depending on substrate specificity (Argüello, 2003). The ATPases that are in charge of heavy metal-translocation have a conserved proline amino acid pursued by a cysteine amino acid. These pumps maintain equilibrium of crucial metals such as Cu^{+}, Co^{2+}, and Zn^{2+}, and they are responsible for toxic metal (Pb^{2+}, Cd^{2+} and Ag^{+}) resistance. CPxtype ATPases carry metals to the periplasm with no further transport from the periplasm to the outside. The substrates of CPxtype ATPases are thought to be metal-thiolate combined structures rather than free metals (Sharma et al., 2000; Hynninen, 2010).

The cation diffusion facilitator family (CDF) of carriers organize cellular entrance and exit of heavy metals. They are exclusively operated for metal transportation, which is contrary to the functions of the P-type ATPases and other transporters. Transportation is mainly accomplished by a potassium gradient and proton motive force (Hynninen, 2010).

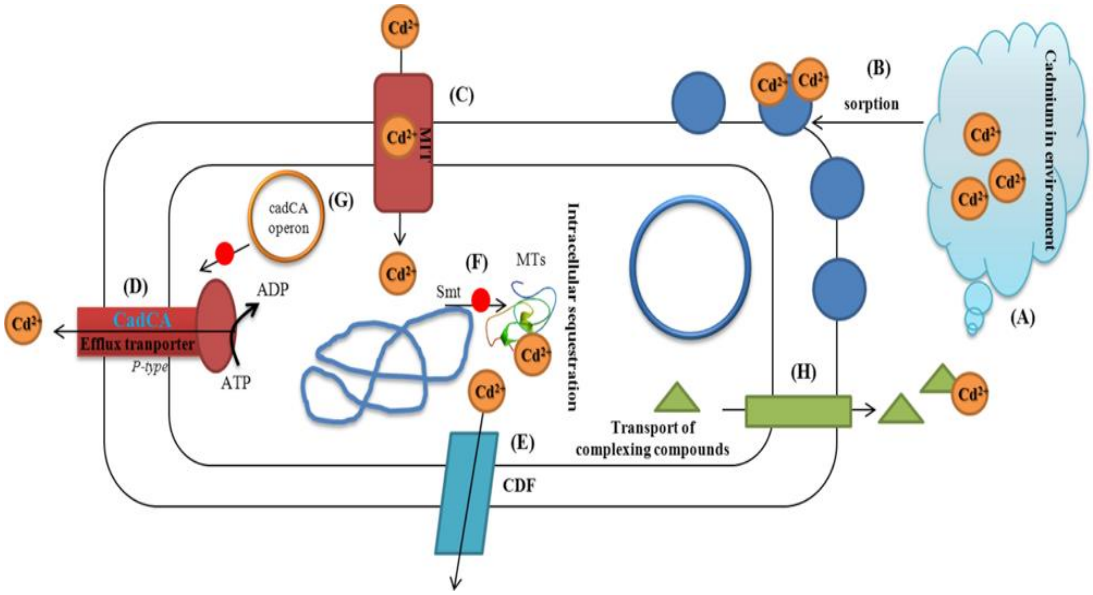

Figure 1.1 Cd resistance systems in bacteria (Taken from Sochor et al., 2011).

Lead precipitation processes are divided into three stages: binding of metal to the cell surface, process-grounded uptake within the cell, and eventual condensation of lead ions as lead phosphate crystals (Levinson et al., 1996). For example, in *Staphylococcus aureus*, lead is intracellularly condensed as lead phosphate. Therefore, *S. aureus* can resist 600-fold higher dose of lead ions compared to a sensitive strain (Levinson et al., 1996; Levinson and Mahler, 1998). In *Vibrio harveyi*, lead ion is condensed as $Pb_9(PO4)_6$, which is an unusual and complex lead phosphate salt. Although the details of the regulation is not known completely, this condensation process is controlled partially by quorum sensing. In this model, quorum sensing is proposed to control the availability of inorganic phosphates (Mire et al., 2004). The $Pb_9(PO4)_6$ form of lead ion condensation was also seen in *Providencia alcalifaciens* 2EA, due to the inorganic phosphates generated by phosphatase (Naik et al., 2012). Intracellular sequestration of lead ion was also shown by *in situ* experiments. In various strains of terrestial bacteria, lead containing discharges were identified (Perdrial et al., 2008). In addition, treatment of *Synechocystis* PCC 6803 strain of a cyanobacterium with lead ions caused an increase in cell quantity and in cell size (Arunakumara and Xuecheng, 2009).

Class II Metallothioneins, which are generally employed proteins in heavy metal removal processes, are expressed by *smtA* gene. SmtB protein is known to inhibit *smtA* transcription. This gene functions in zinc homeostasis, and *smtA* deletion increases sensitivity to zinc ion in *Synechococcus* PCC 7942 (Blindauer, 2011). Lead was also reported as one of the few cations that turns on *smtA* expression. Lead was shown to trigger *smt* gene expression and MT biosynthesis not only in *Synechococcus* PCC 7942, but also in *B. cereus*, *Streptomyces* sp., *Salmonella choleraesuis* 4A and *Proteus penneri* GM-10 (Huckle et al., 1993; Rifaat et al., 2009; Murthy et al., 2011; Naik et al., 2012). *BmtA* is another Class II Metallothionein gene, which expresses a lead ion binding metallothionein in *Pseudomonas aeruginosa* WI-1 (Naik et al., 2012). Moreover, a metallothionein-like protein is shown to interact with lead ion in *B. megaterium* (Roane, 1999). Besides, some extracellular enzymes, such as *S. subrutilis* superoxide dismutase, supposedly bind to lead ion (So et al., 2001).

MerR family of regulator proteins are in charge of controlling the intracellular concentration of cadmium, silver, lead and other metals in bacteria. These proteins function in the regulation of genes that play crucial roles in bacterial defense systems against toxic metals or increased levels of microelements. Distinct C-terminal ligand binding domains of MerR proteins make them capable to apprehend a specific metal (Chen and He, 2008). PbrR from *C. metallidurans* CH34 (CmPbrR) is a MerR-like protein encoded by *pbrR* gene, located on pMOL30. PbrR regulates the expression of *pbr* lead resistance genes whose products are the components of lead efflux pump (Borremans et al., 2001; Taghavi et al., 2009). CmPbrR is very specific to lead ions (more than 1000-fold specificity over other metals) (Chen et al., 2005). In fact, CmPbrR and its two homologues are the only lead specific regulatory proteins identified until now (Chen and He, 2008; Taghavi et al., 2009).

ZntR is the other regulator belonging to the family of MerR proteins. It is in charge of the regulation of *zntA* gene expression. ZntR is known to transport lead as well as zinc and cadmium ions out of the cell in *E. coli* (Brocklehurst et al., 1999, Binet and Poole, 2000). It was claimed that zinc and cadmium stimulate *zntAR*, and this regulation of *zntA* is also driven by ZntR (Binet and Poole, 2000). Although the interplay of zinc ions and ZntR is well known, ZntR and lead ion relationship has not been clearly shown, yet (Binet and Poole, 2000).

A Pbr efflux system, which functions in the clearance of lead from the cell, was first demostrated by Borremans et al., (2001) in *C. Metallidurans* (next two ones as well) CH34. Since *C. metallidurans* CH34 is known to be resistant to several heavy metal ions, the bacterium is a common model organism for metal resistance and tolerance studies (Janssen et al., 2010). *Pbr*, located within a *mer-pbr-czc* island on pMOL30, expresses the Pbr efflux system (Monchy et al., 2007). The seven genes, which are located on *pbr* locus (*pbrUTRABCD*), appear as two transcription units, namely *pbrUTR* and *pbrABCD* (Hynninen et al., 2009). The transcriptional activator PbrR regulates these *pbr* promoters (Permina et al., 2006; Hynninen et al., 2009). Until now, *pbr* has been found only in plasmids of lead-resistant bacteria (Permina et al., 2006; Hynninen et al., 2009).

In *C. metallidurans* CH34, *pbr* encoded proteins are: the membrane protein PbrT; a PIB-type ATPase PbrA (the main efflux transporter); undecaprenyl pyrophosphate phosphatase PbrB (an integral membrane protein); a putative signal peptidase PbrC; a putative intracellular lead binding protein PbrD and positive regulator PbrR (Borremans et al., 2001; Monchy et al., 2007; Hynninen et al., 2009). As a member of iron/lead transporter superfamily, PbrT (lead uptake permease) functions in lead influx together with other certain transporters (Debut et al., 2006). PbrD is proposed to bind to lead ions in the cytoplasm to reduce the toxic effect of these ions. It also functions as a chaperone for lead. Moreover, binding of lead to PbrD is suggested to activate *pbrA* expression (Jarosławiecka and Piotrowska-Seget, 2014). PbrD has a cysteine rich metal binding motif containing proline and serine residues. Given the fact that PbrD is not necessary for complete lead resistance, PbrD deficient organisms exhibit decreased level of intracellular lead (Borremans et al., 2001; Jarosławiecka and Piotrowska-Seget, 2014).

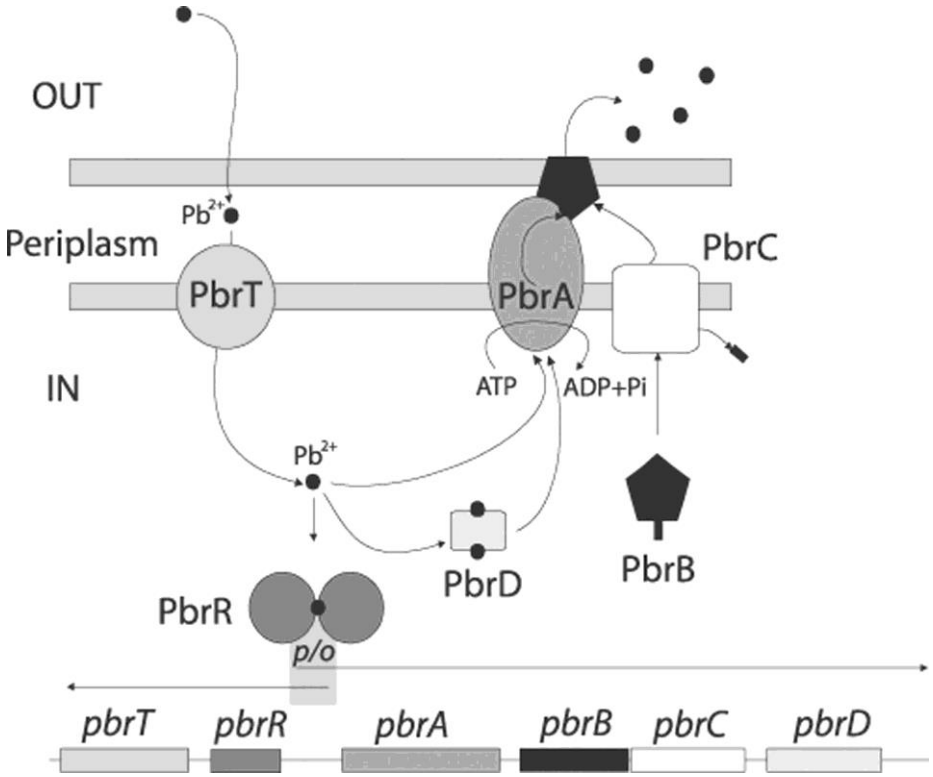

Figure 1.2 The lead-resistance efflux system Pbr (Taken from Mergeay et al., 2003).

Ag-resistant microorganisms have also been isolated from habitats with natural Ag. The first and well characterized Ag-resistance mechanism in bacteria is encoded on pMG101 plasmid of *Salmonella typhi*, which was responsible for killing several patients in Massachusetts General Hospital (McHugh et al., 1975). pMG101 is a 180 kb plasmid of the IncHI incompatibility group. The plasmid gives resistance to silver, mercury, tellurite, and to a broad range of antibiotics (Gupta et al., 1999). The locus for Ag resistance which is the *sil-CFBA(ORF105aa)PRSE* region, which contains nine genes, eight of which are homologs to other known metal resistance genes (Silver 2003; Silver et al., 2006; Mijnendonckx et al., 2013). A P-type ATPase efflux pump, called SilP, transports silver from the cell cytoplasm to the periplasm. The periplasmic protein SilF is proposed to be a chaperone that is responsible for Ag ion transportation from SilP to the SilCBA complex. The SilCBA complex is a member of heavy metal

efflux-resistance nodulation cell division family of efflux pumps. This complex is formed by a three-polypeptide membrane-potential dependent cation/proton antiporter system that spans the cell membrane. The system is formed by an efflux pump (SilA), an outer membrane factor (SilC), and a membrane fusion protein (SilB). This organization transports Ag ions from the periplasm to the exterior of the cell (Silver, 2003; Franke, 2007; Mijnendonckx et al., 2013). The other gene called *orf105*, found between *silA* and *silP* genes, expresses a 105 amino acid uncharacterized peptide (Gupta et al., 1999). It is known that the products of *silRS* control the *silCFBA(ORF105aa)P* genes. *SilRS* expresses a two-component regulatory system consisting of a transmembrane histidine kinase SilS and a response regulator SilR. Having its own promoter, *silE* gene is mapped downstream of *silRS*, and it encodes a periplasmic protein. SilE expression is found to be highly enhanced during growth in the presence of Ag ions (Mijnendonckx et al., 2013). The Ag detoxification systems are frequently conserved on transposons, which accelerate the dispersion of resistance (Mijnendonckx et al., 2013).

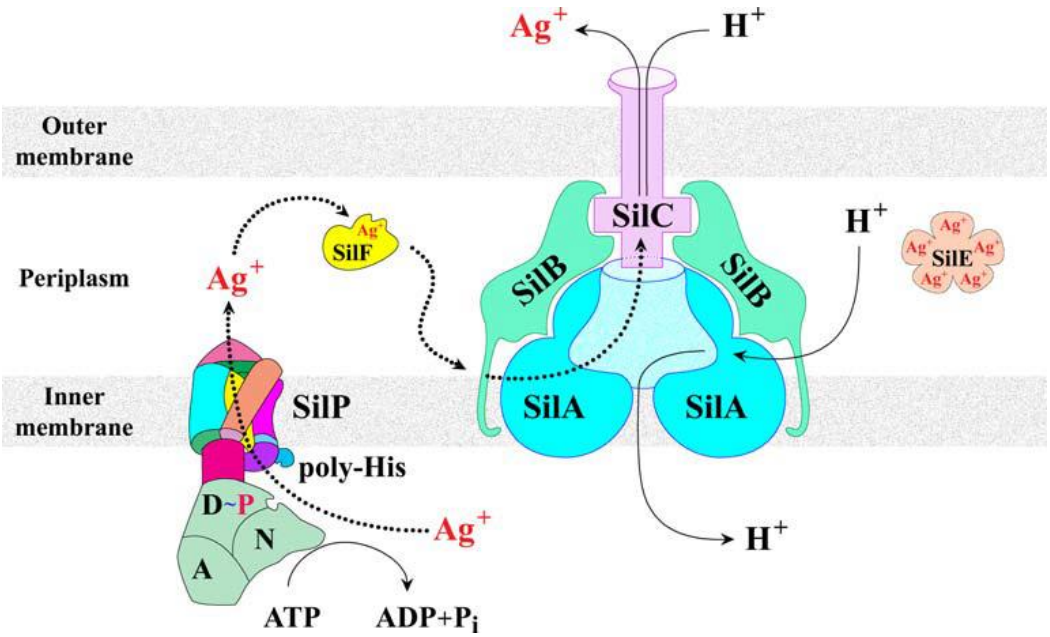

Figure 1.3 Bacterial Ag resistance mechanism (Taken from Silver et al., 2006).

1.3 Biological remediation technologies for heavy metal pollution

Appropriate restoration strategies for heavy metal contaminated habitats are realized using traditional applications like surface wadding and draining, land digging and flushing (Wuana et al., 2010; Vesely et al., 2012). However, large-scale application of these implementations led to accumulation of sewer water, which subsequently pollutes the subterranean water resources (Nouri et al., 2008). Therefore, the creation of original and unconventional strategies are imperative for these hazardous wastes (Sivakumar, 2013; Mani and Kumar, 2014). The contaminated environments can also be redressed via physical and chemical operations like charge substitution and electron-gaining reactions, condensation, membrane filtration and phase change (boiling) (Tang et al., 2007). Nevertheless, drawbacks like excessive cost and energy demand, filter clogging and insufficient productivity turned all these strategies into insufficient procedures for manufacturing enterprises.

In order to pick up the proper manipulations for the decontamination of metal pollutants, common criteria such as low expenditure, industrial pertinency and clarity are being searched (Acheampong et al., 2010). In this context, more recent and capable applications such as light induced chemical substitution, fluid and wetter filters, have been employed (Malaviya and Singh, 2011; Xu et al., 2013; Mani and Kumar, 2014). Another promising technology, bioremediation, is the application of microscopic life forms or their components exerting great capacity in scavenging and breakdown of pollutants (Gadd, 2000; Malik, 2004; Radhika et al., 2006; Farhadian et al., 2008; Guo et al., 2010).

The economical aspect of bioremediation is the principal superiority over traditional technologies. It is possible to perform it on a polluted site in which the technical staff are less exposed to the toxic substances. To summarize, low cost, long-lasting detoxification capacity, minimal invasiveness and possibility to be employed together with traditional remediation methods make bioremediation an attractive tool in environmental sciences (Vidali, 2001; Bai et al., 2008; Mani and Kumar, 2014).

The major drawback for bioremediation is the selection, identification and characterization of the appropriate microorganisms from the vast majority of

microorganisms for the remediation of heavy metal polluted habitats. In addition, low decontamination capacities due to the difficulties during the adaptation stage is another disadvantage of this technique. Finally, the lack of knowledge about the background molecular processes happening in microorganisms interrupts its routine utilization for field implementation (Guo et al., 2010).

Up to date, bioremediation studies have evolved a lot and continue expand vigorously. Nevertheless, enhancements in the taxonomic precision, methodological measurements, qualitative and quantitative techniques are mandatory (Mani and Kumar, 2014).

1.4 Infrared spectroscopic techniques in microbiology

1.4.1 Infrared spectroscopy

The information retrieved from bacterial cell is abundant and contains the structural and functional data of its biomolecules. Many analytical techniques such as wet biochemistry tools, mass spectrometry and various types of Raman and infrared spectroscopy have been perfected to acquire this knowledge from a bacterial cell. Infrared spectroscopy has been broadly utilized to gather molecular information from the bacterial cells (Huffman et al., 2003).

In this method, infrared light spans the molecules and alters their vibrational attitude by transferring radiation that causes them to vibrate and rotate. In other words, IR light or radiation arouse the particular chemical groups of biomolecules and as a result, they start to oscillate or turn exclusively at permanent penetration length (wavelength). This event causes intake of radiation at wavelengths, which is analogous to the wavering state of related functional groups (Alvarez-Ordóñez et al., 2011).

During the IR radiation of a sample a heterogeneous/integrated absorption spectrum is acquired, in which the intensities of the observed bands arise from the multiple scans recorded while IR light traverses a sample. The intensities and positions of these expansive and unique infrared peaks can be resolved to identify, characterize and

quantify the sample. Throughout the radiation, the IR bands resounds the chemistry of bacteria, which enables taxonomical differentiation or characterization of molecular alterations that they endured during environmental stress (Alvarez-Ordóñez et al., 2011). Even though this method is an accepted chemical approach in microbiology, only recently it has been employed to investigate the relationship between microbes and their environments or to study the ecology of microorganisms (Ammann and Brandl, 2011).

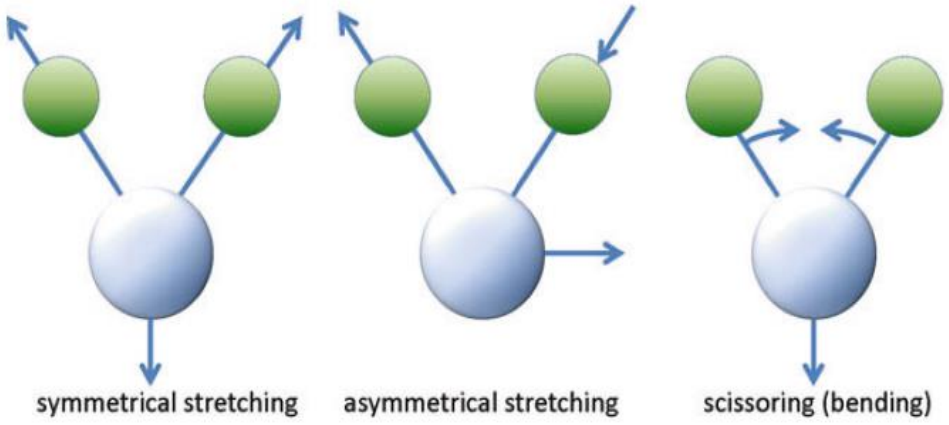

Figure 1.4 IR radiated molecular vibrations (Stretching and bending vibrational modes) (Taken from Alvarez-Ordóñez and Prieto, 2012).

The most widely used method among infrared techniques is Fourier Transform Infrared Spectroscopy (FTIR). The main component of FTIR is a light converting apparatus, known as interferometer/beam splitter. The beam splitter generally evaluates the collision model between two radiation poles. Particularly, it first divides the radiation into two parts and forces them to traverse diverse routes. Subsequently, these radiations come together as a single light and finally this light exits the interferometer (Smith, 2011).

The beam splitter transmits the radiation to the specimen and after the intensification of the wave, the information is translated to the digital mode in a computer for Fourier-transformation (Stuart, 2004).

The Michelson interferometer is a most utilized collision meter in FTIR experiments, in which a scan is produced by the relocation of the reflector (mirroring glass). Herein, the collision model is examined by the detecting component for radiation (detector) and by the application of arithmetic approach (Fourier-transformation); the final spectrum emanates (Stuart, 2004; Smith, 2011).

FTIR is a reliable and accurate chemical fingerprint technique, due to its rapidity, versatility and sensitivity. Low cost and reduced sample preparation steps make FTIR a very convenient tool for analytical measurements. Abundant data can be obtained about the specimen through the interpretation of a spectrum. Furthermore, definite wavenumber detections, supreme signal-to-noise ratios, outputs, and multiplex advantage, put the FTIR in a favorable position amongst other biochemistry techniques (Smith, 2011).

Bacterial components can be characterized using various IR spectroscopy based methods. Transmittance FTIR is among the most widely used IR spectroscopic techniques in which the sample is fixed/positioned in the course of IR light and examined. Another FTIR technique is diffuse reflectance FTIR (DRIFT) spectroscopy, in which the infrared light is sent to the specimen and returned or dispersed back from the specimen to the light detector. It is possible to interpret lyophilized bacteria using this technique, which is considered as supreme scattering specimen.

In attenuated total reflectance (ATR)-FTIR spectroscopy, a small amount of specimen is placed to an impenetrable and highly pure crystal with comparatively high-refractive index. Extensive specimen preparation is not required. In addition, the existence of the particular ATR cells permits the analysis of numerous specimens at a short time period. The interior facet of the crystal reflects the IR light generating transient influx that expands away from the exterior of the crystal and penetrates into the specimen. The specimen takes a part of the transient influx light and the reflected light moves onward to the light detector. Several studies have compared the abilities of the DRIFT and ATR-FTIR techniques in differentiation of bacteria and concluded that ATR-FTIR has finer resolution power for the distinction of subspecies compared to DRIFT technique (Alvarez-Ordóñez and Prieto, 2012).

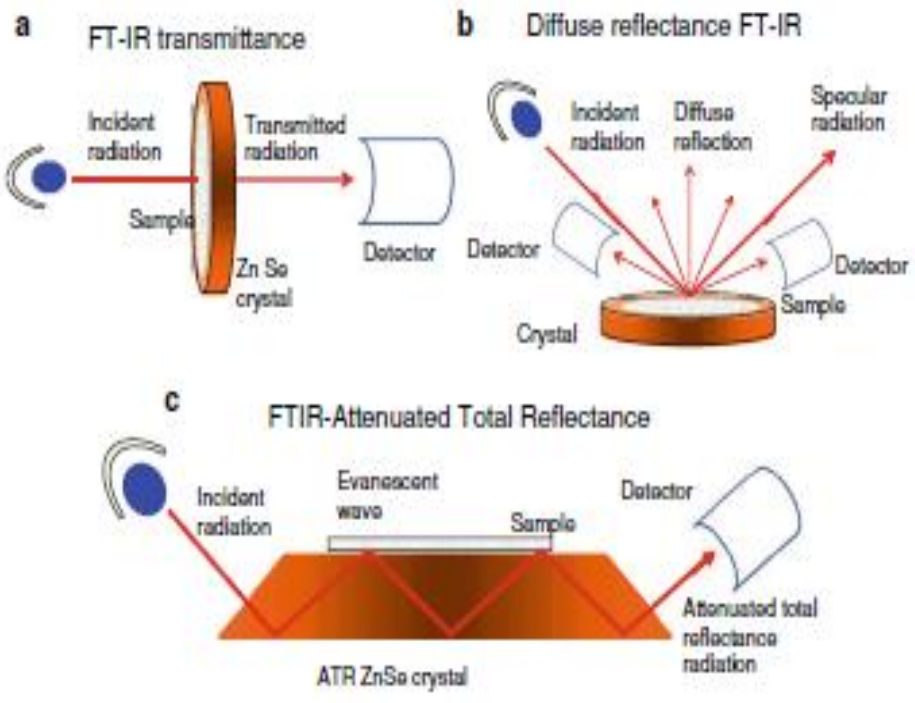

Figure 1.5 The fundamental FTIR techniques for the characterization of the bacterial biomolecules and stress response: (a) transmittance FTIR, (b) diffuse reflectance FTIR, (c) attenuated total reflectance ATR-FTIR (Taken from Alvarez-Ordóñez and Prieto, 2012).

