Evaluation of the Antimicrobial Effects of *Laurus nobilis* leaf Extract as a Fresh Meat Preservative

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نموذج التفويض

أنا محمود سبتي ابراهيم حمدان أفوض جامعة فلسطين التقنية خضوري بتزويد نسخ من رسالتي /أطروحتي للمكتبات والمؤسسات أو الهيئات أو الأشخاص عند طلبهم حسب التعليمات النافذة في الجامعة.

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Dedication

This work is dedicated to everyone who wanted this thesis to pass and complete.

To the soul of my dear father, who has always been a positive role model in my life.

To my family, friends, and colleagues who brought me to the Highway.

To my manager at work Dr. Husam Twier who supported me and provided the facilities to fulfill this work.

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Evaluation of the Antimicrobial Effects of *Laurus nobilis* leaf Extract as a Fresh Meat Preservative

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Abstract

Fresh red meats especially sheep meats, by their nature are nutritious and easily metabolisable and therefore offer suitable substrates for the growth and metabolism of microorganisms. Foodborne disease is a cluster of microbial or toxic gastrointestinal diseases, which results from consuming contaminated food with certain microbial agents or their toxins. Prevention of food spoilage and food poisoning pathogens is usually achieved by the use of chemical preservatives which have negative effects on human health. The purpose of this thesis is to find a potentially valuable, healthy safer and natural alternative preservative. The objectives of present work are concentrated on the screening of sheep meat samples for the presence of lipid food poisoning bacteria, investigating the antimicrobial activity of *Laurus nobilis* extracts against lipid food poisoning bacteria, and detecting the duration that can the fatty food be preserved by antioxidant and antimicrobial action of *Laurus nobilis* leaf. Samples of sheep meat were collected and bacterial

contaminants were isolated and identified by specific biochemical techniques, and sequence of the 16SrRNA gene. Antimicrobial activity of *Laurus nobilis* extract was investigated against all isolates using agar well diffusion technique. Thirty four bacterial isolates were isolated and identified. Methanol extraction of *Laurus nobilis* leaves produced the maximum essential oil yield than ethanol extraction. Gram-positive isolates were more affected by antimicrobial effect of *Laurus nobilis* extract than gram-negative isolates. In addition, *Laurus nobilis* extract has the ability to increase the shelf-life of sheep meat to 13 days with accepted features at refrigerator, and to three days at room temperature by using spraying method. The thesis results showed that *Laurel* extract has the potential to be used as natural alternative preventive to control food poisoning diseases and preserve foodstuff avoiding health hazards of chemically antimicrobial agent applications.

Chapter 1
Introduction

Chapter 1

1- Introduction

Food poisoning is considered as one of the universal reasons for sickness and death in developing countries and represents a major problem in industrialized countries (Mostafa et al., 2016). Food poisoning is defined as a cluster of microbial or toxic gastrointestinal diseases results from consuming contaminated food with certain microbial agents or their toxins (Rahman & Othman, 2017). Food poisoning symptoms vary with the source of contamination. The most common symptom of food poisoning is watery or bloody diarrhea, which annually affects approximately 550 million people worldwide, and causes about 230,000 deaths case per year (Tomaszewska, Trafialek, Suebpongsang, & Kolanowski, 2018). Foodborne illness can take place at any point of processing chain which include: growing, harvesting, processing, storing, and transporting (Davis & Pavia, 2015). Most of the foodborne diseases are caused by microbial contamination, mainly gram positive and gram negative bacteria (Mostafa et al., 2016). In addition, chemicals that entering the human body through the eating of polluted food and water can lead to long-term disability and even death (Tomaszewska et al., 2018).

Fresh red meats, especially sheep meat is considered as a high-energy type of food with rich nutritional value, which makes it one of the

main items in our meals (Jamilah, Abbas, & Rahman, 2008). Fresh sheep meat provides an important source of high-quality protein which also contains a large number of vitamins and minerals (Jamilah et al., 2008). The shelf life of fresh meat is extremely depended on several factors such as pH, water activity, microbial contamination including pathogens, lipid oxidation, and color changes (Jamilah et al., 2008). Sheep meat has a short shelf-life of about one day or less at ambient temperature (15-30°C), and a few days at refrigerating temperature (0-10°C) (Lucera, Costa, Conte, & Nobile, 2012). This is mainly due to microbial spoilage caused by both pathogenic and non-pathogenic microorganisms, and/or lipid oxidation (Lucera et al., 2012).

The major strategic problem of food manufactures, mainly lipid foods, is free radical oxidation of the fatty component; due to the chain reaction of lipid peroxidation (Conforti, Statti, Uzunov, & Menichini, 2006). Chemical structure of the fatty acid, nature of food processing, cooking and storage temperature, and minor constituent antioxidants are the main factors that have the ability to change and control the extensity of oxidation of fatty acids (Conforti et al., 2006). Recent medical research indicates that many chronic diseases, including cardiovascular diseases and at least some types of cancer, are initiated by free radical oxidation of lipids, nucleic acids, or proteins (Nehir El, Karagozlu, Karakaya, & Sahın, 2014).