1.4.2 Advantages of IR spectroscopy for microbiology

The selected main advantages of IR spectroscopy in microbiology are listed below: (Naumann, 2006).

1. IR spectroscopy is consistently applied to every culturable microbe without an exception. Even single colony (< 20 mm) is sufficient to be measured though this technique in a very short time (several minutes).

2. It is possible to detect, enumerate, classify and identify microorganisms in one single apparatus and interpret the outcomes of the experiment in 24 hours along with their desolation (isolation) and growth.

3. Taxonomic classification of microbes can be made, free from the particular preselection methods. The technique is exceptionally specific to differentiate the strains, species and genus contrary to some other usual identification methods.

4. The above-mentioned features empower IR spectroscopy to be applied for robust discrimination of pathogenic microbial life forms, epidemiology and sanitation studies, and clarification of infectious diseases. In addition, it enables to characterize and screen the environmental microbes, regulate the biotechnological operations and microbial nature in different industrial facilities and support type/strain accumulations. Furthermore, *in situ* identification of specific cellular constituents is accomplished for the microorganisms in several studies.

1.5 Chemical pattern-recognition methods in environmental chemistry and microbiology

Earth's ecosystem operates within a sophisticated harmony among the organic and inorganic elements. The analytical description of these components is compulsory for the comprehension of ecological events (Hopke, 2015). Contemporary progress in the field of environmental science requires attaining higher and unbiased knowledge about the intricate environmental chemistry based systems (Christy et al., 2013). Specimens such as biological organisms can be gathered from their habitats and their features can be simultaneously examined via biological detectors. Therefore, the application of convenient analytical tools in order to describe the bulk chemical data for the qualitative and quantitative interpretation of organisms is a mandatory step in ecological studies (Hopke, 2015). In the meantime, the magnificent improvements in chemical pattern recognition techniques have allowed scientists to accomplish this task, successfully. The main idea behind these techniques is to explore algorithmic and probabilistic tools in order to illuminate the analytical questions, to derive the most knowledge from the presented measurements and most importantly to reduce/remove the miscalculations and inaccuracies emerged during the environmental examinations. In addition, the delivery of pertinent knowledge about the contamination levels, geographical reorientation of contaminants and the discrimination of pollutants in a proper way from the vast number of gathered information are within the scope of chemometrics (Christy et al., 2013).

Intricate FTIR spectra of the microorganisms also allow the superior data mining and processing techniques in order to achieve dependable discrimination and differentiation. The FTIR spectra of chemical substances are simple and several bands can be examined by single manual measurements. However, the spectra for microorganisms are intricate fingerprints and should be interpreted by chemometric tools. In these chemical pattern recognition techniques, the establishment of adequate generality is necessary in order to classify the microbes correctly. Since the variance within the species of microbes is large, it is required to withdraw the characteristic components of the species from the overall difference. At the same time, strain characteristic components should be expelled (Wenning and Scherer, 2013).

Proper and reliable data mining assays should be selected for classification and discrimination. For this purpose, unsupervised and supervised pattern recognition techniques can be explored. Unsupervised techniques such as hierarchical cluster analysis (HCA) and principal component analysis (PCA) are most popular to blindly characterize and discriminate the spectra. Anonymous information is identified according to its resemblance, variety and implicacy (Tindall et al., 2000; Samelis et al., 2011; Schmidt et al., 2012). In addition, these methods are convenient for the examination of data repeatability and total segregation of the experimental groups, when there is a preliminary information about the spectrum. Moreover, the identification and elimination of the outliers/deviations from the data set are easily performed by unsupervised tools, which is an important procedure in order to implement the supervised tools for classification purposes. Although the differentiation, discrimination and characterization of molecular constituents of cells are easily examined through unsupervised chemometrics, the accurate identification and correct classification of samples (species, strain) based on the utilization of prior information are usually confirmed using supervised chemometric tools (Wenning and Scherer, 2013).

HCA describes the resemblance and discrepancy among the microorganisms by calculating the interval (dimension) among their spectra and clustering algorithms. The most utilized dimensions are Pearson's correlation and Euclidian distance. In case of clustering algorithms, Ward's and the average linkage algorithms are widely employed in the differentiation of microorganisms (Beveridge and Graham, 1991). The algorithms generate a final dendrograms, which a tree-like structures in order to understand the sample clustering. The discrepancy or heterogeneity is described by looking to the left side of Y-line. The quantity of clustered spectra and resemblance among them determine the weight of the heterogeneity (Helm et al., 1991). In order to understand whether the bacterial spectra symbolizing the identical species, genus, strains, sero and haplogroups are similar to each other or not, these HCA dendrograms are used (Nauman et al., 1991). Most importantly, it is possible to define and

characterize an anonymous bacterium by comparing the interval between its spectrum with the spectra of familiar bacteria (Davis and Maurer, 2010).

PCA is the main Exploratory Data Analysis (EDA) approach and most utilized multivariate analysis method used in chemometrics. Biological and chemical applications of PCA have been started over the last decades and it provides the basis of numerous contemporary chemometric techniques (Brereton, 2007). Moreover, PCA is an extensively employed versatile unsupervised pattern-recognition method (Bro and Smilde, 2014) for the differentiation of microorganisms using large and complex FTIR spectroscopy data (Preisner et al., 2008). PCA illustrates the items through variables generated after the linear integrations of authentic variables. These linear integrations termed principal components (PCs) are estimated along the course of most extreme change and opposite to one another. PC1 and PC2 describe the maximum information directions of the analytical data (Ferreira, 2002; Luna et al., 2013). In spite of the fact that PCA grants an outlined representation of the information set, its fundamental utility is to show the structure in the information (Muehlethaler et al., 2011). The particular scores obtained for each information set can be utilized to group the information in the PC-based coordinate system or to relapse back against the pre-established concentration matrix for quantitative assessment. On the other hand, PCA is just equipped for perceiving total variance in regards to an entire information set and not suitable for distinguishing intra-group changes (Wang and Mizaikoff, 2008). These score plots can be utilized to translate the similitudes and contrasts between microscopic organisms (bacteria). The closer the specimens are inside of a score plot, the more comparable they are regarding the principal component score assessed (Davis and Maurer, 2010).

Soft Independent Modelling by Class Analogy (SIMCA) is an extensively employed supervised chemometric tool in order to classify numerous spectra. Training sets are needed with an arrangement of traits and their class participation (like bacteria group). The phrase soft attributes to the evidence that the method is able to recognize the specimens as having a place in many classes. Prior to SIMCA, the examples having a place with every class should be investigated utilizing PCA in which just the noteworthy elements are held and subsequently identified (Davis and Maurer, 2010).

1.6 Aim and scope of the study

In this thesis study, it was aimed improve understanding of the resistance mechanisms that bacteria develop against heavy metals.

The first and second part of the study were performed to characterize and discriminate Cd, Pb and Ag-resistant *E. coli* and *S. aureus* via ATR-FTIR spectroscopy coupled to chemometrics. In order to achieve this, resistant *E. coli* and *S. aureus* bacteria were used. These bacteria were examined since they are extensively studied representatives of gram-negative and gram-positive bacteria, respectively. Furthermore, in these sections, unsupervised chemometric methods, namely PCA and HCA were used, since they are broadly applied methods for the differentiation of microbial communities with impressive accuracy.

The last part of the study was conducted for the instantaneous and accurate differentiation and most importantly classification of Ag, Cd and Pb-resistant *E. coli* and *S. aureus* and environmental *Microbacterium oxydans* FS45 and *Gordonia* sp. FS18 from their non-resistant counterparts. Within this particular context, the supervised classification pattern-recognition tool SIMCA was used in light of FTIR spectra of these bacterial isolates.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Growth Conditions

Laboratory (*E. coli* ATCC 8739 and *S. aureus* ATCC 6538) and environmental (freshwater) bacterial strains (*Gordonia* sp. FS18 *and Microbacterium oxydans* FS45) were grown at 28˚C in an orbital shaker at 200 rpm under aerobic conditions. Environmental bacterials strains were isolated from the mucus of freshwater fish, *Alburnus alburnus* (bleak) caught from the Lake Mogan, Ankara. Nutrient broth (5 g peptone from meat and 3 g meat extract per liter, *Merck*) and nutrient agar (5 g pancreatic digest of gelatin, 3 g beef extract, and 15 g agar per liter, *Becton Dickinson*) were used as culture media. Cadmium chloride and lead nitrate (*Sigma*) stock solutions were prepared by dissolving in dH_2O and in 1:10 diluted nitric acid (in dH_2O), respectively. Silver nitrate ($AgNO_3$ *Sigma*) stock solution was prepared by dissolving in dH_2O. For the sterilization of the stock solution, 0.22 μm filter (*Pall*, USA) was used. The standard metal solutions were added to the autoclaved growth media after cooling to 45-50˚C. All bacteriological procedures were performed in sterile conditions under a laminar flow hood (*Esco*, USA).

2.2 Determination of Minimum Inhibitory Concentrations (MICs) of cadmium, lead and silver for heavy metal-resistant bacterial strains.

Laboratory (*E. coli* ATCC 8739 and *S. aureus* ATCC 6538) and environmental (freshwater) bacterial strains (*Gordonia* sp. FS18 and *Microbacterium oxydans* FS45) were incubated for three days in different concentrations of cadmium, lead and silver to analyze the growth inhibitory effects of these metals. Metal concentrations ranging

between 1.9 and 2000 µg/ml were used to identify the MICs via broth dilution. Several passages of bacteria with inocula from previous cultures (serial passaging) were performed before MIC determination tests. After the MIC test, the bacteria were acclimated to their corresponding MICs. The grown bacteria (both in NB and NA media) were considered as resistant (mutant). European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodologies (established in 2003) were followed for all experiments. *E. coli* control group (N=15) were compared to *E. coli* cultured in the presence of cadmium (N=15), in the presence of lead (N=15) and in the presence of silver (N=15). The same grouping and samples sizes applied to *S. aureus* as well.

2.3 Sample preparation for ATR-FTIR measurements

"The absorbance value of 0.5 OD at 600 nm in 2 ml of total volume was used to adjust the bacterial concentrations for UV-VIS spectrophotometric measurements (*UV-2600/2700, Shimadzu,* Japan). The cellular concentrations were arranged with distilled water and the bacteria were collected by centrifugation at 10,000 g (*Sigma 1-14 Microfuge, SciQuip,* UK) for 10 minutes. After removal of supernatant, the pellets were suspended by pipetting up and down in 10 µl of distilled water" (Gurbanov et al., 2015).

2.4 ATR-FTIR Spectroscopy

"The infrared spectra of control and metal resistant bacterial strains were collected using a Spectrum 100 FTIR spectrometer (*Perkin-Elmer Inc*., Norwalk, CT, USA) equipped with a Universal ATR accessory. The spectrum of air was used as a reference. Each sample (5 µl) was placed on a diamond/ZnSe crystal plate (*PerkinElmer*) and dried with a mild N_2 flux for 2 minutes. The samples were scanned over a 4000 to 650 cm^{-1} spectral range, for 100 scan with a resolution of 4 cm^{-1} at room temperature. Data collection and manipulation were carried out using Spectrum 100 software (*PerkinElmer*)" (Gurbanov et al., 2015).

2.5 IR data analysis and chemometrics

"In data analyses, the second derivative and vector-normalized FTIR spectra were used in order to increase accuracy in the determination of band positions. Absolute intensities of spectral bands were calculated using *OPUS 5.5 software* (*Bruker Optics, GmbH*)" (Gurbanov et al., 2015).

"PCA was performed for discrimination of spectral groups. In this chemometric approach, the spectra of each group was imported into *Unscrambler X* 10.3 (*Camo Software AS)* multivariate analysis (MVA) software. The PCA software was applied to mean-centred, second derivative and vector-normalized spectral data. PCA was performed in the fingerprint (1800-650 cm^{-1}) and whole IR regions (4000-650 cm^{-1}). The results were presented as score and loading plots. The score plots summarize the general valuable information, discriminate them according to the alterations of molecular components and discard the trivial ones. The loading plots deeply identify and relatively quantify the leading alterations related to the most affected data" (Gurbanov et al., 2015).

HCA was performed with *OPUS 5.5 software* (*Bruker Optics, GmbH*) and *Unscrambler X* 10.3 (*Camo Software AS)*. Each cluster corresponds to a whole spectrum, either a control or a resistant bacterial group. HCA was applied to vector-normalized second derivative of absorbance spectra for each sample. The dendrograms were generated using Ward's and Single-linkage clustering algorithms. Clustering was based on the magnitude of similarities or distance among the spectra. Spectral distances were obtained using Euclidean and Squared Euclidean distances.

"The sensitivity and specificity of FTIR spectroscopy were calculated based on the HCA results. These terms were calculated using previously described methods as given in Table 2.1. The sensitivity measures the proportion of actual positives, which are correctly identified, e.g., the percentage of Pb and Cd-resistant bacteria as being resistant. The specificity measures the proportion of negatives, which are correctly identified, e.g. the percentage of control bacteria as not being resistant (Table 2.1)" (Gurbanov et al., 2015).

Table 2.1 Definitions for sensitivity and specificity

Cluster analysis results based on ATR-FTIR data			
Positive*		Negative *	
Heavy Metal Resistant	A	B	Sensitivity=A/(A+B)
Control	C	D	Specificity=D/(C+D)

* Positive and negative values are determined as follows:

A: the number of heavy metal resistant bacteria identified in resistant groups (true positive).

B: the number of heavy metal resistant bacteria identified in control groups (false negative)

C: the number of control bacteria identified in heavy metal resistant groups (false positive).

D: the number of control bacteria identified in control groups (true negative).

2.6 Soft Independent Modeling of Class Analogy (SIMCA) analysis

Supervised pattern recognitions of control and heavy metal resistant bacterial groups were accomplished using Soft Independent Modeling of Class Analogy (SIMCA) approach. SIMCA was conducted in the whole IR (4000-650 cm^{-1}) region of analytic (spectral) data via *Unscrambler X* 10. 3 (CAMO Software, Inc.). In this supervised classification method, each class of data set was mathematically modeled via PCA as a first step to generate a training set. Subsequently, samples were tested to each class set to develop an appropriate discrimination model for control and heavy metal resistant bacterial groups.

2.7 Statistical data analysis

The result uncertainties were expressed as ± standard error of mean (SEM). *E. coli* heavy metal resistant groups (cadmium and lead) vs. *E. coli* control group and *S. aureus* heavy metal resistant groups (cadmium and lead) vs. *S. aureus* control group were analyzed using *one-way ANOVA* test in *GraphPad Prism 6.01 (GraphPad Software, Inc.).* The same test was applied to compare *E. coli* Ag-resistant group vs *E. coli* control group and *S. aureus* Ag-resistant group vs *S. aureus* control group.

The absolute intensities of the spectral bands in the control groups were normalized to 100 and changes of the intensities in the heavy metal resistant groups were compared relative to this value. A ‘p’ value less than or equal to 0.05 was considered as statistically significant. The degree of significance was denoted as less than or equal to $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, $p < 0.0001$ ****.

CHAPTER 3

RESULTS AND DISCUSSION

This thesis is composed of three parts. In first part, rapid differentiation and characterization of Cd and Pb-resistant *E. coli* and *S. aureus* were studied using ATR-FTIR spectroscopy together with unsupervised chemometric analyses tools. The second part summarizes the study showing the detailed molecular characterization of Ag-resistant *E. coli* and *S. aureus* based on their infrared spectra coupled with unsupervised chemometrics. In third part, we focused on the classification of the Ag, Cd, and Pb-resistant laboratory (*E. coli* and *S. aureus*) and Mogan lake (Ankara) derived environmental bacterial (*Gordonia* sp. FS18 and *Microbacterium oxydans* FS45) strains using supervised pattern recognition SIMCA technique in light of their ATR-FTIR spectra. The different laboratory and environmental bacterial strains were used in order to understand whether the SIMCA technique can be applied to classify bacteria originating from various sources or not.

3.1 The rapid differentiation of Cd and Pb-tolerant bacteria by ATR-FTIR spectroscopy coupled with unsupervised chemometric techniques.

3.1.1 Minimum inhibitory concentration (MIC) for cadmium and lead

"Minimum Inhibitory Concentrations (MIC) for cadmium chloride were determined as 31.25 μg/ml (171 μM) for both *E. coli* and *S. aureus* strains. For lead nitrate, the MIC was 75 μg/ml (226 μM) for *E. coli* and 100 μg/ml (302 μM) for *S. aureus* strains. These results showed that *S. aureus* has a higher resistance to lead compared to *E. coli*. Different MIC values for cadmium and lead have been reported for *E. coli* and *S.*

aureus in the literature. MIC values for Cd range between 0.5 and 500 μM in *E. coli* and MIC for Pb ranges between 5 and 12.5 μM. In the case of *S. aureus*, MIC values for Cd range between 62.5 and 4364 μM and MIC for Pb ranges between 33nM and 3321 μM (Gelmi et al., 1994; Tauriainen,1998; Nies, 1999; Hossain et al., 2012; Nath et al., 2012). Obviously, the determined MIC values for these two bacteria differ considerably between species and even between different strains. The cause of variations in MIC between previous studies and ours may be the differences in the environments where the samples were collected and differences in media composition or growth conditions (Kardas et al., 2014)" (Gurbanov et al., 2015).

3.1.2 ATR-FTIR Spectroscopy and chemometrics

"Research on the characterization and discrimination of microorganisms by FTIR spectroscopy has been conducted for many years (Naumann, 1988; Garip et al., 2009; Li et al., 2010; Kardas et al., 2014). In previous studies, transmission mode of FTIR spectroscopy was considerably used in the characterisation and discrimination of microorganisms; the use of ATR mode has been limited (Mariey et al., 2001). In the current study, we performed ATR-FTIR spectroscopy to differentiate lead and cadmium resistant *E. coli* and *S. aureus* from their respective control strains. The use of ATR mode in IR spectroscopic studies provides several advantages over transmission mode such as; the ability to analyze small samples as one drop, few or none sample preparation steps and being unaffected from sample thickness (Kendall et al., 2009)." (Gurbanov et al., 2015).

"Figure 3.1A, B and C show the representative second derivative, vector-normalized spectra of the control *E. coli* and *S. aureus* in the 2980-2820, 1750-1250 and 1250-950 cm^{-1} regions. The bands labeled in the figure were assigned according to related literature (Table 3.1). These complex spectra include several bands originating from lipids, carbohydrates, proteins, etc. As seen from the figure, the spectra consist of two main regions; C-H stretching and fingerprint" (Gurbanov et al., 2015).

"The C-H region mainly includes lipid-associated spectral bands. The representative second derivative and vector-normalized spectra of the control, cadmium and lead resistant *E. coli* in the 2980-2820 cm^{-1} region are presented in figure 3.2A. The spectra of *S. aureus* control, *S. aureus*-Cd and *S. aureus*-Pb in the same region are given in figure 3.2B. Remarkably discriminative features of lipid-oriented spectral bands for both heavy metal resistant bacteria strains can be seen from the figures. As clearly seen from figure 3.2A, B and Table 3.2, there was a reduction in the intensity of the lipid bands in the C-H stretching region [CH_2 antisymmetric stretching (band number 2), CH_2 symmetric stretching (band number 4)] in the Cd and Pb-resistant *E. coli* and *S. aureus* groups as compared to the control ones. In an absorption spectroscopy, according to the well-known Beer-Lambert law, absorption band intensity is proportional with the concentration of the sample. Therefore, the reduction in the intensity of the lipid bands implies a decrease in lipid concentration, e.g. lipid biosynthesis" (Gurbanov et al., 2015).

Table 3.1 General band assignment of an ATR-FTIR spectrum of a bacterium (Adapted from Gurbanov et al., 2015).

Band number	Wavenumber (cm^{-1})	Spectral Bands
1	2958	**CH_3 antisymmetric stretching**: lipids, protein side chains, with minor contribution from carbohydrates and nucleic acids
2	2924	**CH_2 antisymmetric stretching:** mainly lipids, with minor contribution from proteins, carbohydrates, nucleic acids
3	2873	**CH_3 symmetric stretching:** mainly protein side chains, with minor contribution from lipids, carbohydrates and nucleic acids
4	2852	**CH_2 symmetric stretching:** mainly lipids, with minor contribution from proteins, carbohydrates, nucleic acids
5	1743	**Ester CO stretch:** polyester storage compounds, polysaccharides, polyhydroxyalkanoates (PHAs)
6	1722	**Ester CO stretch**: polyester storage compounds, polysaccharides, poly-3-hydroxybutyrate (PHB)
7	1635	**Amide I:** protein (80% C=O stretching, 10% N—H bending, 10% C—N stretching)
8	1534	**Amide II:** protein (60% N-H bending, 40% C—N stretching)
9	1468	**CH_2 scissoring:** lipids
10	1453	**CH_2 bending**: mainly lipids, with minor contribution from proteins
11	1392	**COO^- symmetric stretching**: fatty acids
12	1233	**PO_2^- antisymmetric stretching:** mainly nucleic acids with minor contribution from phospholipids
13	1170	**CO—O—C antisymmetric stretching**: phospholipids, triglycerides and cholesterol esters
14	1118	**C-O stretching:** RNA ribose
15	1082	**PO_2^- symmetric stretching:** nucleic acids and phospholipids
16	1057	**C–O stretching**: polysaccharides
17	966	**C-C stretching:** DNA backbone

Table 3.2 The relative changes in the absolute intensities of the main second derivative spectral bands in the control, Cd and Pb-resistant *E.coli* and *S.aureus* (Adapted from Gurbanov et al., 2015).

Band Number	***E.coli* Control (n=15)**	***E.coli* Cd (n=15)**	***E.coli* Pb (n=15)**	***S.aureus* Control (n=15)**	***S.aureus* Cd (n=15)**	***S.aureus* Pb (n=15)**
1	100 ± 2.59	91.25 ± 7.18	64.90 ± 4.47 ****	100 ± 5.31	95.44 ± 2.78	42.69 ± 1.72 ****
2	100 ± 3.50	77.36 ± 2.98 ****	79.85 ± 0.54 ****	100 ± 0.62	93.33 ± 3.46	82.77 ± 2.34 ****
3	100 ± 1.09	88.81 ± 4.92	46.45 ± 5.07 ****	100 ± 2.33	89.24 ± 1.21 ****	83.19 ± 1.16 ****
4	100 ± 1.90	73.12 ± 0.46 ****	66.41 ± 3.68 ****	100 ± 1.23	97.59 ± 0.64	86.39 ± 3.41 ***
5	100 ± 15.73	218.09 ± 23.41 *	424.00 ± 52.01 ****	100 ± 8.28	105.84 ± 5.97	111.34 ± 4.66
6	100 ± 16.37	195.63 ± 29.16 *	145.64 ± 31.15 ****	100 ± 19.11	118.01 ± 6.52	175.67 ± 12.23 ***
7	100 ± 7.72	69.81 ± 9.38 *	74.08 ± 5.02 *	100 ± 8.16	95.05 ± 2.87	74.36 ± 3.58 **
8	100 ± 1.95	88.21 ± 3.58 *	73.12 ± 3.81 ****	100 ± 1.47	90.62 ± 3.01	79.40 ± 5.37 ***
9	100 ± 8.41	54.21 ± 2.09 ****	53.18 ± 0.70 ****	100 ± 1.90	97.53 ± 4.03	84.38 ± 4.10 **
10	100 ± 2.82	94.20 ± 3.71	90.03 ± 3.73	100 ± 4.74	93.98 ± 5.27	86.90 ± 2.02
11	100 ± 15.55	69.97 ± 4.98	50.62 ± 1.06 **	100 ± 4.10	80.40 ± 6.36 **	81.11 ± 2.38 **
12	100 ± 5.51	85.46 ± 6.90	81.74 ± 1.68 *	100 ± 1.59	43.43 ± 1.25 ****	49.13 ± 2.84 ****
13	100 ± 2.74	80.06 ± 4.43 **	63.50 ± 5.09 ****	100 ± 3.22	70.54 ± 2.34 ****	49.44 ± 1.80 ****
14	100 ± 4.85	78.86 ± 2.48 **	75.53 ± 6.70 **	100 ± 1.50	77.69 ± 8.30 **	78.17 ± 2.59 **
15	100 ± 6.88	98.41 ± 5.49	50.16 ± 12.57 ***	100. ±2 .70	99.14 ± 12.15	78.39 ± 1.29
16	100 ± 15.19	92.42 ± 4.39	48.97 ± 2.60 ***	100 ± 3.95	86.66 ± 11.40	91.31 ± 2.72
17	100 ± 6.66	76.06 ± 4.16 **	66.06 ****	100 ± 5.53	80.52 ± 10.50	65.20 ± 1.57 ***

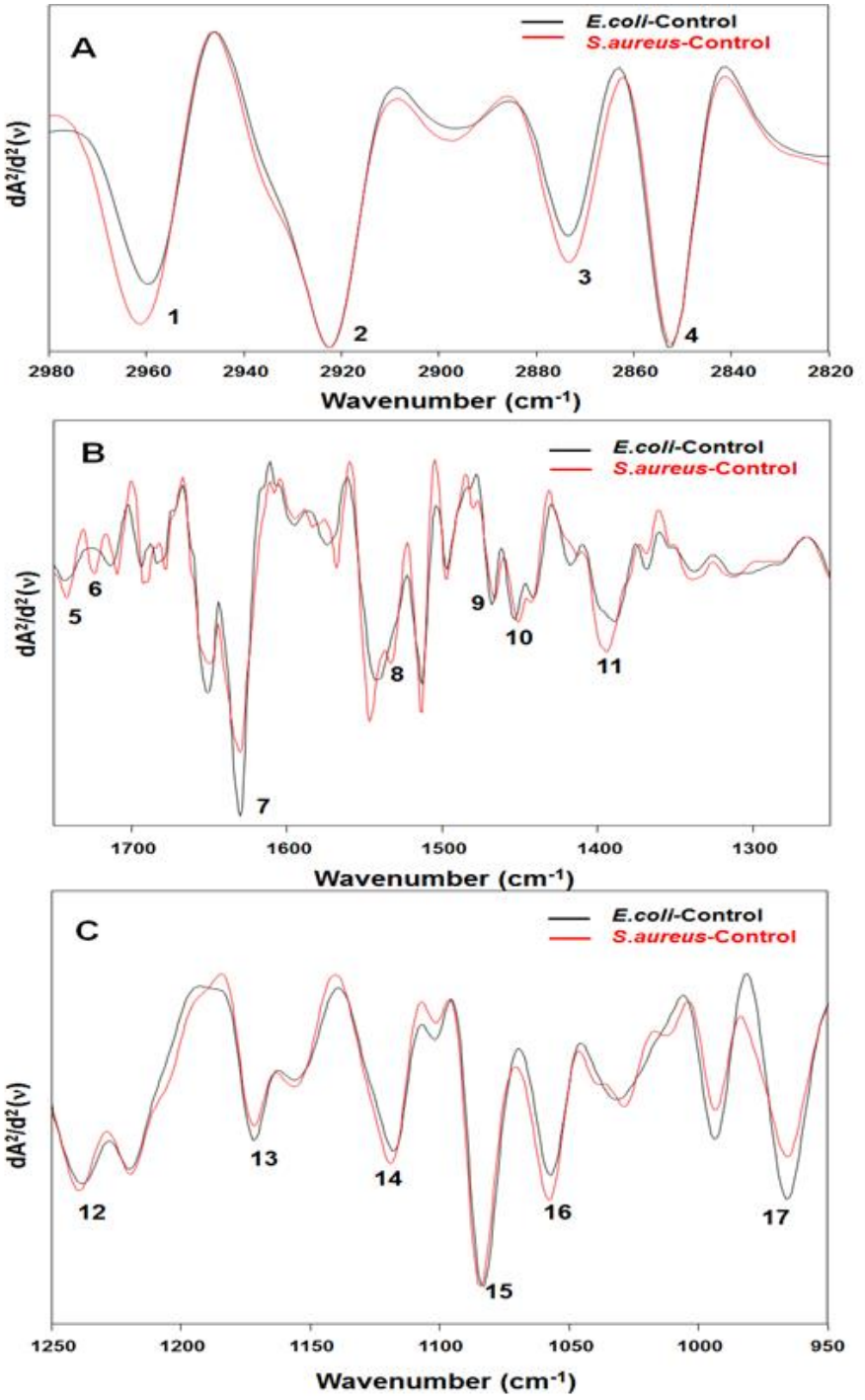

Figure 3.1 The representative second derivative vector-normalized ATR-FTIR spectra of the control spectra of E. coli and S. aureus in the (A) 2980–2820, (B) 1750–1250 and (C) 1250-950 cm^{-1} spectral regions (Adapted from Gurbanov et al., 2015).

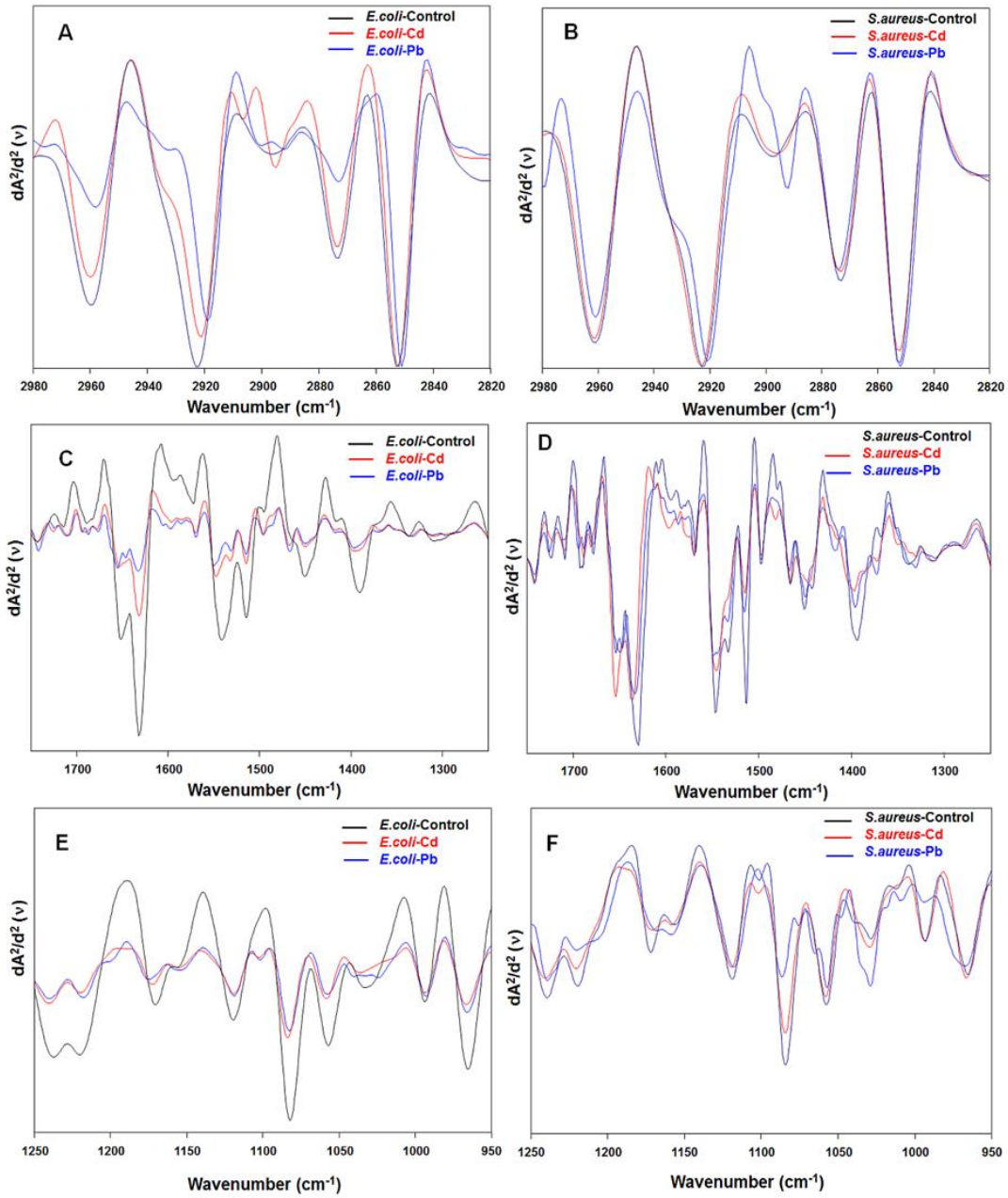

Figure 3.2 The representative second derivative vector-normalized ATR-FTIR spectra of the control, Cd and Pb-resistant *E. coli* (A,C,E) and *S. aureus* (B,D,F) in the 2980-2820, 1750-1250 and 1250-950 cm^{-1} spectral regions, respectively (Adapted from Gurbanov et al., 2015).

"Although there are several studies about the alterations of the bacterial lipids due to temperature, nutrients, pH and aromatic substances (Russell, 2002), a small amount of data is available regarding the interaction of heavy metals with bacterial lipids (Markowicz et al., 2010). As mentioned, structural and compositional changes in lipids, inhibition of lipid synthesis and lipid peroxidation are among the major impacts of metal stress to bacterial communities (Guschina and Harwood, 2006). It is necessary to emphasize that a bacterium changes its lipid composition in order to establish membrane stability and fluidity and to decrease its permeability to protons (Dunkley et al., 1991). A number of studies have shown alterations in the ratios of saturation/unsaturation, cis/trans and branched/unbranched structures of lipids due to stress conditions (Denich et al., 2003; Heipieper et al., 2003). A recent study also demonstrated a decline in bacterial saturated lipids following metal exposure (Markowicz et al., 2010). It was assumed that these alterations protect the cell from the deleterious effects of the heavy metals (Markowicz et al., 2010)" (Gurbanov et al., 2015).

"The fingerprint region consists of spectral bands emerging from the functional groups of proteins, lipids, carbohydrates and nucleic acids. Figure 3.2C and D show the representative second derivative vector-normalized spectra of the control, cadmium, and lead resistant *E. coli* and *S. aureus* in the 1750-1250 cm^{-1} region, respectively. Likewise, the spectra of control, cadmium, and lead resistant groups in the 1250-950 cm^{-1} region are given in figure 3.2E and F. As seen from the figures, there are alterations in the intensities, band shapes and wavenumber values of spectral bands for both heavy metal resistant bacteria strains. Obviously, these alterations can be used to discriminate cadmium and lead resistant strains from the controls. Oust et al., reported that different regions, especially the fingerprint region, of FTIR spectra can be used for the discrimination and identification of bacterial species (Oust et al., 2004). Moreover, many researchers have used FTIR spectroscopy to distinguish and classify a range of microorganisms (Helm et al., 1991; Curk et al., 1994; Amiel et al., 2000)" (Gurbanov et al., 2015).

"Polyhydroxyalkanoate (PHA) is a biological polymeric compound produced in bacteria under growth restricting environmental conditions to provide the cell with

carbon and energy storage substances. One of the common short-chain-length members of the PHA family is known as polyhydroxybutyrate (PHB) (Khanna and Srivastava, 2005). We assigned the spectral bands located at 1743 and 1722 cm^{-1} (band numbers 5 and 6) as polyester storage compounds and polysaccharides according to the literature (Randriamahefa et al., 2003; Kamnev et al., 2005; Kamnev, 2008). A significant increase in the intensity of the CO ester stretching bands located at 1743 and 1722 cm^{-1} occurred in Cd and Pb-resistant *E. coli* (Figure 3.2C, Table 3.2). This implies elevated levels of PHB as well as PHAs in general. The intensity of the 1722 cm^{-1} band increased in the Pb-resistant *S. aureus* group (Figure 3.2D, Table 3.2). Similarly, increased amounts of PHA and PHB were previously shown to be associated with heavy metal stress (Kamnev, 2008; Kardas et al., 2014). It has been previously reported that bacteria seem to deal with environmental stress conditions by remodeling various cellular mechanisms (Markowicz et al., 2010). It was also reported that heavy-metal stress stimulates the production and accumulation of PHA and PHB in bacteria (Kamnev et al., 2005). Our results indicated that under Pb and Cd exposure, *E.coli* increases its polyester synthesis; *S. aureus*, however, synthesizes the polymer under Pb but not Cd exposure" (Gurbanov et al., 2015).

"The intensity changes at the lipid bands fingerprint region confirm the results of lipids at the C-H region. The CH_2 scissoring and bending vibrations of the lipids at 1468 and 1453 cm^{-1} respectively diminished in Cd and Pb-resistant groups for both bacteria (Table 3.2). Similarly, the band emerging from the COO^- symmetric stretchings of fatty acid (band number 11 located at 1392 cm^{-1}) decreased in heavy metal resistant groups for both bacteria (Table 3.2). The band located at 1170 cm^{-1} (band number 13) emerges from the CO—O—C antisymmetric stretchings of phospholipids, triglycerides and cholesterol esters. Likewise, the intensity of this band decreased similarly to other lipid and fatty acid associated bands in Cd and Pb-resistant groups in both bacteria (Table 3.2)" (Gurbanov et al., 2015).

"As clearly seen in figures 3.2A, B, C, D and Table 3.2, there was a decrease in the intensities of the minor and major protein bands (band numbers 3, 7 and 8) indicating a decrease in protein content due to the Cd and Pb resistance for both bacteria species. This reduced protein content might be explained as a common mode of heavy metal

resistance for bacteria by insulating, secreting or reducing Cd and Pb with the help of cellular proteins such as ion/proton efflux pumps (Roane and Pepper, 2000; Kardas et al., 2014). In addition, this sharp decline in the protein concentration of metal-resistant bacterial strains indicates the inhibition of protein biosynthesis and deactivation of sensitive enzymes as aforementioned (Hassen et al., 1998; Nies, 1999)" (Gurbanov et al., 2015).

"Qualitative and quantitave characters of DNA from different bacteria were investigated in a recent study using FTIR spectroscopy (Whelan et al., 2014). In the study, the detailed spectal bands emerging from different structures of DNA were reported. Considering this particular work, we explored the second derivative and vector-normalized spectra for nucleic acid bands. We analysed the absolute intensities of the bands at 1233, 1118, 1082 and 966 cm^{-1} (band numbers 12, 14, 15 and 17 respectively) as shown in Table 3.2. The analysis of the 1250-950 cm^{-1} spectral region (Figure 3.2C and F) indicated that the amount and structure of nucleic acids changed in heavy metal resistant bacteria strains. Reduction in the concentrations of both DNA and RNA were observed in heavy metal resistant groups of *E. coli* and *S. aureus*. These alterations may be associated with heavy metal related nucleic acid methylations based on the findings of a recent study that proposed an association between heavy metal exposure and changes of nucleic acid methylation in different bacteria (Kardas et al., 2014). The relation between heavy metals, e.g. Cd and Pb, and DNA methylation has been reported previously (Bleich et al., 2006; Dolinoy et al., 2007) since environmental factors can modify existing genome-wide components of the epigenetics (Baccarelli and Bollati, 2009). It is known that heavy metals induce the generation of oxidative stress intermediates within a cell that in turn cause DNA damage by binding to DNA methyltransferases (Fowler et al., 2004; Valinluck et al., 2004). Interaction of these reactive oxygen species with DNA methyltransferases causes overall methylation of the cytosine residues at CpG sites (Turk et al., 1995; Baccarelli and Bollati, 2009). Recently, Whelan et al., (2014) investigated the importance of conformational changes of DNA in environmentally stressed bacteria using FTIR spectroscopy, demonstrating a preference for A-form DNA. Accordingly, we assume that the generation of ROS because of heavy metal exposure subsequently activates epigenetic mechanisms such

as DNA methylation and induces alterations in DNA, which allow the cell to survive under such toxic conditions" (Gurbanov et al., 2015).

"The qualitative and quantitative spectral changes induced by Cd and Pb resistance are not sufficient to spot and classify control and heavy metal resistant bacterial groups. This insufficiency led us to use unsupervised chemometric approaches such as HCA and PCA in the current study" (Gurbanov et al., 2015).

"It will be difficult to perform spectral analysis if spectroscopic methods generate hyper-spectral data. In the spectral data analysis, multivariate statistical analyses are necessary to extract specific, meaningful and valuable information. In the present study, we used PCA and HCA, which are unsupervised methods, to explore the spectral data without any preliminary information about the microorganisms. These techniques have been commonly used for microbiological identification and classification studies since they are appropriate for easy and quick analysis (Kansiz et al., 1999; Kirschner et al., 2001; Huang et al., 2004; Xie et al., 2005; Stockel et al., 2012a; Strittmatter et al., 2014)" (Gurbanov et al., 2015).

"PCA can be utilized to the dimensionality of the information without diminishing its scedasticity. Every spectrum is contrasted with the others to make homogeneous sets. A score plot can then be generated to picture the results, every spectrum being represented by a plot" (Gurbanov et al., 2015).

"An alternate method is to perform HCA, utilizing differences between spectra. Pearson's product moment correlation coefficient and Euclidean separation are often used as distances. The most utilized calculations are Ward's (Ward, 1963) and unweighted pair group method with arithmetic mean (UPGMA), additionally called "average linkage". The contrast between the two calculations is the best approach to ascertain separations between groups after every combination cycle (Spath, 1980). HCA has often been applied to microorganisms' spectra in differentiation and identification studies. Furthermore, HCA has also been used to characterize molecular profile changes to evaluate vulnerabilities of microbes to antimicrobial agents (Suci et al., 1998). The recognition and classification of "obscure" spectra can be performed by calculating the separation intervals between this spectrum and those included in the model, or by presenting it in the HCA. In the current study, HCA analysis was based

on the vector-normalized second derivative spectra of all bacteria. FTIR spectroscopy specific to certain spectral regions such as C-H and fingerprint have been widely used for microbiological classisfication studies (Kansiz et al., 1999; Kirschner et al., 2001; Ammann and Brandl, 2011). In our study, in addition to the fingerprint (1800-650 cm^{-1}) region, we also focused on the whole IR region (4000-650 cm^{-1}) for quick and easy discrimination analysis. HCA results for the fingerprint (1800-650 cm^{-1}) region of *E. coli* and *S. aureus* are given in figures 3.3A and B, respectively. As seen from the figures, Pb-resistant *E. coli* and *S. aureus* segregated distinctly from the Cd-resistant and control groups. According to the HCA results, the control and Cd-resistant *E. coli* were differentiated with 100% sensitivity and 100% specificity values. *S. aureus* samples were seperated at 100% sensitivity and 94% specificity levels. For Pb resistance, the sensitivity and specificity values were 100% for both species. These results indicated that Pb-resistant bacteria were successfully discriminated from the Cd and control groups. To support the profound effects of Pb resistance in both bacterial species, the same analyses were applied to the control vs. Cd-resistant groups and control vs. Pb-resistant groups, separately, in the whole IR region (4000-650 cm^{-1}). The HCA results of the control vs. Cd-resistant groups and control vs. Pb-resistant groups for both *E. coli* and *S. aureus* are given in figures 3.4 and 3.5, respectively. Higher heterogeneity values in the dendograms were obtained for Pb resistance compared to Cd resistance, which indicates that Pb resistance is a feature acquired through extensive qualitative and quantitative molecular changes" (Gurbanov et al., 2015).

"In addition to triple HCA comparisons between the control, Cd and Pb-resistant groups for *E. coli* and *S. aureus* species (Figure 3.3), we applied pairwise analysis on the respective controls, Cd-resistant, and Pb-resistant groups of *E. coli* vs. *S. aureus* in the fingerprint region to see whether or not there is a clear discrimination at the species level (Figure 3.6). The heterogeneity values for the control groups and Cd-resistant groups were 2.5 and 3 respectively, whereas the heterogeneity value for Pb-resistant groups was 5.5. The higher heterogeneity value indicates more differences in their spectra. Thus, we can confidently infer that Pb resistance is a property developed through profound changes in biochemical constituents of the bacteria" (Gurbanov et al., 2015).

"PCA was also performed in different spectral regions in order to see whether or not the heavy metal resistant bacteria were segregated separately from the control groups. The score plot results for *E. coli* and *S. aureus* in the fingerprint region are given in figures 3.7A and B, respectively. These plots illustrate the principal components that represent the variation among the samples. As can be seen from figures 3.7A and B, maximum variation values were adequate (*E. coli* PC1+ PC5= 64%; *S. aureus* PC2 + PC3=43%). The same PCA was applied to *E. coli* and *S. aureus* in the whole IR region (4000-650 cm^{-1}) as shown in figures 3.8A and B, respectively. In the whole IR region, maximum variations were similarly adequate (*E. coli* PC1 + PC2= 97%; *S. aureus* PC1 + PC2=94%) as in the fingerprint region. The score plot of *E. coli* showed that Pb and Cd-resistant samples are clearly segregated from the control group. The Pb and Cd groups had some overlapping regions on the boundary. This led us to speculate that Cd resistance occurs in part via alterations of already-existing molecules or biochemical reactions rather than *de novo* synthesis of new molecules (Figure 3.7A). As can be seen from figure 3.7B, both Pb and Cd-resistant *S. aureus* are segregated from the control group. *S. aureus* control and Cd-resistant groups exhibited a small overlap. Similar to *E. coli*, Pb and Cd resistances brought about a clear segregation in components" (Gurbanov et al., 2015).

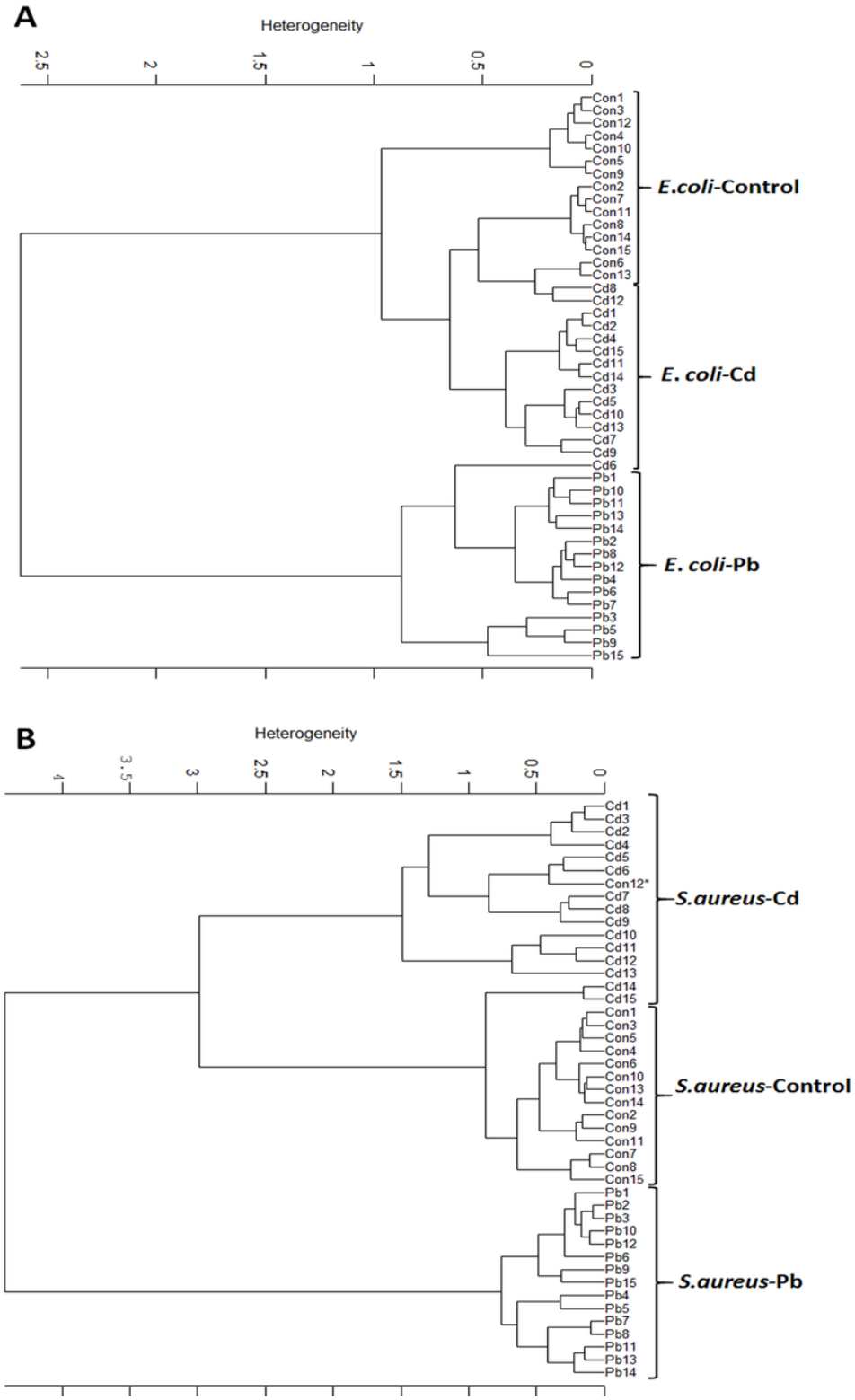

Figure 3.3 Hierarchical cluster analysis of the control, Cd and Pb-resistant of A) *E. coli* B) *S. aureus* in the 1800-650 cm^{-1} spectral region (Adapted from Gurbanov et al., 2015).