The availability of many food products has changed the nature of their production, value, quality, and organoleptic characteristics, increased necessities for the quality and situation of the individual products, and encouraged a demand on all consumer markets, that is focusing more on goods with an elevated market value (Conforti et al., 2006). In addition, many researches have focused on the knowledge and behavior of the consumer's concern about natural products, for natural antioxidants and/or antimicrobial compounds as an alternative to synthetic substances (Nehir El et al., 2014).

An assortment of synthetic antimicrobials has been used to decrease bacterial contamination in meat and fresh products (Rafiq et al., 2016). However, synthetic antimicrobials have been connected with health problems such as hypersensitivity, allergies, asthma, hyperactivity and cancer (Anand & Sati, 2013). Furthermore, synthetic antimicrobials can destroy both harmful and beneficial bacteria in human intestine, and increase bacterial resistance to antimicrobials (Verraes et al., 2013). Natural preservation approaches are an eye-catching as a safety parameter in foods with reduced contents of ingredients and additives that usually render to slow down microbial growth (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016). Biological preservatives are defined as natural substances that are added to fruits, vegetables, prepared food items, cosmetics, and pharmaceuticals in order to increase their shelf life

and maintain their value and safety (Anand & Sati, 2013).

Laurus nobilis, commonly known as Bay, is a plant belonging to the Lauraceae family (Fig 1, Ghadiri, Ahmadi, Moridikyia, Mahdavi, & Tavakoli, 2014), which comprises about 2500 species (Basak & Candan, 2013). It is a native of the southern parts of Europe and the Mediterranean area (Caputo et al., 2017).

Figure 1. *Laurus nobilis* leaves

Laurus nobilis is an aromatic herb used broadly to add a distinctive aroma and flavor to food (Fernández et al., 2018). *Laurus* leaf has been used traditionally as herbal medicine to treat rheumatism, earaches, indigestion, sprains, promote perspiration, and treat a variety of complaints like neuralgia, and intestinal cramps (El Malti & Amarouch, 2009). Additionally, a number of studies have proved the potential

capacity of laurel essential oil as an antimicrobial agent and also the antioxidant property of leaves extracts (Nehir El et al., 2014). Furthermore, the chemical composition of the essential oil isolated from the leaves of *Laurus nobilis* illustrated that eucalyptol (1,8-cineole), sabinene, and linalool are the main components (Caputo et al., 2017). All of these components are classified as monoterpenes or modified monoterpenes which play an important role in the protection mechanism by exhibiting beneficial functions such as antibacterial, antifungal, and antioxidant activities (Basak & Candan, 2013).

The purpose of this research is to find an alternatives to synthetic antibiotics and industrial preservatives, which present in the local market and cause many of the health problems. To our knowledge, no studies have been conducted to examine the antimicrobial activity of *Laurus nobilis* leaf against bacteria present in fresh sheep meat causing lipid poisoning.

The main objectives of this research are:

1) To screen sheep meat samples for the presence of lipid food poisoning bacteria.
2) To investigate the antagonist effects of *Laurus nobilis* extracts against bacterial growth.
3) To determine the duration that the sheep meat can be preserved by antioxidant and antimicrobial action of *Laurus nobilis* leaf.

Chapter 2

Literature Review

Chapter 2

2- Literature Review

Many researchers investigated antimicrobial and antioxidant of *Laurus nobilis* from two aspects, total essential oil or specific active material (active ingredient). Several studies showed the important role of dried leaves in solving several problems related to microbial infection. In addition, this chapter will display the major causes of meat spoilage, and characterize of microorganism that were isolated from meats.

2.1. *Laurus nobilis*: Composition of Essential Oil and Its Biological Activities

The chemical composition of the essential oil from leaves of *Laurus nobilis* was studied by Gas Chromatography GC and Gas Chromatography-Mass Spectrometry GC-MS (Caputo et al., 2017). Fifty five compounds were identified, accounting for 91.6% of the total essential oil. In the composition of the essential oil of *Laurus nobilis*, 1,8-Cineole (31.9%), sabinene (12.2%), and linalool (10.2%) were the main components, with other compounds being present in low percentages or even in traces (Caputo et al., 2017). The essential oil showed significant antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus* 4313, *Bacillus cereus* 4384, *Escherichia coli* and *Pseudomonas aeruginosa*. The essential oil was more effective than 1,8-cineole, which was ineffective against *Escherichia coli*, *Pseudomonas*

aeruginosa, and *Staphylococcus aureus* at a concentration of 0.4 uL/ml (Caputo et al., 2017). The inhibition halos exhibited by different volumes of *Laurus nobilis* essential oil and of 1,8-cineole against different molds (*Aspergillus niger*, *Aspergillus versicolor*, *Penicillium citrinum*, and *Penicillium expansum*) (Caputo et al., 2017). In addition, the treatment of SH-SY5Y human neuroblastoma cells with (1600–50 ug/ml) of 1,8-cineole and *Laurus nobilis* essential oil for 24 h resulted in a low cytotoxic activity, representative by Western blots and quantitative densitometry for adenylate cyclase1 (ADCY1) protein expression in SH-SY5Y (Caputo et al., 2017).