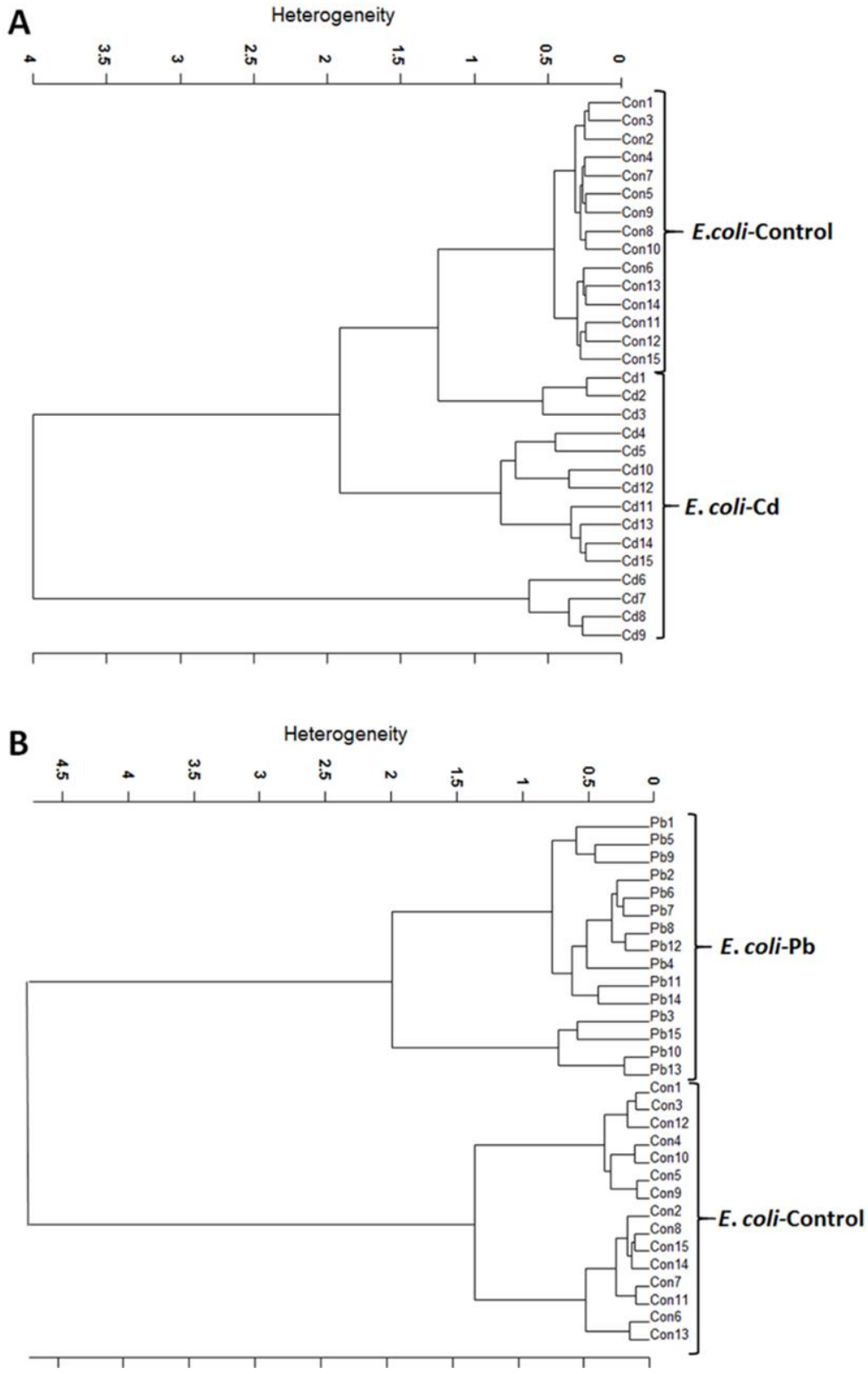

Figure 3.4 Cluster analysis of A) E. coli-control vs. E. coli-Cd and B) E. coli-control vs. E. coli-Pb in the 4000–650 cm^{-1} spectral region (Adapted from Gurbanov et al., 2015).

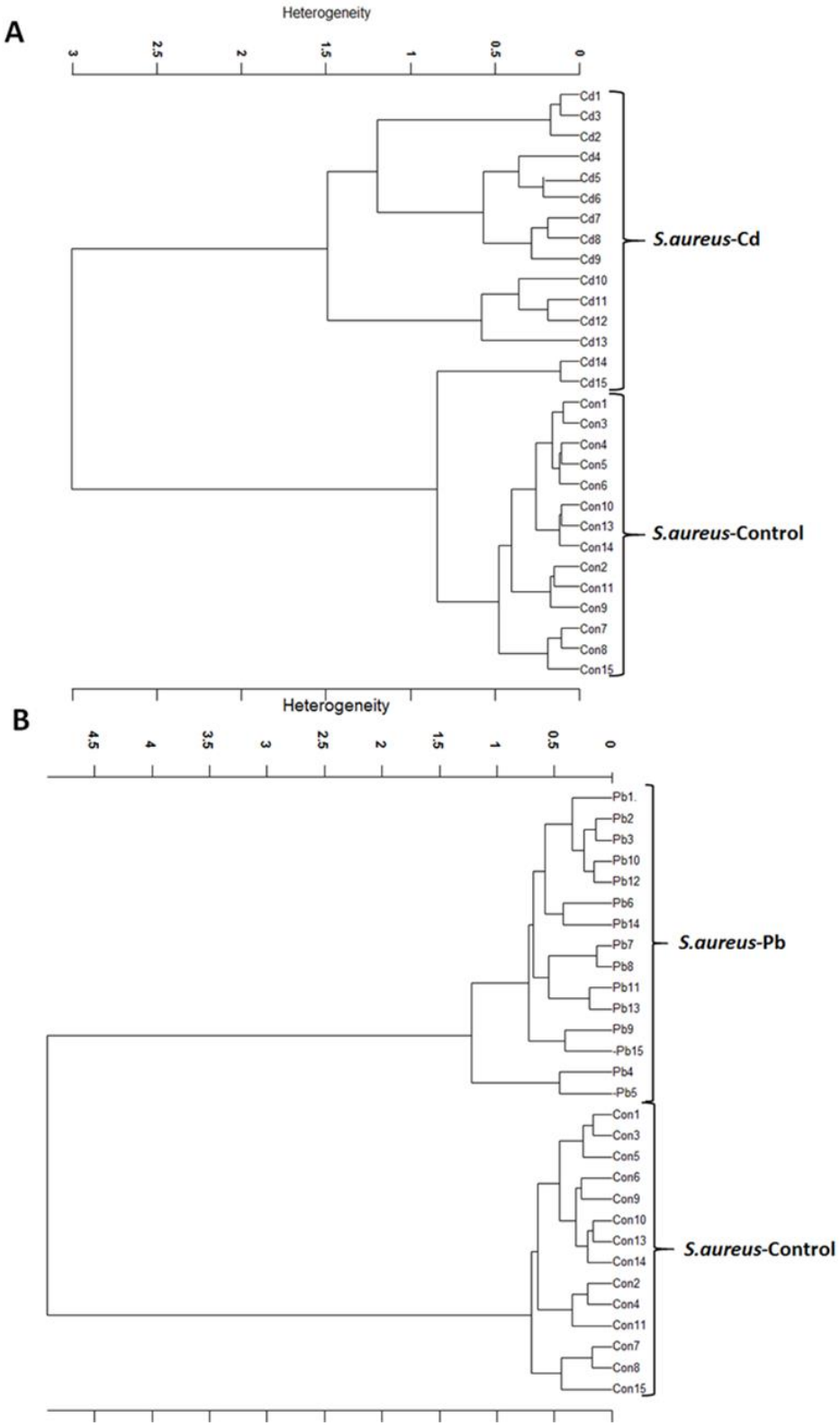

Figure 3.5 Cluster analysis of A) S. aureus-control vs S. aureus-Cd and B) S. aureus-control vs S. aureus-Pb in the 4000–650 cm^{-1} spectral region (Adapted from Gurbanov et al., 2015).

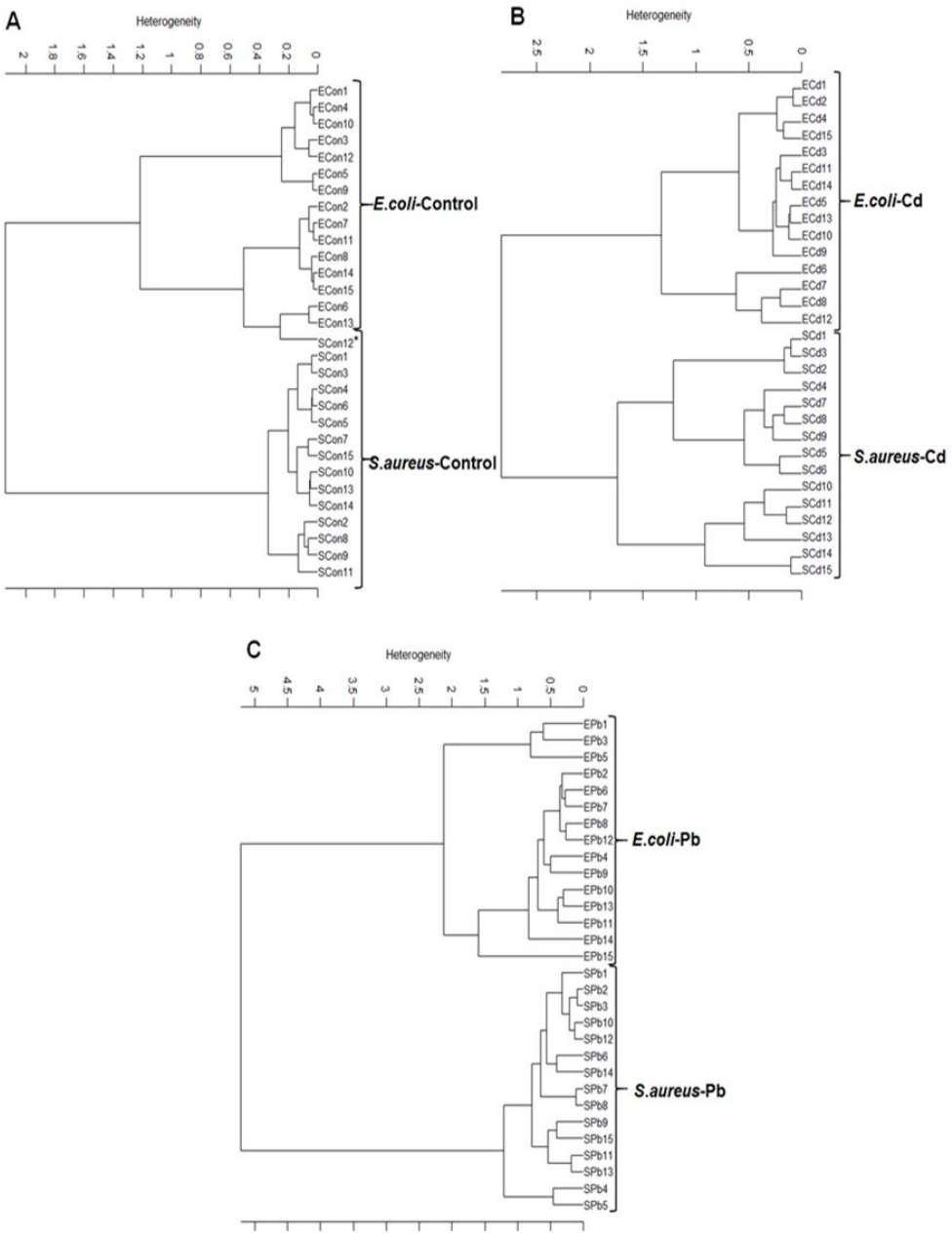

Figure 3.6 Hierarchical cluster analysis of A) *E. coli*-control vs *S. aureus*-control, B) *E. coli*-Cd vs *S. aureus*-Cd, C) *E. coli*-Pb vs *S. aureus*-Pb in the 1800–650 cm^{-1} spectral region (Adapted from Gurbanov et al., 2015).

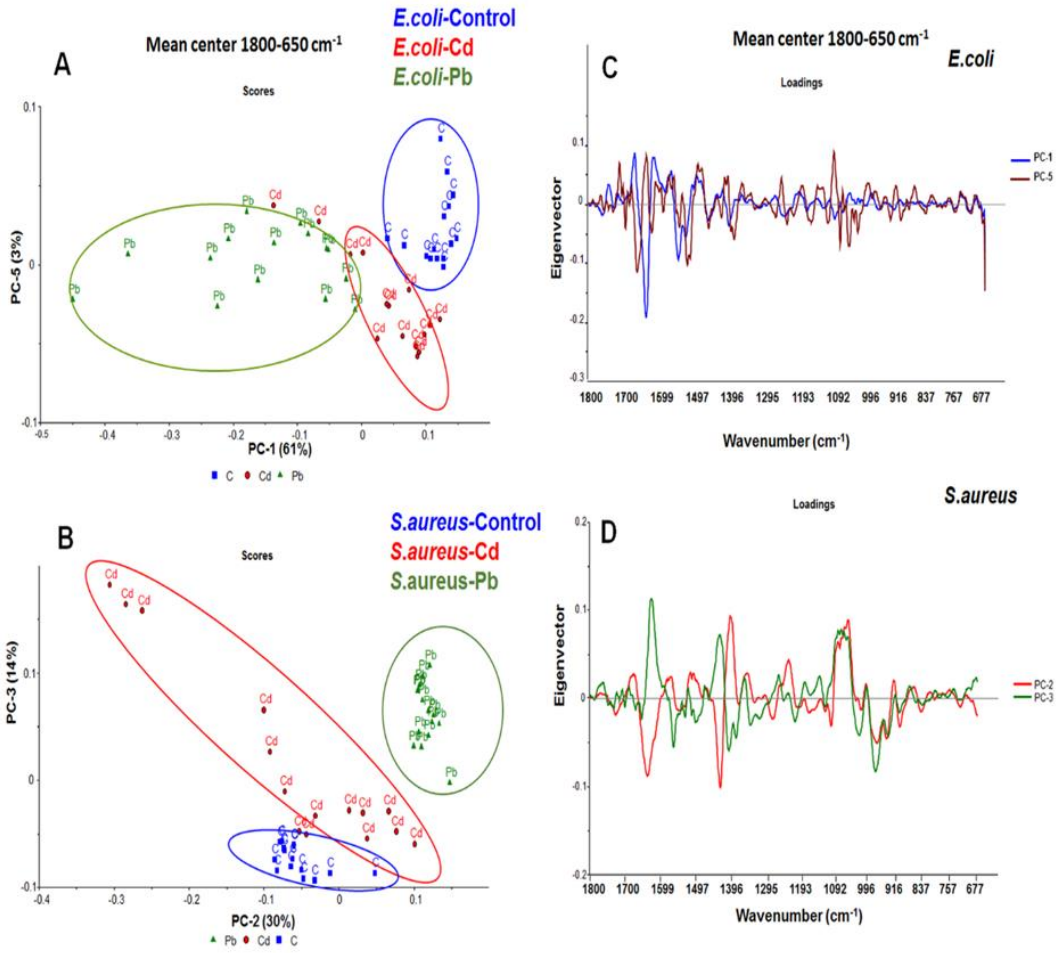

Figure 3.7 PCA scatter plots for all A) E. coli B) S. aureus samples and loading plots for all C) E. coli D) S. aureus samples in the 1800–650 cm^{-1} spectral region (Adapted from Gurbanov et al., 2015).

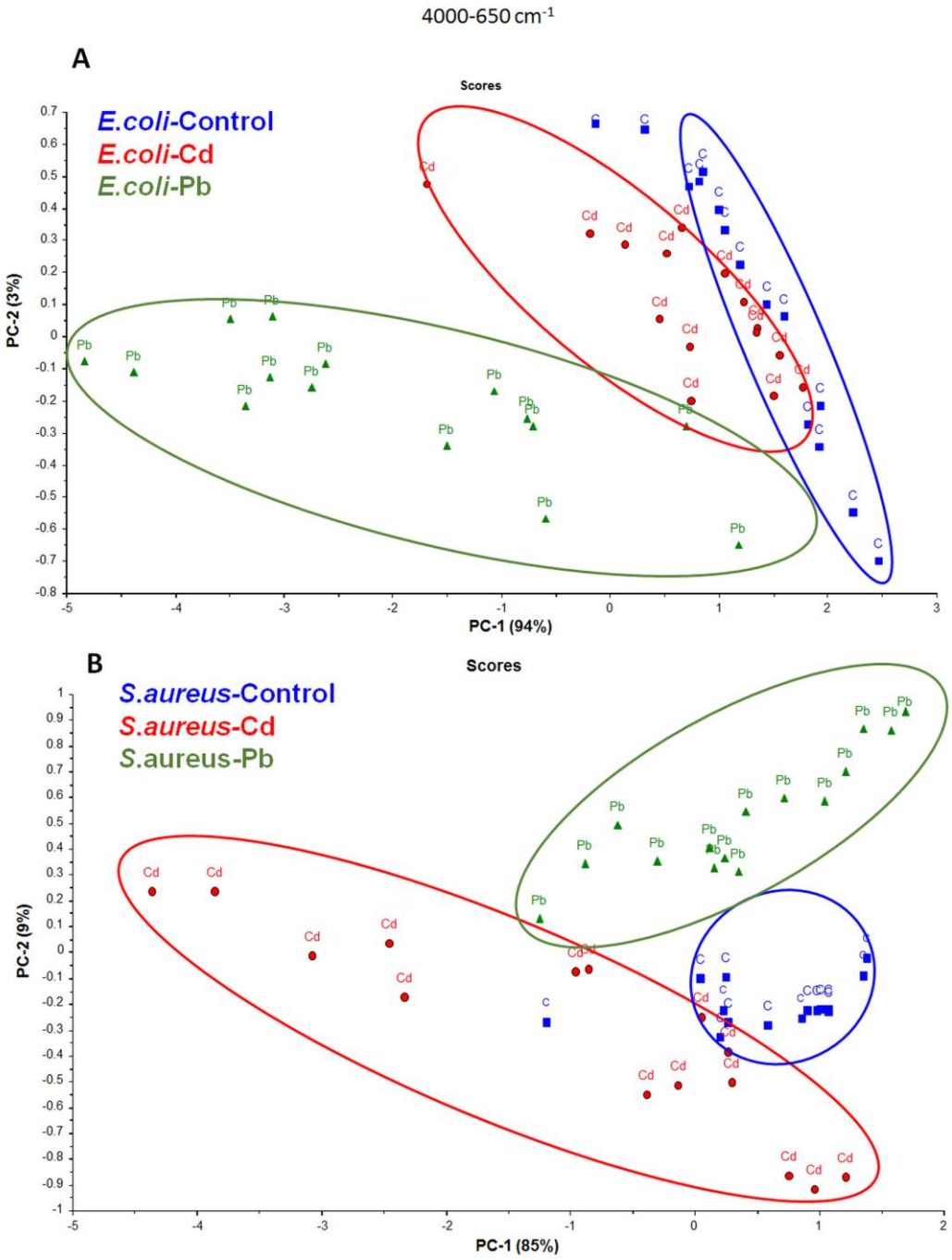

Figure 3.8 PCA scatter plots for all A) E. coli B) S. aureus samples in the 4000–650 cm^{-1} spectral region (Adapted from Gurbanov et al., 2015).

"Loading plots enable analysis of the whole IR spectra and indicate the wavenumbers that contribute most to the variation described in the PC terms. The loading or absolute values at a given wavenumber demonstrate the magnitude of the difference among the tested samples (Kansiz et al., 1999). The loading plots obtained from PCA results of *E. coli* and *S. aureus* are shown in figure 3.7C and D, respectively. The high loading values for the 1800-650 cm^{-1} region represent this region's considerable contribution in the discrimination of *E. coli* and *S. aureus* groups and also indicates the Cd and Pb resistance-induced alterations in molecular content of both bacteria species" (Gurbanov et al., 2015).

"As can be deduced from the results of this study, Cd and Pb-resistant bacteria were almost totally differentiated from the controls. We attempted to reveal the connection between our results and molecular alterations that occurred in heavy metal resistant bacteria. As previously mentioned, Cd resistance mainly occurs by efflux pumps (Bruins et al., 2000). For Pb, the most predominant resistance mechanisms are adsorption through extracellular polysaccharides, cell exclusion and ion efflux to the outside of the cell (Jarosławiecka and Piotrowska-Seget, 2014). Additionally, after entering the cell through the essential metal transporters, Pb can be deactivated by binding to the metallothioneins (MT) or precipitated as insoluble phosphates (Jarosławiecka and Piotrowska-Seget, 2014). It is also well known that bacteria can easily change their metabolism extensively in terms of content and structure of cellular macromolecules under environmental stress (Harrison et al., 2007; Hense et al., 2007). Generally, environmental changes force bacteria to change the regulation of many unassociated genes (Madigan et al., 2010). Nutritional starvation interrupts rRNA and tRNA biosynthesis and eventually stops ribosome production, and ultimately DNA and protein synthesis (Madigan et al., 2010). Correspondingly, in our case, we assume that nutritional starvation and heavy metal stress led to the development of the main metabolic alterations in proteins, lipids, nucleic acids and other cellular components in the heavy metal resistant bacteria. In short, induction of complex genetic response systems following Cd and Pb exposure for the aforesaid complex heavy metal resistance mechanisms significantly alter bacterial metabolism, further modifying cellular biomolecules as well. These alterations and modifications may be determining

factors for the clear differentiation of heavy metal resistant strains of *E. coli* and *S. aureus* from the controls" (Gurbanov et al., 2015).

3.2 Investigation of gross molecular alterations in silver-resistant bacteria by IR spectroscopy and unsupervised chemometrics.

3.2.1 Inhibitory concentrations for Ag

In this study, silver nitrate ($AgNO_3$) inhibitory concentration values were determined, as 22 μg/ml (130 μM) for *E. coli* ATCC 8739 strain and 16 μg/ml (94 μM) for *S. aureus* ATCC 6538 strain.

3.2.2 ATR-FTIR spectroscopy

Figure 3.9A and B illustrate the representative IR spectra of the control, and Ag-resistant *E. coli* and *S. aureus* groups at complete IR region (4000-650 cm^{-1}), respectively. As shown in the figure, there are obvious differences between the spectra of Ag-resistant and control groups in both bacteria. In order to elucidate these observable changes, we applied chemometric analyses to the infrared spectra.

3.2.3 An exploratory PCA approach

First, PCA was performed to the control and Ag-resistant *E. coli* and *S. aureus* groups in the whole (4000-650 cm^{-1}) IR regions as an exploratory analysis tool. PCA loading plots for all *E. coli* and *S. aureus* samples in the whole IR region (4000-650 cm^{-1}) are shown in figure 3.10A and B, respectively. The PC-1 value is shown in blue while the PC-2 value is shown in red. Loading plots allow the analysis of the whole IR spectra and they indicate the wavenumbers, which contribute mostly to the variation described in the PC terms. The magnitude of the difference among the tested samples is demonstrated by loading or absolute values at a given wavenumber.

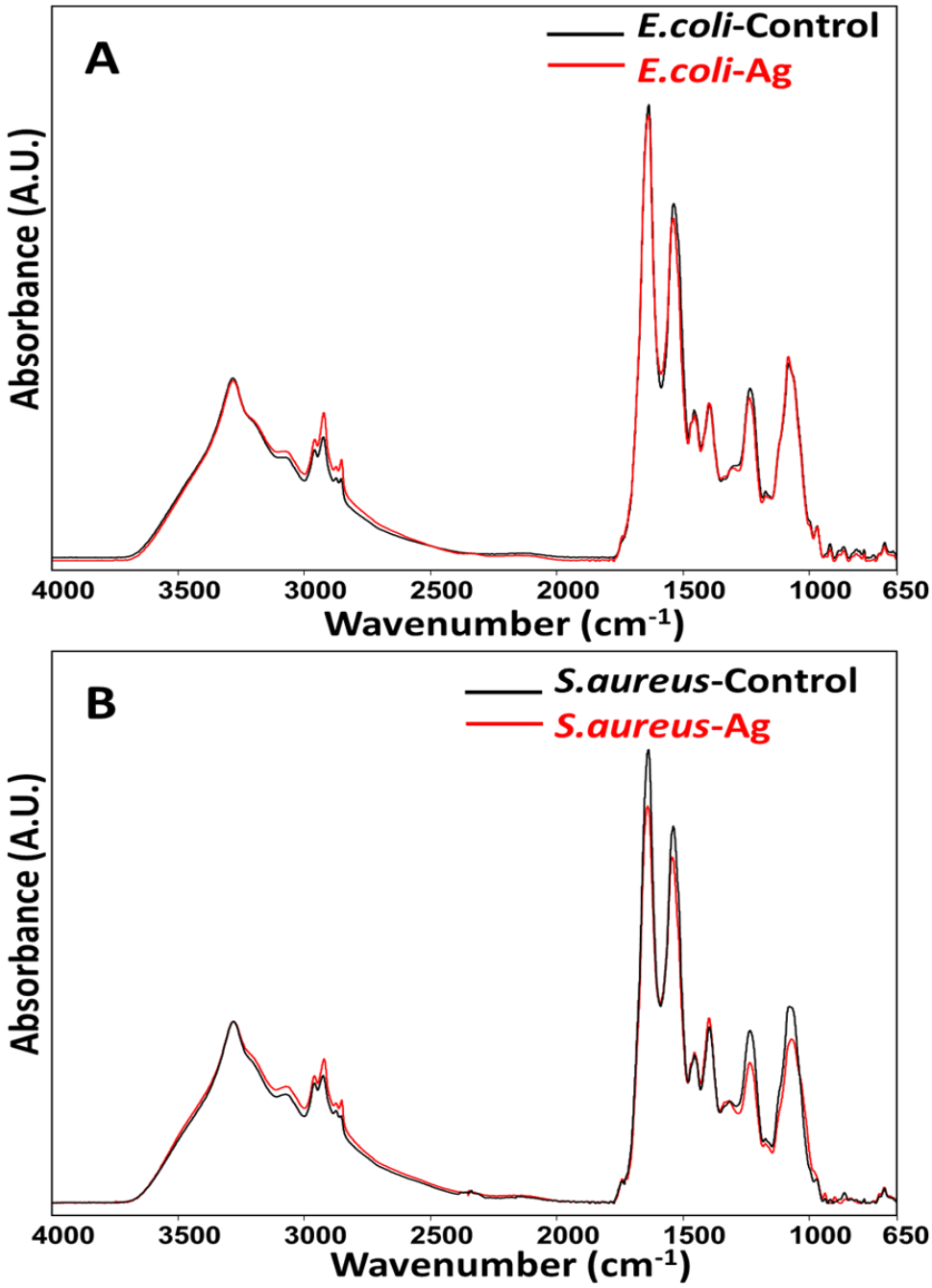

Figure 3.9 IR spectra of control and Ag-resistant *E. coli* (A) and *S. aureus* (B) samples in the 4000–650 cm^{-1} spectral region. Spectra were normalized to Amide A band located at 3282 cm^{-1}.

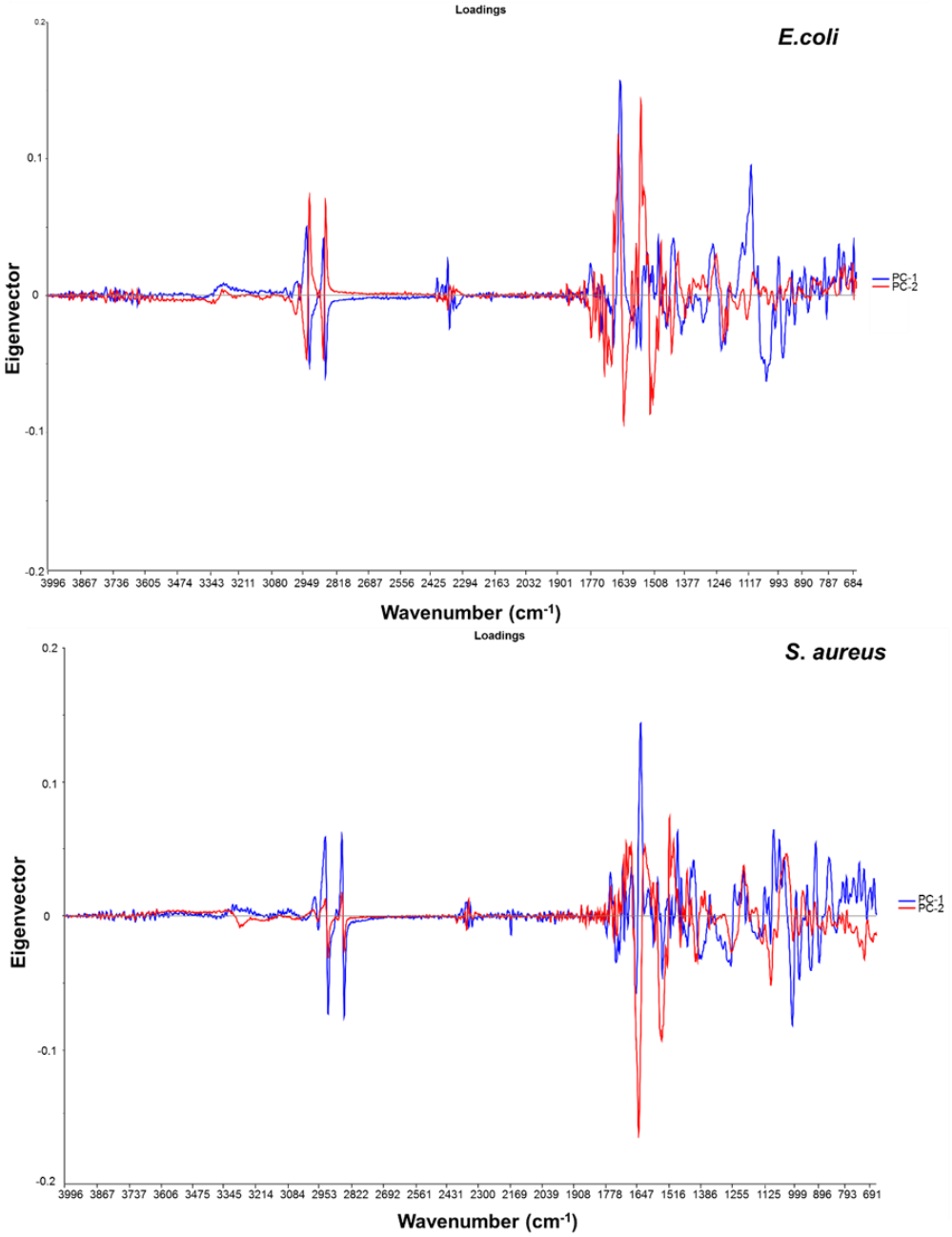

Figure 3.10 PCA loading plots for control and Ag-resistant *E. coli* (A) and *S. aureus* (B) samples in the 4000–650 cm^{-1} spectral region.

As can be seen from the loading plots, there are remarkable differences in both of the IR spectra. These differences are prominent at 3050-2800 cm^{-1} and 1800-690 cm^{-1} spectral regions for both bacteria. In addition, high Eigen vector values were observed for these spectral regions, which indicate the presence of measurable variations in the biomolecular profile of *E. coli* and *S. aureus* upon acquisition of Ag-resistance.

Since the spectral differences were observed in both lipid and finger-print regions, score plots were obtained in the whole spectral region (4000-650 cm^{-1}). The score plot results for *E. coli* and *S. aureus* in the whole IR region are shown in figure 3.11A and B, respectively. As shown in the figure, the maximum variation value was quite high (*E. coli* PC1+PC2=88%; *S. aureus* PC1+PC2=82%). The score plots also showed that Ag-resistant samples are clearly segregated from the control samples.

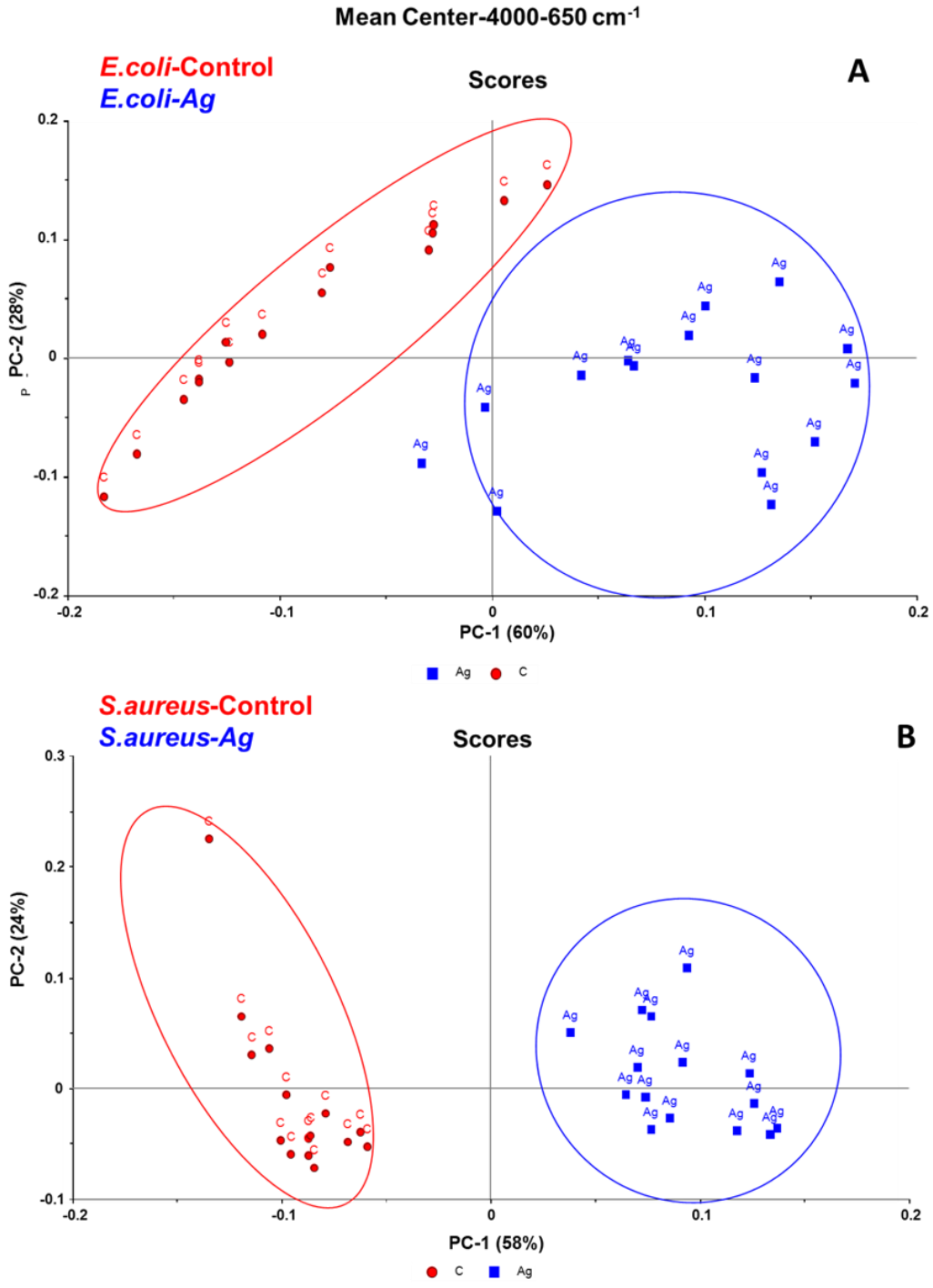

Figure 3.11 PCA score plots for control and Ag-resistant *E. coli* (A) and *S. aureus* (B) samples in the 4000–650 cm^{-1} spectral region.

3.2.4 HCA

HCA results of control, and Ag-resistant *E. coli* and *S. aureus* in the 4000-650 cm^{-1} spectral region are presented in figure 3.12A and B, respectively. As shown in dendrograms, the Ag-resistant and control groups were totally differentiated from each other (15/15) with highest distance values. The distance values were 10 for both bacteria, which is maximum value in HCA.

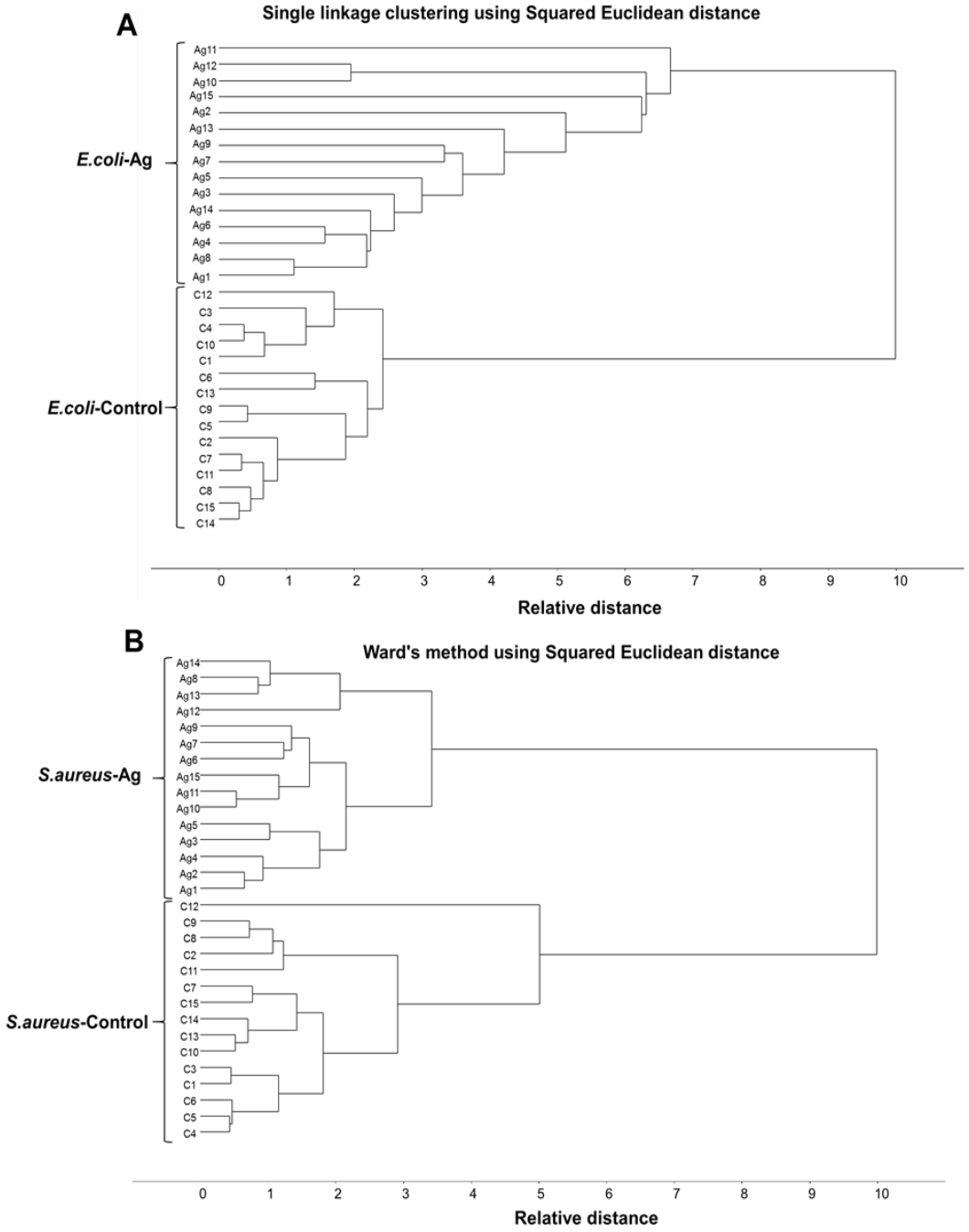

Figure 3.12 Hierarchical cluster analysis of control and Ag-resistant *E. coli* (A) and *S. aureus* (B) samples in the 4000–650 cm^{-1} spectral region.

3.2.5 ATR-FTIR spectral data analysis

Subsequent to PCA and HCA exploratory analyses, a detailed ATR-FTIR data analysis of control and Ag-resistant bacterial groups was performed for more profound evaluation of biomolecular alterations. The detailed ATR-FTIR analysis includes precise band wavenumber, band intensity and bandwidth calculations. The results of these calculations are shown in figures 3.13-3.15 for different spectral regions.

Figure 3.13A and B represent the second derivative and vector-normalized infrared spectra of the control, and Ag-resistant *E. coli* and *S. aureus* in the C-H stretching (2980-2830 cm^{-1}) region. The spectra contain several peaks, which arise from the vibrations of different functional groups of biomolecules such as lipids and proteins. All the main spectral bands are labelled in the figure, accordingly.

The comparison of absolute intensities of the CH_2 antisymmetric band between the control and Ag-resistant bacterial groups is shown in figures 3.13C and D. The intensities of the CH_2 antisymmetric band increased significantly in the Ag-resistant *E. coli* and *S. aureus* compared to the control groups (Figures 3.13C and D, respectively).

Figures 3.13E and F illustrate the comparison of the bandwidth values for the CH_2 antisymmetric band in the control and Ag-resistant bacterial samples. The bandwidths of the CH_2 antisymmetric band significantly decreased for Ag-resistant *E. coli* and *S. aureus* as compared to the control groups (Figure 3.13E and F).

The changes of the band wavenumber values for the CH_2 antisymmetric band in the control and Ag-resistant *E. coli* and *S. aureus* are given in figures 3.13G and H. The band wavenumber values of the CH_2 antisymmetric band significantly shifted to a lower value for Ag-resistant *E. coli* and *S. aureus* as compared to the control groups (Figure 3.13G and H).

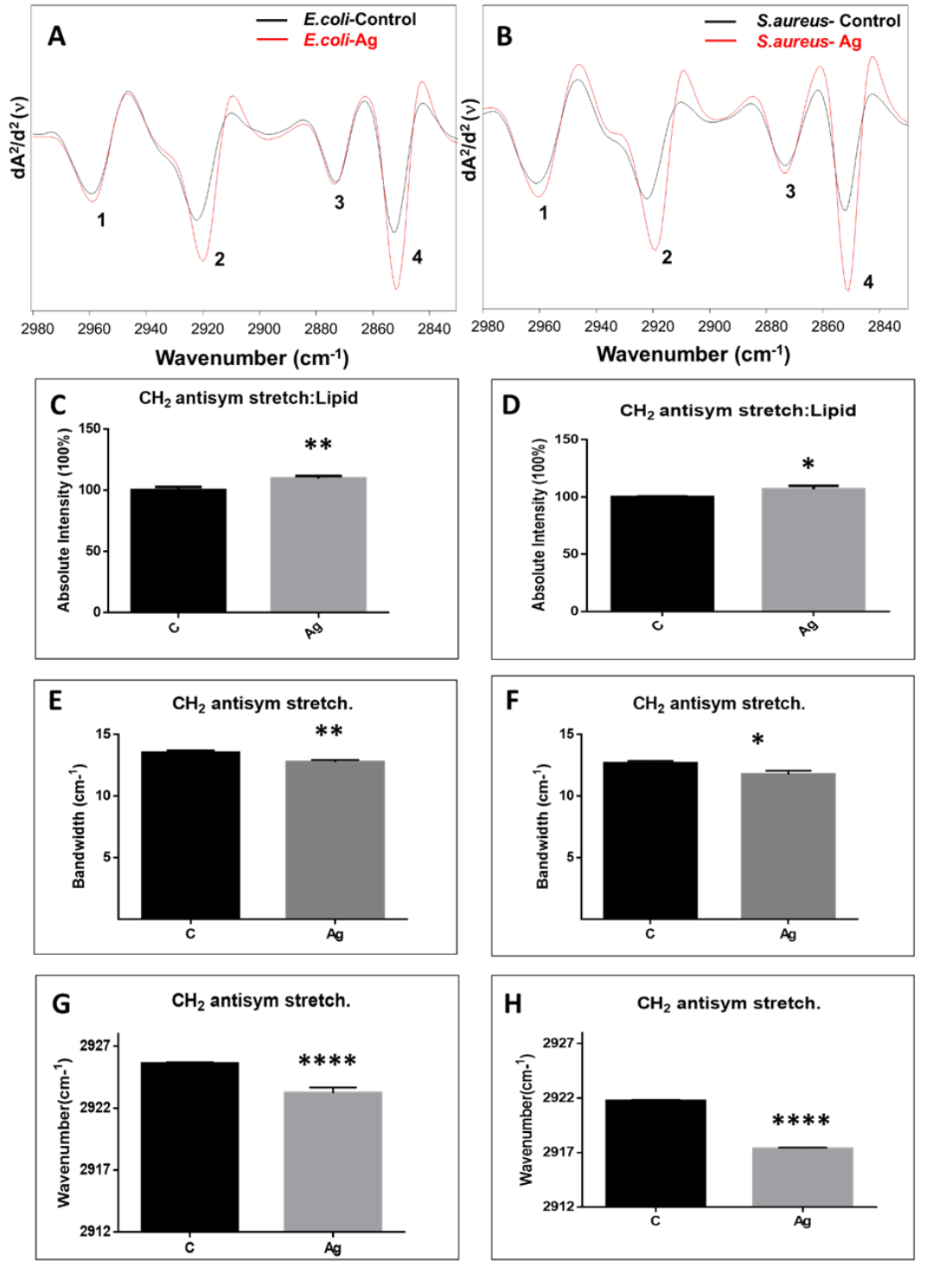

Figure 3.13 The representative second derivative IR spectra of control and Ag-resistant *E. coli* (A) and *S. aureus* samples at 2980–2830 cm^{-1} spectral region (B). The absolute intensity (C, D), bandwidth (E, F) and wavenumber (G, H) values of CH_2 antisymmetric stretching bands in *E. coli* and *S. aureus* groups.

Figure 3.14A and B show the second derivative and vector-normalized infrared spectra of control, and Ag-resistant *E. coli* and *S. aureus* at the 1750-1350 cm^{-1} region. The main bands have been labelled in the figure, accordingly.

The alterations of the Amide I band intensities of the control, and Ag-resistant *E. coli* and *S. aureus* groups are demonstrated in figure 3.14C and D, respectively. As can be inferred from these figures, the intensities of the Amide I band decreased significantly in Ag-resistant groups compared to the control groups.

Figure 3.14E and F show the comparison of Amide I band wavenumber values in the control and Ag-resistant *E. coli* and *S. aureus*. The band wavenumbers of the Amide I band significantly shifted to higher values for the Ag-resistant *E. coli* and *S. aureus* as compared to the control groups.

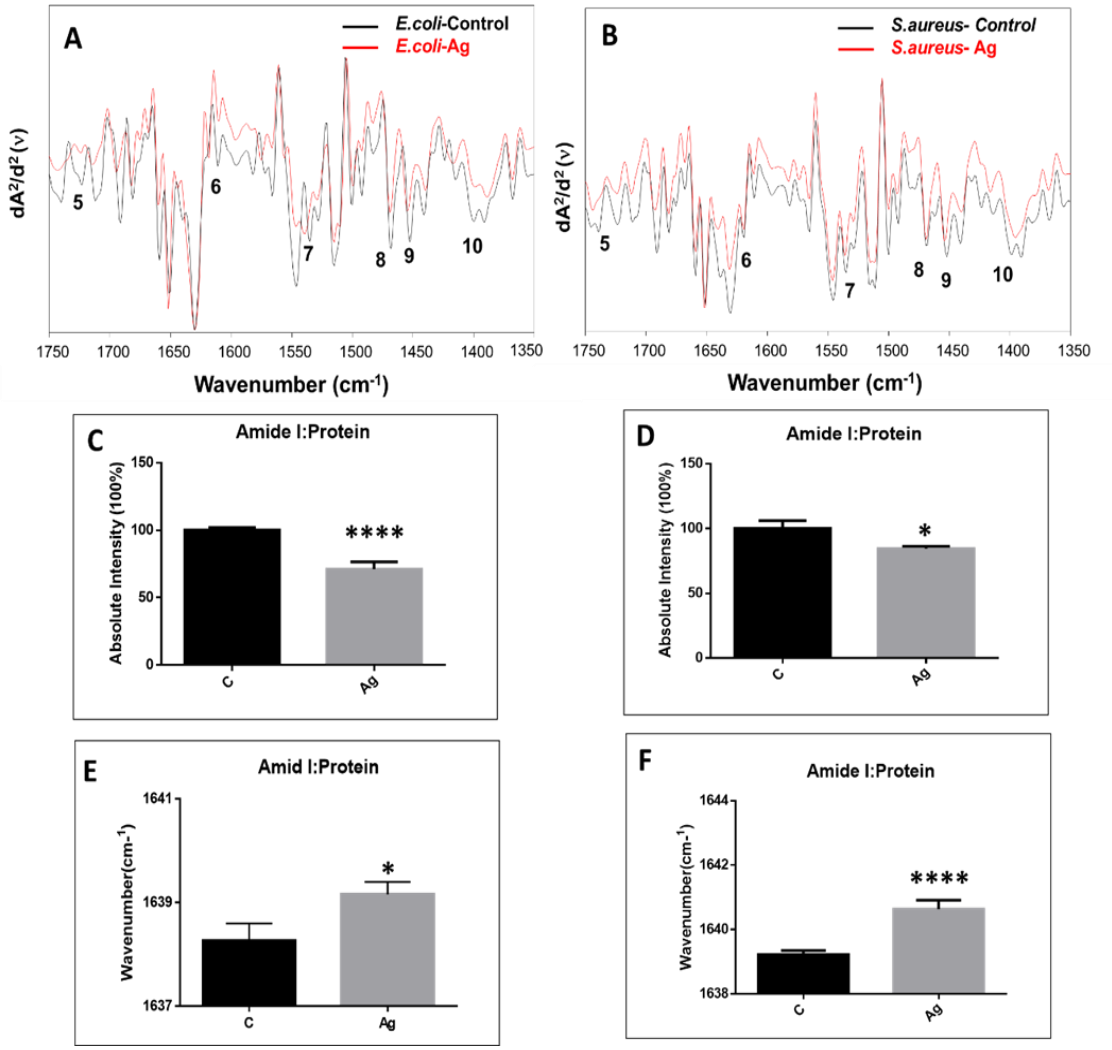

Figure 3.14 The representative second derivative IR spectra of control and Ag-resistant *E. coli* (A) and *S. aureus* samples at 1750–1350 cm^{-1} spectral region (B). The absolute intensity (C, D) and wavenumber (E, F) values of Amide I bands of the *E. coli* and *S. aureus* groups.

The second derivative and vector-normalized IR spectra of the control and Ag-resistant *E. coli* and *S. aureus* at 1350-940 cm^{-1} spectral region are shown in figure 3.15A and B, respectively. Figure 3.15C and D show the comparison of the absolute intensities of the PO_2 antisymmetric stretching band emerging from nucleic acids and phospholipids, for both control and Ag-resistant groups of *E. coli* and *S. aureus*. There is a significant decrease in the band intensities of the PO_2 antisymmetric stretching band for the Ag-resistant *E.coli* and *S. aureus* as compared to the control groups.

A comparison of the band intensities of the C-O stretching RNA band for both control and resistant groups of *E. coli* and *S. aureus* are shown in figure 3.15E and F, respectively. A significant decrease in the band intensities of the C-O stretching of RNA was observed for both Ag-resistant groups as compared to the control groups. Figure 3.15G and H illustrate the comparison of the band intensities of the C-O stretching band that belongs to polysaccharides in both control and Ag-resistant bacteria samples. A significant decrease in the band intensities for this C-O stretching band was observed for Ag-resistant *E. coli* and *S. aureus*, compared to the control groups.

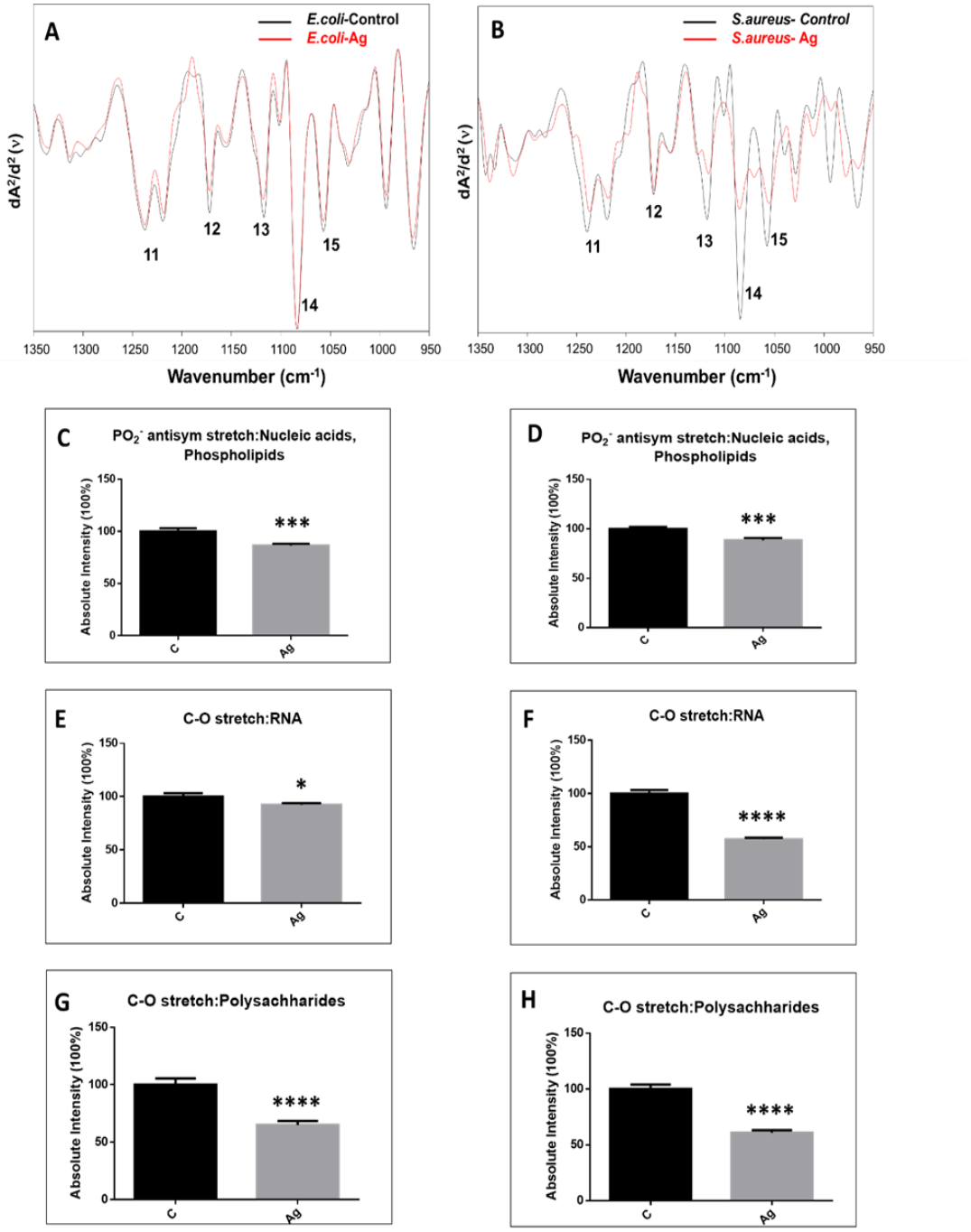

Figure 3.15 The representative second derivative IR spectra of control and Ag-resistant *E. coli* (A) and *S. aureus* samples at 1350–940 cm^{-1} spectral region (B). The intensities of PO^{2-} antisymmetric stretching (C, D), C-O stretching (E, F), C-O stretching (G, H) bands in these bacteria.

Heavy metals induce toxicity inside the cells in several ways. They may block the functional groups of biomolecules, displace the essential metal ions in biomolecules or modify their conformation, leading thus to function loss (Rajendran et al., 2003). They may cause oxidative damage to cells by over production of reactive oxygen species, and some heavy metals may deplete the cell's antioxidants (Ercal et al., 2001). Therefore, the recuperation of heavy metal polluted areas is of outmost importance. Due to their ability of developing heavy metal resistance, bacteria have been proposed as good candidates for heavy metal bioremediation (Valls and Lorenzo, 2002; Iyer et al., 2005). In order to select the most suitable bacterial strains, it is therefore necessary to understand heavy metal resistance mechanisms in bacteria. In the current study, the Ag resistance mechanisms for *E. coli* and *S. aureus* were investigated. For this purpose, first, the inhibitory concentrations of Ag for *E. coli* and *S. aureus* were identified and obtained as 130 and 94 μM, respectively. In our previous study, the inhibitory concentrations for Cd and Pb in *E. coli* ATCC 8739 were found as 171 and 226 μM, respectively. Moreover, the inhibitory concentrations of Cd and Pb were acquired as 171 and 302 μM, respectively for *S. aureus* ATCC 6538 strain (Gurbanov et al., 2015). Even though *E. coli* and *S. aureus* showed resistance against all three of the heavy metals at the above-mentioned concentrations, the bacteria appeared to be less tolerant to Ag and Cd than it was to Pb. In other words, the MIC results showed that *E. coli* and *S. aureus* have a higher resistance to Pb compared to Ag and Cd and a higher sensitivity to Ag compared to Cd and Pb.

To elucidate the bacterial heavy metal resistance mechanisms in a holistic way, we then applied ATR-FTIR spectroscopy together with chemometric tools. Environmental circumstances can change the chemical composition and structure of biological entities at the molecular level. These molecular components can be marked and described through the specific vibrational elements in the IR spectroscopy (Wang and Mizaikoff, 2008). Therefore, FTIR spectroscopy has been extensively used as a tool for the classification, identification and discrimination of microorganisms by producing unique finger-prints of whole-organism (Wenning and Scherer, 2013). For example, Dziuba et al. (2007) used FTIR spectroscopy to identify several lactic acid

bacteria at the genus level, while Erukhimovitch et al. (2005) showed that FTIR can be used to quickly discriminate between bacterial and fungal infections. Similarly, recent studies by our group which were investigated cobalt-acclimated and Cd, and Pb-resistant bacterial strains (Kardas et al., 2014; Gurbanov et al., 2015), indicated that ATR-FTIR is a powerful technique that can be used for a variety of studies on bacteria, including heavy metal resistance. Therefore, our present study aims to expand our current understanding of heavy metal resistance in bacteria by comparing infrared spectra of control, and Ag-resistant *E. coli* and *S. aureus*.

Chemical and physical features of the complex systems such as whole bacterial cells can be modelled using chemometrics to their IR spectra (Gabrielsson et al., 2002). Among the chemical pattern recognition techniques, PCA is most popular exploratory method because it reduces and transforms the huge analytical information to explainable variables (Brereton, 2007; Luna et al., 2013). These variables are known as principal components (PCs) and three of them (PC1, PC2 and PC3) should be utilized in order to demonstrate the foremost information and eliminate the negligible ones (Teye et al., 2013). PCA reflects the summarized presentation of the data set; however, its main function is to explore the variations in the spectral data (Muehlethaler et al., 2011). Furthermore, when the loadings of these PC's are plotted as a component of diverse variables, they demonstrate which variable stands for the foremost dissimilarity (Pichardo-Molina et al., 2007). There are two main advantages of PCA. First, it is an effective data compression technique. Second, PCA helps to resolve the overlapping spectral parameters, since it considers variation across the chosen whole spectral region instead of considering an individual wavelength (Wang and Mizaikoff, 2008).

The applications of PCA in microbiology have been reported in several studies (Stockel et al., 2012b; Strittmatter et al., 2014). In addition to those studies, for example, various heavy metal resistant sediment bacteria were differentiated using PCA and HCA (Nithya et al., 2011). Another study reported the ability of PCA in the differentiation of bacterial species using single microbial cells (Huang et al., 2004). Similarly, PCA was used in other study to distinguish the live and dead food-borne *Salmonella* cells (Sundaram et al., 2012). Moreover, this method was performed to

differentiate Cd and Pb-resistant *E.coli* and *S.aureus* from their control counterparts in our previous study (Gurbanov et al., 2015). Therefore, to be able to clarify spectral differences between control and Ag-resistant *E.coli* and *S.aureus*, PCA analysis was applied to the finger-print and whole IR spectral regions. We have seen remarkable differences in PCA score and loading plots of the studied groups. The loading spectra represented the relative spectral changes due to the Ag resistance in studied bacteria. These variations were prominent in the C-H and finger-print regions which were revealed by high Eigen vector values observed for these regions. The score plot disclosed almost totally segregated clusters of the Ag-resistant groups from the control groups.

HCA was utilized, in order to identify whether the spectral differences obtained from PCA analysis enable the discrimination and classification of Ag-resistant bacteria from control ones. This analysis is a well-optimized unsupervised pattern recognition technique to achieve accurate discrimination and classification of similar samples (Brereton, 2007). In this chemometric approach, IR band positions and intensities generate input data for the segregation of samples into distant clusters (Severcan et al., 2010; Demir et al., 2015). While PCA establishes the common association, HCA detects the similarities/dissimilarities among the samples (Brereton, 2007). Similar to PCA, HCA has been widely applied in the discrimination of microbial populations (Bosch et al., 2006; Nithya et al., 2011). Spore and vegetative forms of different bacteria have been rapidly discriminated via HCA (Bombalska et al., 2011). Recently, HCA was applied in order to differentiate various strains of food-related *E. coli* (Wenning et al., 2014). This analysis was also used to classify Cd and Pb-resistant *E.coli* and *S.aureus* in our recent study (Gurbanov et al., 2015). We applied HCA as an unsupervised chemometric method in order to support the differentiations obtained through PCA. According to HCA dendrograms, all samples (15) in the Ag-resistant group were differentiated from the all samples (15) in the control group for both *E. coli* and *S. aureus*. In other words, HCA analysis demonstrated the clear clustering of the control and Ag-resistant with a high distance value, implying the large spectral differences among the studied groups.

Both PCA and HCA analyses confirmed the large differences in the biochemical constituents of Ag-resistant and control bacteria. To identify these alterations in a precise and quantitative manner, the detailed spectral band analyses, such as wavenumber, intensity, and bandwidth calculations were performed. In IR spectroscopy, according to the Beer-Lambert law, band intensity is proportional to the concentration of the sample. Therefore, an alteration in the intensity of the lipid bands implies a change in lipid concentration. Elevated lipid concentrations in Ag-resistant bacterial groups mean the altered lipid metabolism due to the Ag-stress. The heavy metal resistance induced alterations in bacterial lipid metabolism have been reported in several studies (Guschina and Harwood, 2006; Markowicz et al., 2010; Kardas et al., 2014). Moreover, we have indicated the diminished lipid amount in Cd and Pb-resistant *E.coli* and *S.aureus* in our prior study (Gurbanov et al., 2015). However, in case of Ag resistance, an increased lipid concentration was acquired in the same bacteria. The differences in lipid amount of Pb, Cd and Ag-resistant bacteria may be associated with the variation in the heavy metal resistance mechanisms. Furthermore, the earlier studies have reported that the increased concentrations of heavy metals further elevated the content of several phospholipids in bacterial communities isolated from heavy metal polluted soils and these findings support the increased lipid concentration induced by Ag resistance (Frostegard et al., 1993; Pennanen et al., 1996). These elevated lipid content could arise from the well-documented phenomena of intracellular lipid vesicle formation (Kulp and Kuehn, 2010; Lee, 2012).

Lipids as an essential structural part of the biological membranes play crucial roles in the regulation of various cellular activity such as fluidity, viscosity, order etc. (Murata and Siegenthaler, 1998). The alteration in membrane lipid dynamics and orders can be evaluated by calculating the bandwidths and the shifts in the wavenumber of the CH_2 stretching bands, respectively. In the current study, decreased bandwidth values of the CH_2 antisymmetric stretching band in all Ag-resistant groups, implied a decrease in the dynamics of membrane lipids for Ag-resistant *E. coli* and *S. aureus*. Moreover, the shift of the wavenumber of the same band to lower values indicated the increased membrane lipid order in Ag-resistant bacterial groups, which demonstrated structural alteration in the lipid skeleton. Similar to these results, it has been stated that Ag interacts with membrane lipids further decreasing membrane fluidity, altering

membrane properties and disrupting membrane proteins in *E .coli* (Cabiscol et al., 2000; Mijnendonckx et al., 2013).

These structural and quantitative alterations of lipids in resistant bacteria might be promoted by heavy metal exposure induced oxidative stress. The relationship between metal related oxidative stress and bacterial lipids has been reported only in a few studies (Markowicz et al., 2010; Gurbanov et al., 2015). It was reported that, heavy metal stress exerts a great influence on lipid metabolism of resistant organisms (Guschina and Harwood, 2006). Heavy metals generate reactive oxygen species (ROS) and increase membrane lipid peroxidation (Markowicz et al., 2010). The resistant microorganisms exhibit the ability to acclimatize themselves according to the altered lipid metabolism (Guschina and Harwood, 2006). Markowicz et al. (2010) suggested that the metal stress associated alterations in the saturated lipids help the bacterial cell to deal with the toxic action of metals. Previously, Dibrov et al. (2002) mentioned that, cytoplasmic membrane is a major target for Ag. Similarly, Ag-induced production of ROS causes an increase in membrane permeability for *E. coli* and *S. aureus* (Park et al., 2009; Morones-Ramirez et al., 2013). Furthermore, environmental stress has been shown to bring into existence the alterations in the saturation/unsaturation, cis/trans and branched/unbranched structures of lipids as well as the changes in membrane bilayer stability and fluidity (Kim et al., 2001; Denich et al., 2003; Heipieper et al., 2003).

Since lipids are not the only molecules affected from the heavy metal stress, we performed the analysis of bacterial proteins as well. In IR spectroscopic analysis, the quantitative and qualitative information about the proteins can be obtained by evaluating amide bands (Severcan and Haris, 2012). Our results demonstrated a decrease in the concentration as well as structural alterations of the proteins in Ag-resistant groups. The decreased protein content in Cd and Pb-resistant *E.coli* and *S.aureus* has been reported in our previous study, which confirms the results presented in this current study (Gurbanov et al., 2015). The heavy metal induced changes in protein structure and content are generally associated with the modifications in the protein synthesis such as, inhibition and deactivation of sensitive enzymes (Hassen et

al. 1998; Nies, 1999). For example, Feng et al. demonstrated that the interaction of Ag ions with proteins' thiol functional groups caused to the inactivation of bacterial proteins (Feng et al., 2000). Moreover, the contextual alteration in bacterial protein content caused by heavy metal exposure may be related to the stimulation of ion/proton efflux transporters by the heavy metals since this stimulation enforces the cell to alter and reduce its proteins, in order to discard the metals away (Roane and Pepper, 1999). Soft metal transporting P_{IB}-type ATPases can be given as an example of these transporters and it has been recently reported that these proteins orchestrate the resistance mechanism and halt the accumulation of environmentally unfriendly metals such as Pb, Cd and Ag by carrying out these metals across the cell membrane (Naik and Dubey, 2013). It is known that, P-type ATPase efflux pump, SilP pumps Ag ions from the cytoplasm to the periplasm. Another periplasmic protein/ chaperone SilF carries Ag ions from SilP to the SilCBA cluster. This cluster forms the basis of membrane-potential dependent cation/proton antiporter system, covering the cell membrane. The group of proteins, particularly efflux pump (SilA), an outer membrane factor (SilC), and a membrane fusion protein (SilB) transport Ag ions from the periplasm to the outside of the cell (Mijnendonckx et al., 2013). At this point, the role of bacterial metallothioneins (MTs) should also be taken into consideration. As known, these metal binding proteins function to immobilize the metals in order to maintain intracellular homeostasis (Hamer, 1986).

Heavy metal resistance induced changes in bacterial nucleic acid and polysaccharide contents have been stated in several studies (Kardas et al., 2014; Whelan et al., 2014). Therefore to ascertain these changes in the case of Ag resistance, FTIR spectroscopy was performed since the capacity of this technique in the efficient inspection of nucleic acids have been demonstrated in several recent studies (Kardas et al., 2014; Whelan et al., 2014; Gurbanov et al., 2015). According to our results, Ag-stress exerted notable influence on the above-mentioned macromolecules. In our earlier study, we have demonstrated the decreased nucleic acid content in Cd and Pb-resistant *E. coli* and *S. aureus* (Gurbanov et al., 2015). Remarkable reductions in the DNA and RNA concentrations also in Ag-resistant bacteria can be explained by the modulated nucleic

acid biosynthesis mechanisms. Regarding this, it was shown that DNA converted from the relaxed state to condensed state in Ag-treated *E. coli* and *S. aureus* indicating loss of replication ability of DNA (Feng et al., 2000), which indicated the decreased nucleic acid biosynthesis. The decrease in this biosynthesis can be explained by the state of nutritional starvation during heavy metal exposure. It is well known that, the expression of the distinct and populous genes can be modulated by the ambient determinants. Nutritional starvation disturbs the production of rRNA and tRNA subsequently halting ribosome assembly, DNA and protein fabrication (Madigan et al., 2010). Since oxidative stress extensively damage the lipids, proteins and nucleic acids in all aerobic organisms including bacteria (Cabiscol et al., 2000), we also should not overlook the detrimental effects of heavy metal induced ROS on nucleic acids.

Practically all the microorganisms possess polysaccharides on their cell surfaces (Yi et al., 2009). These high molecular weight microbial sugar molecules situated in the outer cell membrane as lipopolysaccharides (LPS) (Cuthbertson et al., 2009). On the other hand, extracellular lipopolysaccharides (EPS) secreted outside the cell to cope with the environmental stresses including heavy metals (Kazy et al., 2002; Kumar et al., 2007). Anionic character of these bacterial carbohydrates enable them to bind the metal cations efficiently and alleviate their toxicity. They play a principal role in order to develop a resistance against Pb in gram-negative bacteria (Jarosławiecka and Piotrowska-Seget, 2014). Accordingly, these macromolecules are advised as strong scavengers and biosorbents for heavy metal bioremediation (Kazy et al., 2002). FTIR spectroscopy has been used in several studies to characterize these microbial polysaccharides (Kazy et al., 2002; Marcotte et al., 2007; Kardas et al., 2014). Herein we present the significantly decreased concentrations of the bacterial polysaccharides in Ag-resistant groups, which may depend on their direct interactions with heavy metals. In other words, after facing the heavy metals as a part of resistance system the quantitative changes happen to these polysaccharides as shown similarly in a recent study (Kardas et al., 2014).

In the light of all the facts mentioned above, we hypothesize that nutritional starvation and heavy metal stress led to the induction of intricate genetic response mechanisms further remodeling the main molecular constituents of bacteria. The bacteria develops these defense strategies in order to survive under heavy metal stress conditions by inhibiting all the regular metabolic activities and adjusting the cellular metabolism.

3.3 IR coupled supervised pattern recognition technique for the classification of heavy metal resistant bacteria

This part discusses the classification results for several heavy metal resistant bacteria using supervised pattern recognition technique-SIMCA, based on their IR spectroscopic data. Since the building of appropriate PCA models are necessary step for running SIMCA analysis succesfully, we first presented these PCA models for Cd, Ag and Pb-resistant bacteria and then their SIMCA models. Moreover, besides the laboratory strains (*E. coli* and *S. aureus*), we presented the SIMCA results of two environmental isolates (*Microbacterium oxydans* and *Gordonia* sp.) deriven from Mogan lake (Ankara), in order to ensure the power and robustness of IR coupled supervised chemometric tools for the classification of these environmental bacterial strains also.

3.3.1 SIMCA Classification

SIMCA, is an important supervised pattern recognition method developed in the early 1970s (Brereton, 2007). Construction of confidence intervals for each experimental group is a principal mission of SIMCA, in which unclassified samples are placed into the PC margins and organized according to the most appropriate class (Stanimirova et al., 2010). For that reason, conduction of PCA is a mandatory step for SIMCA classification (Cruz et al., 2013). To clarify, SIMCA computes PC models for every class of the data set and classifies the unknown samples. The unknowns are identified and discriminated by contrasting the residual variance of the modeled class with the

residual variance of the unknown specimen (Sabin et al., 2004; Hernandez-Martinez et al., 2010; Luna et al., 2013). Biggest interval or distance among the classes means the excellent classification for SIMCA (Luna et al., 2013).

In order to obtain SIMCA models, first, PCA was performed for control and heavy metal resistant (Cd, Ag and Pb) groups of *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *Microbacterium oxydans* FS45 and *Gordonia* sp. FS18 experimental groups in the whole (4000-650 cm^{-1}) IR regions.

The score plot results for *E. coli and S. aureus* in the whole IR region (4000-650 cm^{-1}) are shown in figure 3.16A and B. As shown in the figure 3.16A, the maximum variation value was quite high (*E. coli* PC1+PC2 =97%). The score plot shows that Pb and Ag-resistant samples are clearly segregated from the control and Cd-resistant samples. The plot furthermore reveals that the Pb-resistant samples have aggregated much more clearly from the control samples than the Ag-resistant samples. The control and Cd-resistant groups have some overlapping regions between them, on the boundary. Figure 3.16B shows the score plot result for *S. aureus* in the same region. Similarly, the maximum variation value was high for this bacteria as well (*S. aureus* PC1+PC2=93%). The score plot shows that Pb and Cd-resistant samples are successfully segregated from the control and Cd-resistant samples in this region. The control and Ag-resistant groups have some overlapping regions between them, on the boundary.

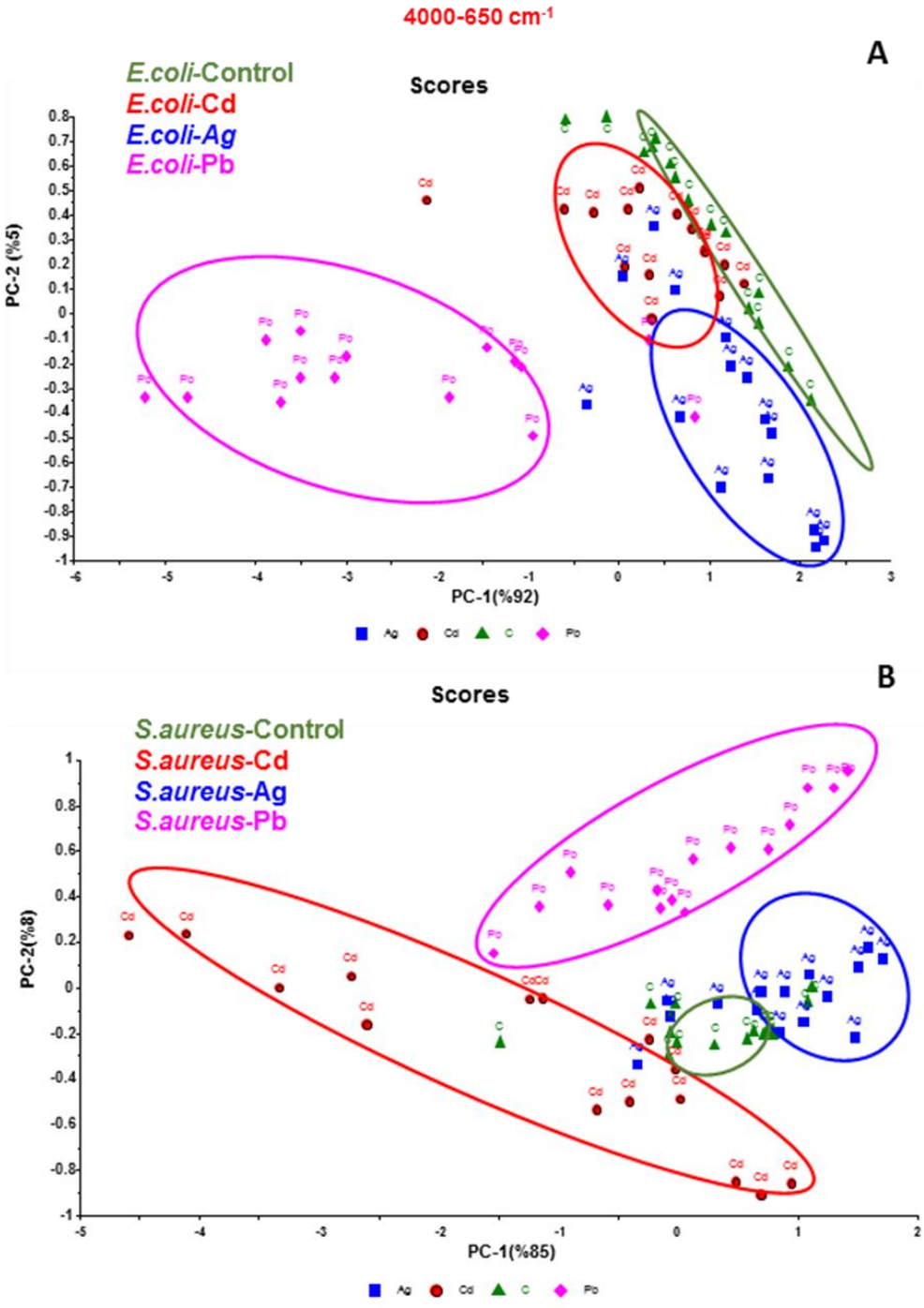

Figure 3.16 PCA score plots for control and Ag, Cd and Pb-resistant *E. coli* (A) and *S. aureus* (B) samples in the 4000–650 cm^{-1} spectral region.

Figure 3.17A and B demonstrate the score plots of *Microbacterium oxydans* FS45 and *Gordonia* sp. FS18 bacteria, respectively. The maximum variation value for *Microbacterium oxydans* was 52% (PC1+PC5); whilst it was 74% (PC1+PC2) for *Gordonia* sp. (Figure 3.17A and B). All the groups are clearly discriminated from each other in these environmental bacterial isolates.

It is not long since, ATR-FTIR spectroscopy was utilized together with pattern recognition techniques to maintain a novel and routine identification of different *S. aureus* strains in less than a day (Xie et al., 2012). Similarly, a number of current investigations analyzing taxonomic discrimination and classification of different foodborne bacteria using FTIR spectroscopy combined with chemometric tools have been reviewed by a recent study (Davis and Maurer, 2010). The study concluded that, these techniques offer routine and broad applications in food microbiology, along with identification, discrimination, enumeration, and classification of bacteria (Davis and Maurer, 2010). Moreover, the application of PCA to the microorganisms isolated from petroleum-contaminated terrestrial tropical soils revealed the differentiation and classification of these strains based on their biodegradation abilities (Chaillan et al., 2004). Furthermore, a recent study by our group demonstrated the application of IR-based chemometric tools (HCA and PCA) for the rapid differentiation of bacteria (Gurbanov et al., 2015).

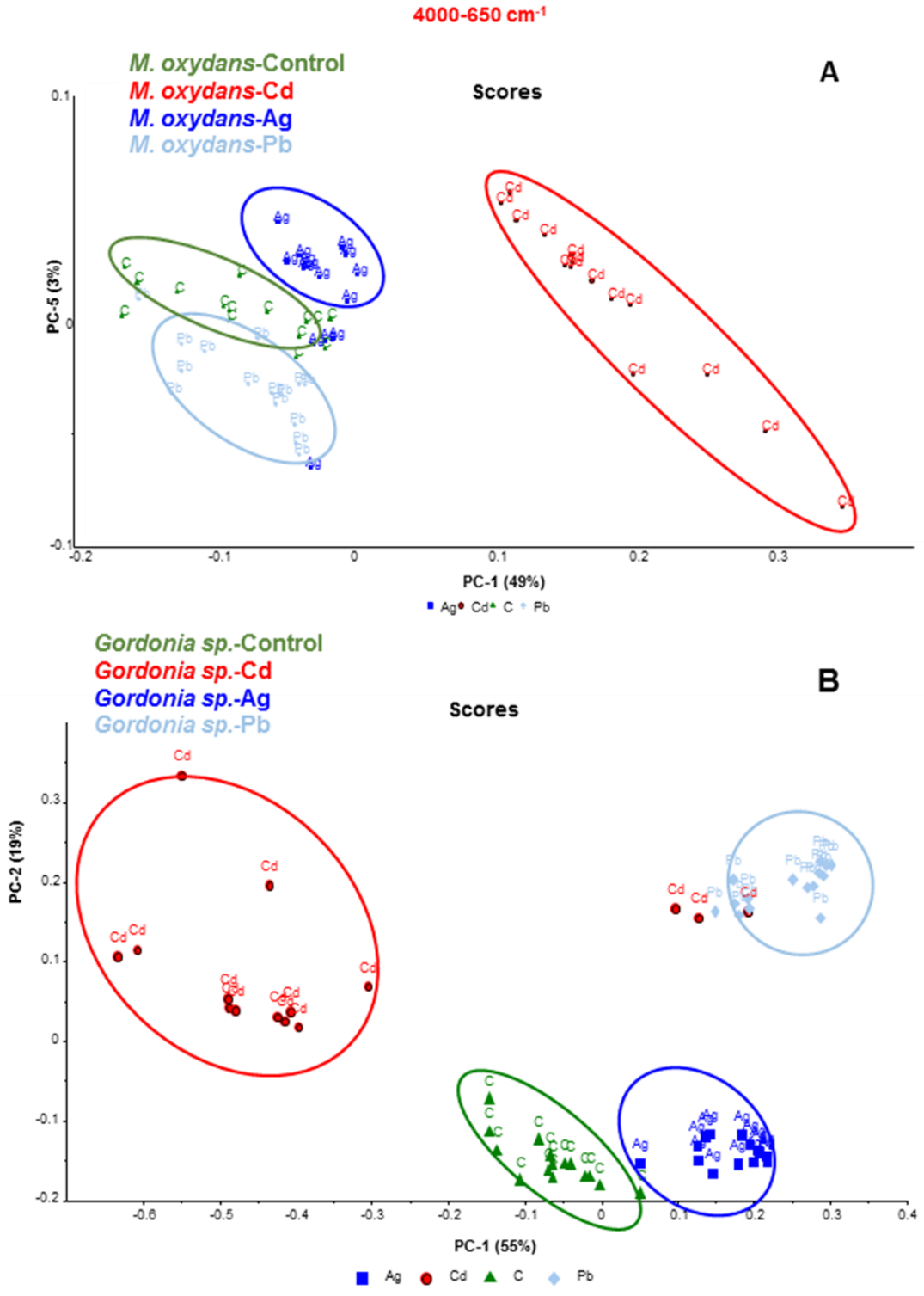

Figure 3.17 PCA score plots for control and Ag, Cd and Pb-resistant *Microbacterium oxydans* FS45 (A) and *Gordonia* sp. FS18 (B) samples in the 4000–650 cm^{-1} spectral region.

SIMCA was applied to the whole IR region (4000-650 cm^{-1}) spectra of *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *Microbacterium oxydans* FS45 and *Gordonia* sp. FS18 experimental groups. The results are summarized in figures 3.18-3.21.

Figure 3.18 summarizes the SIMCA results for *E. coli* ATCC 8739. Figure 3.18A shows the distance in PC space of the Ag, Cd and Pb calibration models from the control model. In general, a distance of 3-4 in the PCA space indicates a clear segregation of the models from each other. As shown in the figure, the distances of the Ag and Cd calibration models from the control model lie between 80 and 84, while the distance of the Pb calibration model from the control model lies between 58 and 60. The large distances indicate that there is a large difference between the Ag, Cd and Pb calibration models and the control model. The results from the SIMCA analysis may be also presented in a plot called the Coomans plot, where distances between two classes are plotted against each other in a score plot. Figures 3.18B, C and D show the Coomans plots for *E. coli* control-Ag, control-Cd and control-Pb models, respectively.

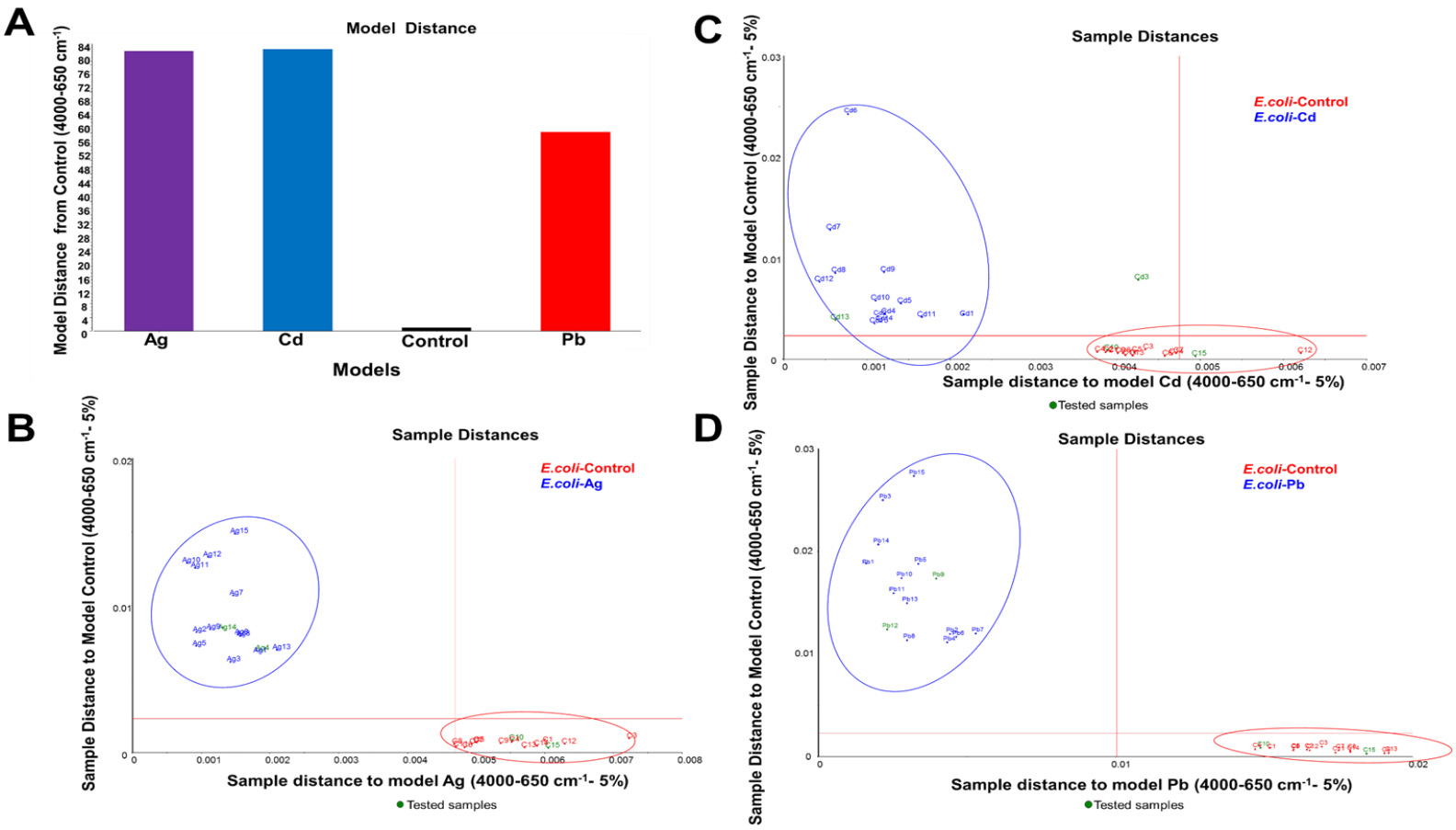

Figure 3.18 The SIMCA results for *E. coli* ATCC 8739. A), Model distances between control, Ag, Cd and Pb-resistant *E. coli*. B), C), D), Coomans plots for *E. coli* control-Ag, control-Cd and control-Pb models, respectively.

Figure 3.19 illustrates the SIMCA results for *S. aureus* ATCC 6538. Similar to *E. coli*, large differences (from 38 to 77) were obtained between the Ag, Cd and Pb calibration models and the control model in *S. aureus* (Figure 3.19A). Coomans plots for *S. aureus* control-Ag, control-Cd and control-Pb models have been shown in figures 3.19B, C and D, respectively.

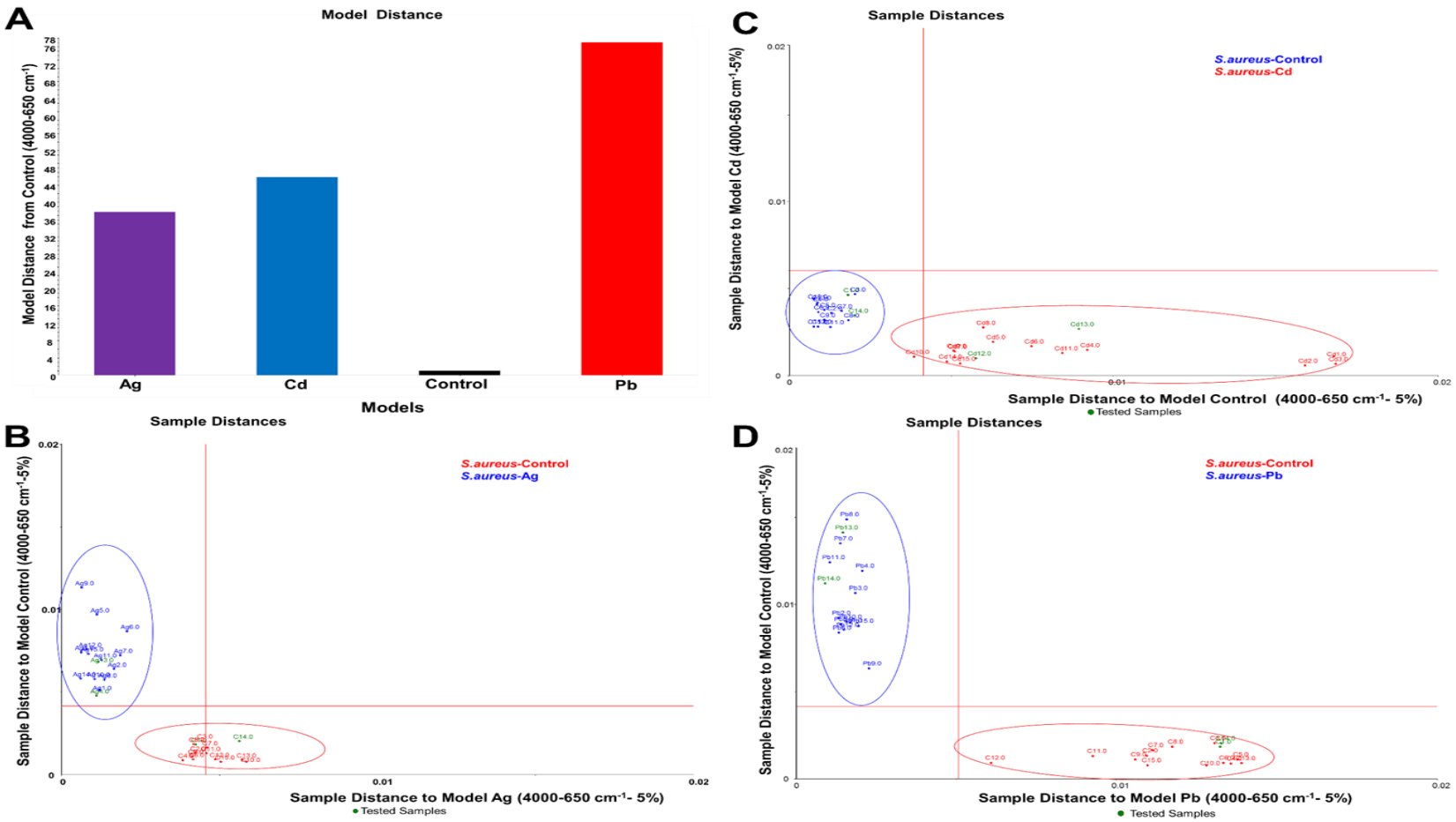

Figure 3.19 The SIMCA results for *S. aureus* ATCC 6538. A), Model distances between control, Ag, Cd and Pb-resistant *S. aureus*. B), C), D), Coomans plots for *S. aureus* control-Ag, control-Cd and control-Pb models, respectively.

The model distances between control, Ag, Cd and Pb-resistant environmental *M. oxydans* FS45 obtained in SIMCA are shown in figure 3.20A. As shown in the figure, the distance between control and Cd models was 68, whilst the distances between the control-Ag and control-Pb models were 13 and 6 respectively. Figure 3.20B, C and D represent the Coomans plots for Ag, Cd and Pb-resistant *M. oxydans*, respectively.

Figure 3.21A demonstrates the model distances for control, Ag, Cd and Pb-resistant environmental *Gordonia* sp. FS18. The distances between control-Ag, control-Cd and control-Pb models were 62, 115 and 93, respectively. The Coomans plots for Ag, Cd and Pb-resistant *Gordonia* sp., are shown in figures 3.21B, C and D, respectively.

The large phenotypic variety between the same species of bacteria requires robust classification methods for the detection of unknown spectra. Therefore, it is obligatory to validate the already trained model. In other words, independent information set of unknown bacteria, which was not used to calibrate the model, should be ascertained by simulating the ordinary operation. This predicts the accuracy of a model in line with the routine circumstances to estimate the percentage of correct assignments (Wenning and Scherer, 2013).

The plots show the discriminations of the Ag, Pb and Cd-resistant laboratory and environmental bacterial groups from the control groups with 5% significance level. In addition, the tested samples chosen randomly from each group (shown in green in the Coomans plot) were identified correctly in their corresponding groups. These results together with high model distances between bacterial groups demonstrate that, FTIR spectroscopy coupled to proper chemometric tools are good substitutes for molecular /immunologic bacterial identification and strain typing methods by displaying rapidity and superior robustness (Wenning and Scherer, 2013).

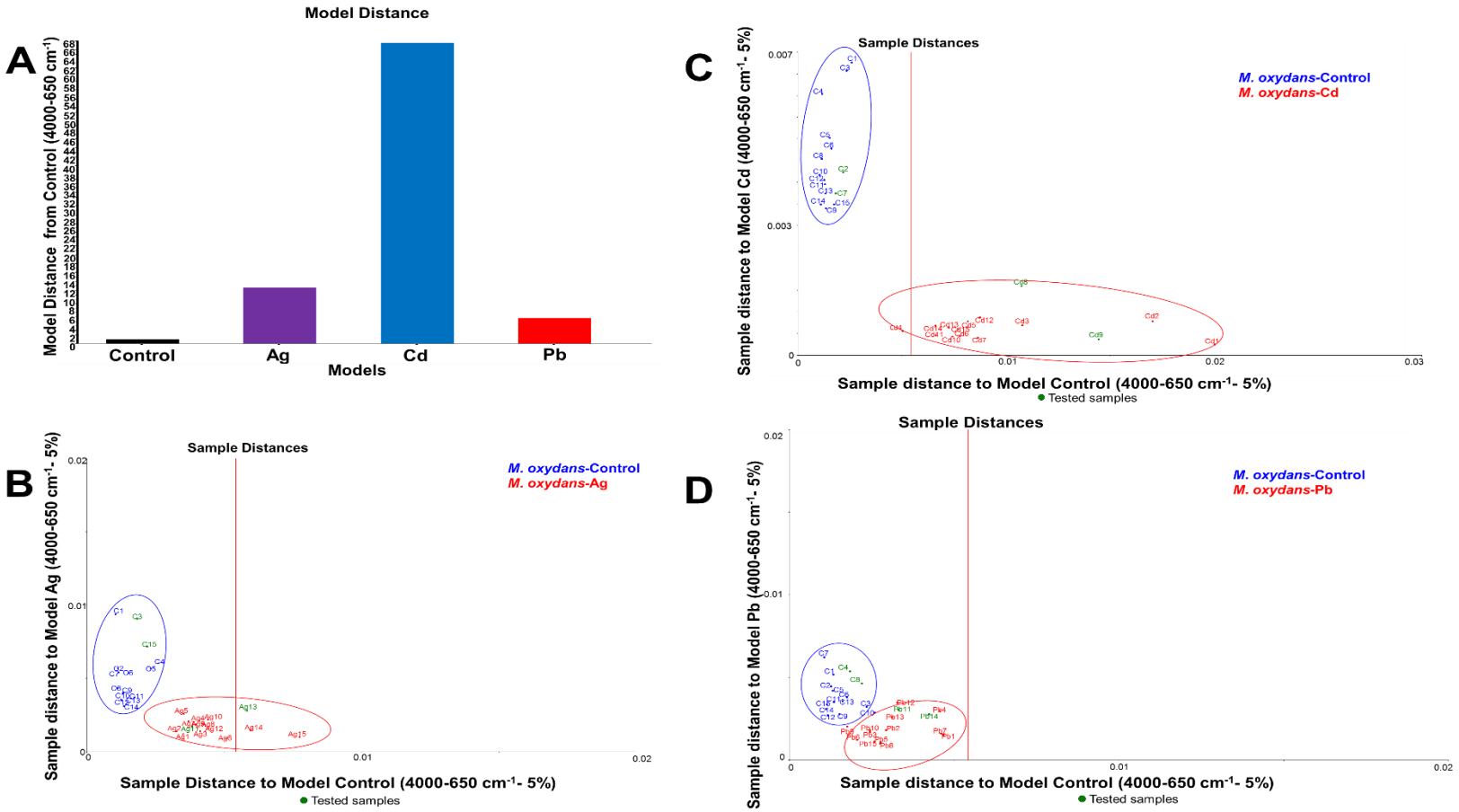

Figure 3.20 The SIMCA results for *Microbacterium oxydans* FS45. A), Model distances between control, Ag, Cd and Pb-resistant FS45. B), C), D), Coomans plots for FS45 control-Ag, control-Cd and control-Pb models, respectively.

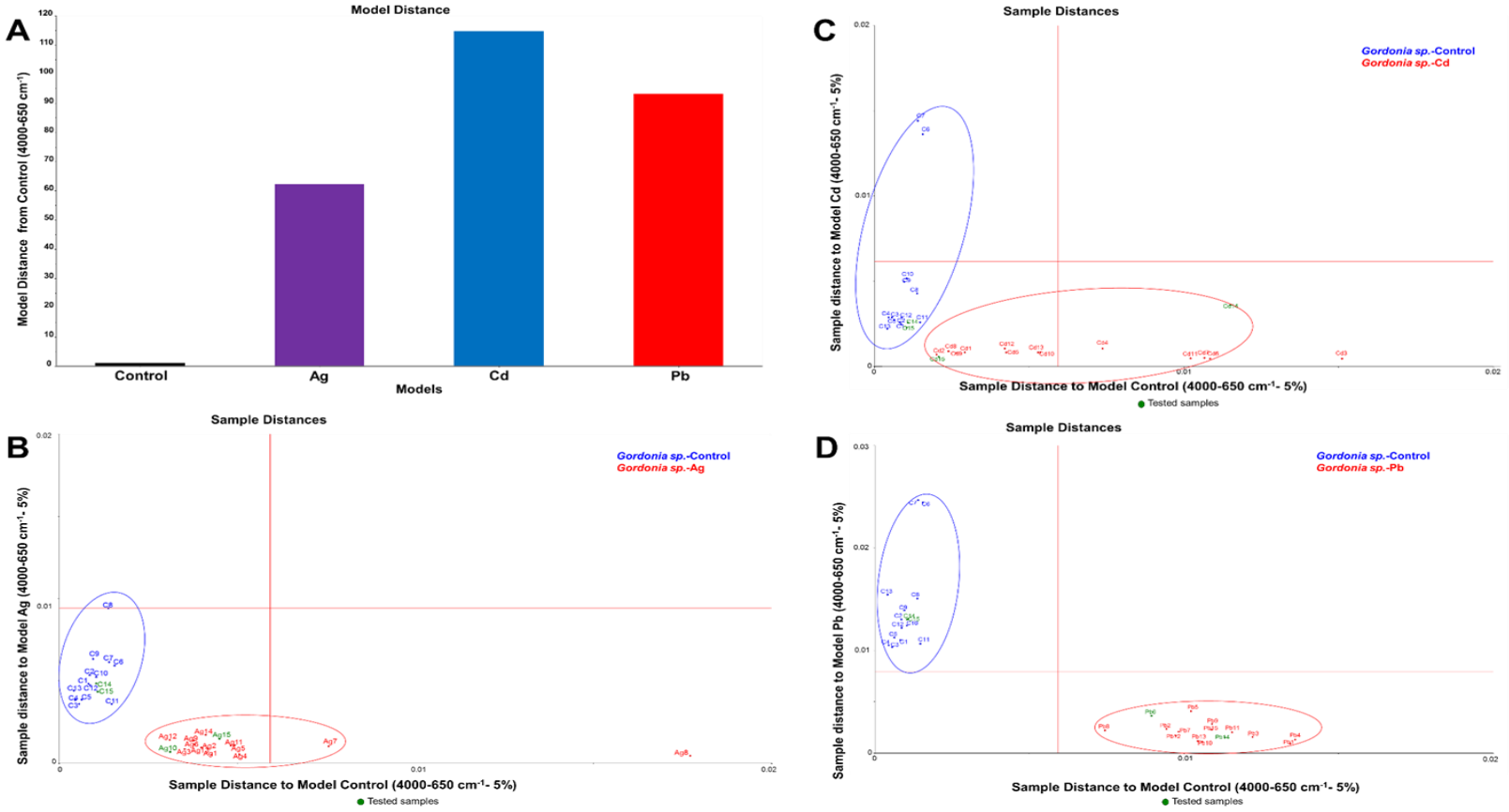

Figure 3.21 The SIMCA results for *Gordonia* sp. FS18. A), Model distances between control, Ag, Cd and Pb-resistant FS18. B), C), D), Coomans plots for FS18 control-Ag, control-Cd and control-Pb models, respectively.

Recent application of IR spectroscopy along with chemometrics (PCA, OPLS-DA and PLSR) as a novel technology for the monitoring, differentiation and selection of probiotic bacterial strains with different phenotypic characteristics, demonstrated very accurate, rapid and non-destructive identification of these strains (Slavchev et al., 2015). Mariey et al., discussed the numerous studies performed over the past decade, which used FTIR spectroscopy and multivariate pattern-recognition methods including PCA and SIMCA toward the discrimination, classification and identification of a wide range of microorganisms (Mariey et al., 2001). Hernandez-Martinez et al. (2010) showed a high potential of PCA and SIMCA based on HATR-FTIR spectroscopy, for the quick characterization and identification of fatty acids in margarines. The study concluded that, broad profile of margarine can be acquired in 10 min using these chemometric approaches, compared to long-lasting traditional analysis technique (Hernandez-Martinez et al., 2010). Another recent study demonstrated that, FTIR spectra of bacteria isolated from the poultry meat can be used to classify the contaminated samples from the control with the help of chemometrics such as PCA, PLS and SIMCA. These techniques were suggested as an effective tool to be further developed for the direct identification of bacteria from the poultry meat (Grewal et al., 2014). In a similar manner, different bacterial strains inoculated into the apple juice were clearly seggregated and classified (77-82%) from each other through FTIR-based PCA and SIMCA analyses, respectively (Al-Holy et al., 2006). FTIR spectroscopy combined with SIMCA technique was utilized to examine the biochemical changes happening to bacterial spores following thermal and pressure-assisted thermal operations for food safety (Subramanian et al., 2007). In past, SIMCA was used together with Visible and Near IR multispectral imaging to develop an efficient method for the automatic detection and good classification of nematodes in valuable fish species. The study revealed adequate differences between the spectral parameters of these parasites and fish flesh (Wold et al., 2001). Furthermore, *Lactobacillus* species were identified successfully from the large FTIR information using PLSR, SIMCA and KNN (Oust et al., 2004).

Environmental microbiology also employs FTIR spectroscopy together with chemometrics for the identification and classification of environmental microorganisms as reported in several studies (Kansiz et al., 1999; Tindall et al., 2000; Behrendt et al., 2002; Winder at al. 2004; Fischer et al., 2006; Bounphanmy et al., 2010; Garon et al., 2010; Bansal et al., 2014). Different strains of cyanobacteria, a bacteria living in freshwater, marine and terrestrial habitats were examined via FTIR spectroscopy together with HCA, PCA, KNN and SIMCA techniques. The study recommended FTIR spectroscopy coupled chemometrics as an alternative distinguishing approach for cyanobacteria based on the correct classifications (100%) of these bacterial strains in KNN and SIMCA analyses (Kansiz et al., 1999). Likewise, saline-tolerant strains of terrestrial cyanobacteria have been differentiated and classified (93%) via ATR-FTIR spectroscopy in combination with PCA and SIMCA (Bounphanmy et al., 2010). FTIR spectroscopy and HCA were applied to categorize a lot of environmental bacterial isolates in faster and much more economical way than any molecular approach including FAME (microbial fingerprinting technique) (Tindall et al., 2000). Similarly, the reliable results were acquired via an FTIR spectroscopy based classification of grass-associated coryneform bacteria species (Behrendt et al., 2002). Previously, the differences between the known genomic species and environmental isolates of *Acinetobacter* from activated sludge were studied using FTIR spectroscopy coupled to HCA and PCA-DFA analyses. The study concluded that, these techniques may contribute for the routine identification of environmental *Acinetobacter* isolates (Winder et al., 2004). In addition to bacteria, microfungi (*Aspergillus* and *Penicillium*) species have been also identified and their strains were characterized using FTIR spectroscopy and chemometrics (Fischer et al., 2006). In another study, different *Aspergillus* species isolated from feed and bioaerosols in agricultural environment, were discriminated and aflatoxin producing *A. flavus* and *A. parasiticus* were differentiated (75 and 100%, respectively) from the controls, based on their FTIR spectra coupled to HCA and DA (Garon et al., 2010).

CHAPTER 4

CONCLUSION

"FTIR spectroscopy has gained serious attention for the discrimination and observation of drug/chemical induced molecular alterations in microorganisms since it enables quick, cost-effective and operator-indepedent monitoring and identification of microbial populations. The use of advanced multivariate statistical analysis tools enhances the discriminative capacity of this technique in microbiology. In our study, we used this approach to enable quick discrimination of heavy metal resistant bacterial populations. The results of the first part demonstrated that there were obvious spectral changes due to the heavy metal exposure, which led to the distinct segragation of both Cd and Pb-resistant bacteria from the control groups. Especially, the Pb-resistant strains clustered apart from the other two groups with 100% sensivity and specificity. This quick discrimination indicates outstanding components of the fingerprint region of the IR spectrum. The outstanding components were detectable in the whole region of the IR spectrum as well. These components may later be detailed in biochemical and genetic studies. The current study using the ability of ATR-FTIR spectroscopy for quick discrimination of heavy metal resistant bacteria will contribute to further bioremediation studies" (Gurbanov et al., 2015).

The second part of our study provided information at the molecular level on what kind of structural and functional alterations that bacteria developed when exposed to Ag. The analysis of the IR spectra revealed a great deal of information about the macromolecules of Ag-resistant bacteria as compared to the non-resistant ones. In addition, we have used chemometric techniques such as HCA and PCA for the fast and reliable discrimination of Ag-resistant bacterial strains from control ones. The information obtained from IR spectroscopy coupled with chemometrics can be then

evaluated to determine the mechanisms that these changes may reflect, and it can be used as a guide to further explore these mechanisms via other techniques. Understanding bacterium resistance to Ag might provide new insight on the potential use of this or similar strains for bioremediation purposes.

In the final part of the study, we present the discrimination and classification of heavy metal resistant bacterial strains, considering their potential for the development of environmental decontamination strategies. These strains showed high resistances againist the most dangerous heavy metals due to their adaption to live in metal toxic environments. However, studying their different responses toward the same exposed toxic metal concentrations can be further utilized to bioremediate the polluted ecosystems. In this context the classification of environmental strains are mostly important since they are isolated from the polluted lake- Mogan. Accordingly, we tried to classify these different strains using supervised chemometric tool- SIMCA. Within this work, we confirmed high classification capacity and superior potential of SIMCA applied to heavy metal resistant bacterial isolates. Promising as they look, this supervised chemometric tool need to be further improved through the abundant FTIR databases of heavy metal resistant bacteria prior to the direct application in field.

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APPENDIX A

IR SPECTRA OF BACTERIA

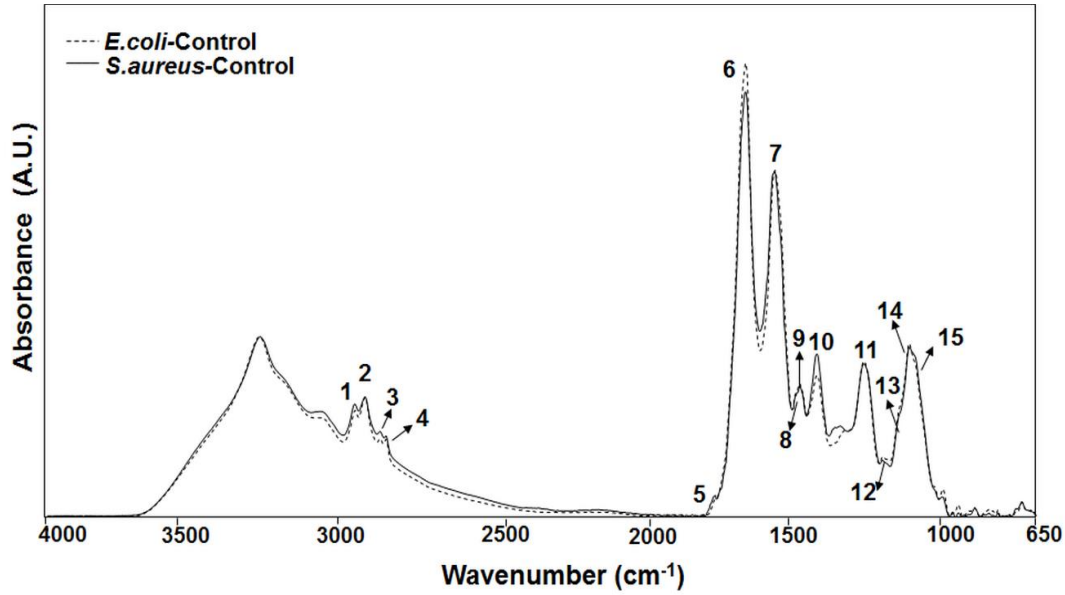

Figure A.1 PCA scatter plots for all A) *E. coli* B) *S. aureus* samples in the 4000–650 cm^{-1} spectral region.

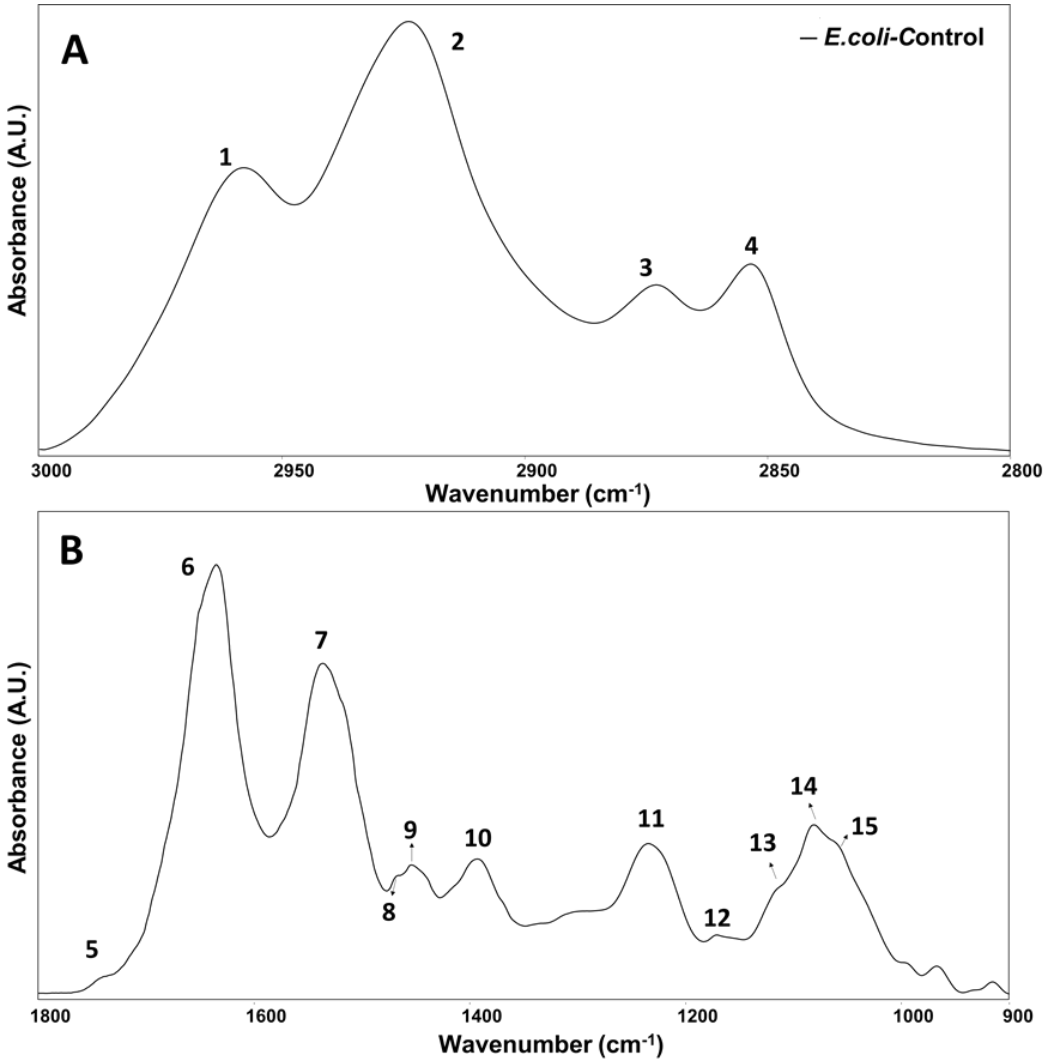

Figure A.2 Average ATR-FTIR spectra of the control spectra of (A) *E. coli* in the 3000–2800 cm^{-1} and (B) the 1800-900 cm^{-1} spectral regions. Both regions were normalized with respect to the amide A band located at 3285 cm^{-1}.

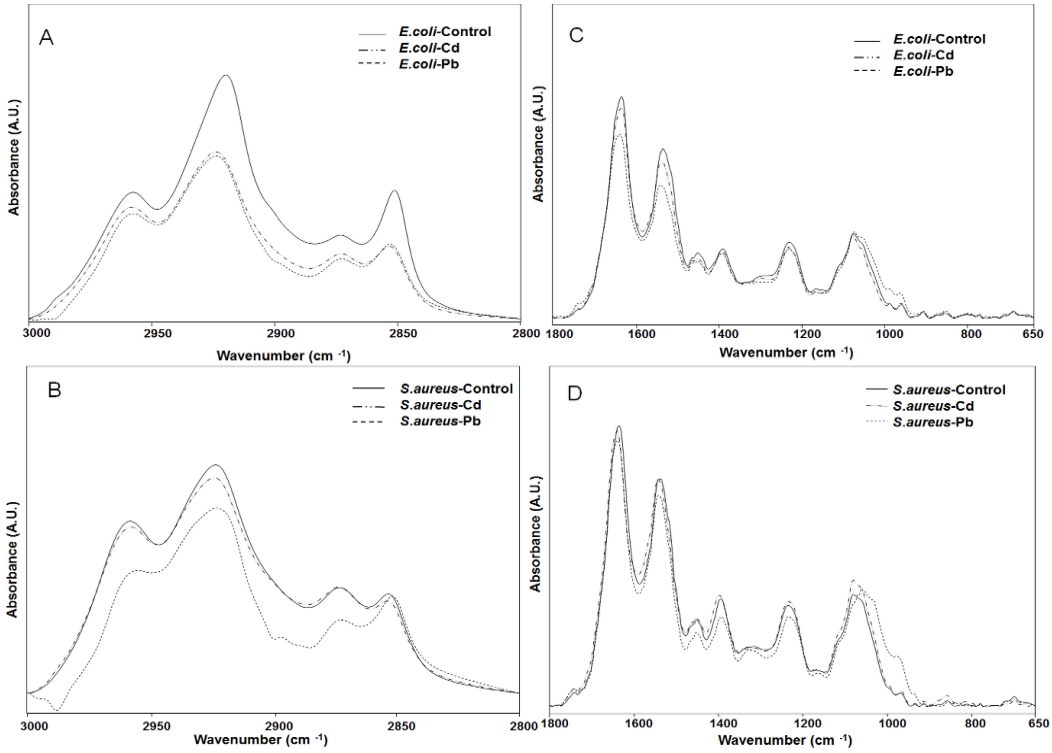

Figure A.3 ATR-FTIR spectra of the control, Cd and Pb-resistant A) *E. coli* B) *S. aureus* in the 3000–2800 cm^{-1} spectral region C) *E. coli* D) *S. aureus* in the 1800–650 cm^{-1} spectral region.

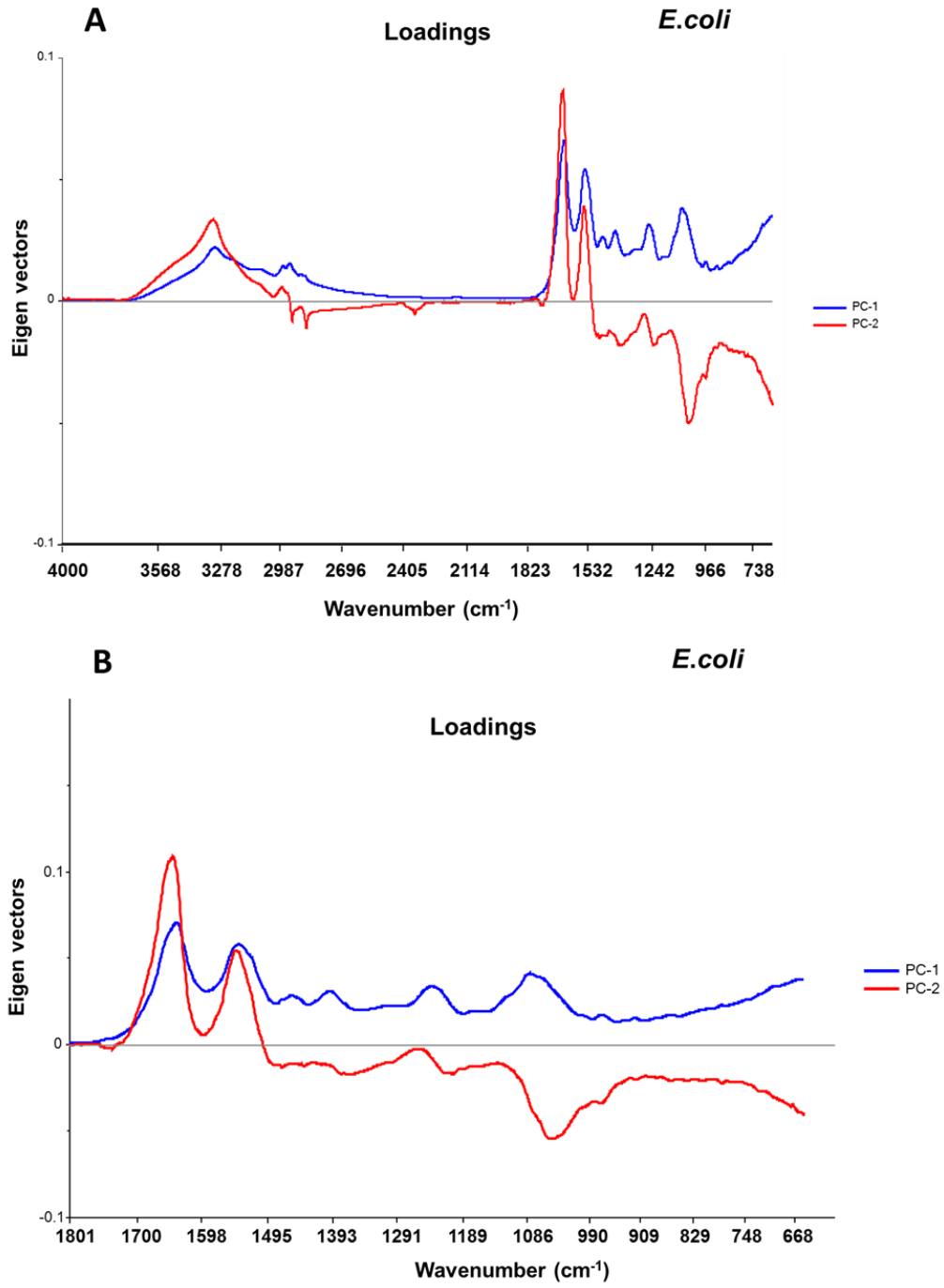

Figure A.4 PCA loading plots for all *E. coli* samples in the (A) 4000-650 cm^{-1} and (B) 1800–650 cm^{-1} spectral regions.

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Education

PhD	Middle East Technical University (METU), Graduate school of Natural and Applied sciences, School of Biochemistry, Ankara (CGPA 3.33/4), **2010- 2016**
MSc	Middle East Technical University (METU), Graduate school of Natural and Applied sciences, School of Biochemistry (MS), Ankara (CGPA 3.21/4), **2006- 2010**
MD	Khazar University School of Medicine, Baku, Azerbaijan (CGPA 83/100), **2000-2005**

Computer knowledge

Microsoft Office Programs, Linux, Ubuntu, Various Statistical and other scientific analysis programs such as GraphPad, MATLAB, OPUS, Spectrum and Unscrambler.

Language skills

YDS-2015 English- **85**

- English- fluent
- Russian-fluent
- Turkish-fluent
- Italian- elementary

Other skills

Training and tutoring for 10 students (graduate and undergraduate) in the research laboratory.

Developed succinct Report Writing Skills through project assignments.

Learnt to speak efficiently in formal meetings through my course representative duties.

Gained insight into how teams can develop strategies to deal with problems through participating as a team member in project assignments.

Independent conduction of scientific experiments
Possess experience in molecular biology, biochemistry, microbiology and biotechnology

First-class research and analytical skills, capable of interacting at all levels to devise innovative solutions in a research/industrial environment.

Memberships

Biophysical Society-2015
American Society for Microbiology (ASM)-2015

Publications

Academic Thesis and Dissertation

- **MS thesis:** The effects of selenium on stz-induced diabetic rat kidney plasma membrane, 2010, Middle East Technical University

- **PhD dissertation:** Characterization of heavy metal resistant bacteria using infrared spectroscopy together with chemical pattern recognition techniques, 2016, Middle East Technical University

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- Feride Severcan, Ozlem Bozkurt, Rafig Gurbanov and Guvenc Gorgulu, "FTIR spectroscopy in diagnosis of diabetes in rat animal model", Journal of Biophotonics, Volume 3, Issue 8-9, 2010, Pages: 621–631

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  DOI: http://dx.doi.org/10.1016/j.bpj.2014.11.3402 **IF: 3.972**

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- Rafig Gurbanov, Nihal Simsek Ozek, Ayse Gul Gozen, Feride Severcan, "Discrimination of Lead and Cadmium Resistant Bacteria by ATR-FTIR Spectroscopy", "IICBE 2014 International Conference on Earth, Environment and Life sciences (EELS-2014)", December 23-24, 2014 Dubai, UAE. Paper ID: C1214017

- R. Gurbanov, N. Simsek Ozek, A. G. Gozen, F. Severcan, "An ATR-FTIR Spectroscopic Approach to Elucidate Biochemical Mechanisms in Heavy Metal Resistant *Escherichia coli*", "115th General Meeting of the American Society for Microbiology (ASM2015)", May 30 – June 2, 2015, New Orleans, Louisiana, USA. 054 (Division Q), p.136, poster #847.

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