Staphylococcus aureus infection is of great importance from clinical view and highly prevalent in medical care centers. *Laurus nobilis* extract was examined for its antibacterial activity against *Staphylococcus aureus* by agar well diffusion and agar dilution methods (Ghadiri et al., 2014). The hydroalcoholic solution of *Laurus nobilis* extract showed antibacterial activity against *Staphylococcus aureus*. The finding proposed the use of the *Laurus nobilis* extract for the treatment of *Staphylococcus aureus* infection (Ghadiri et al., 2014).

Laurel essential oils were obtained by using solvent-free microwave extraction (SFME) and Hydrodistillation methods from *Laurus nobilis* leaves and their antioxidant and antimicrobial activities were determined

(Nehir El et al., 2014). Reduction of extraction time by about 43% in SFME at 622 Watt and 67% in SFME at 249 Watt was compared to Hydrodistillation (Nehir El et al., 2014). The essential oil of *Laurus nobilis* was extracted by SFME power levels and Hydrodistillation inhibited oxidation generated by 2-2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS radical), Linoleic Acid Peroxidation, and 2,2-diphenyl-1- picrylhydrazyl (DPPH radical) (Nehir El et al., 2014). Essential oils were found to display the antimicrobial activity against *Staphylococcus aureus* 6538P, *Escherichia coli* O157: H7 and *Salmonella typhimurium* NRRL E 4463 but not against *Listeria monocytogenes* (Nehir El et al., 2014).

An aqueous extract of *Laurus nobilis* leaf and chitosan was used as a natural edible coating to increase the shelf life of cashew (Azimzadeh & Jahadi, 2018). This natural edible coating successfully delayed lipid oxidation in comparison with the uncoated cashew. Furthermore, the chitosan coating with *Laurus nobilis* extract placed on the surface of the product can decrease microbial load and demonstrated antimicrobial effects on mesophilic bacteria and fungi. This coverage does not have any impact on color, solidity, bitterness, taste, and general accessibility (Azimzadeh & Jahadi, 2018).

2.2. Anti-Quorum Sensing Activity of *Laurus nobilis*

Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density (Zhang & Dong, 2004). Quorum sensing bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density (Miller & Bassler, 2001). Quorum sensing is the key regulator of virulence factors of *Pseudomonas aeruginosa* such as biofilm formation, motility, productions of proteases, hemolysin, pyocyanin, and toxins (Al-Haidari, Shaaban, Ibrahim, & Mohamed, 2016). *Pseudomonas aeruginosa* is an opportunistic pathogen that causes serious human infections (Zhang & Dong, 2004). The quorum inhibition activity of some medicinal plants like, *Laurus nobilis* appears to be a potential mode action of tested extracts to control bacterial pathogenicity (Al-Haidari et al., 2016). Anti-quorum sensing could offer an alternative mode of action against opportunistic pathogenic bacteria. The extracts of *Laurus nobilis* leaves exhibited strong anti-quorum sensing activity like a virulence suppressors of *Pseudomonas aeruginosa* (Al-Haidari et al., 2016). The importance of these medicinal plants is considered as a rich source of compounds that able to inhibit quorum sensing, slow down quorum sensing related virulence processes, and could manage *Pseudomonas* pathogenesis and hinder its dissemination (Al-Haidari et al., 2016).

2.3. Isolation and Characterization of Microorganisms from Raw Meat

Foodborne diseases are the main public health problem leading to increase the morbidity and mortality worldwide (Thanigaivel & Anandhan, 2015). Foods, by their character, are nutritious and simply metabolisable, and therefore offer suitable substrates for the growth and metabolism of microorganisms (Thanigaivel & Anandhan, 2015). Meat is a perfect medium for many organisms to grow because it is high in moisture, rich in nitrogenous compounds and plentifully supplied with minerals and accessory growth factors (Tassew, Abdissa, Beyene, & Gebre-Selassie, 2010). The microorganisms that ultimately bring about the spoilage of flesh foods are either present at the time of slaughter or introduced by workmen and their cutting tools, or by water and air in the dressing, cooling and cutting rooms (Newman, 2005). According to Thanigaivel & Anandhan. (2015) results, the predominant bacterial pathogens isolated from meat were *Escherichia coli, Salmonella spp.*, *Staphylococcus aureus*, and *Pseudomonas*. The main mold pathogen isolated was *Penicillium spp.*, followed by *Mucor spp., Aspergillus niger, Alternaria spp., Sporotrichum, Aspergillus fumigates* (Thanigaivel & Anandhan, 2015). The major yeast pathogen isolated was *Trichosporon spp*., followed by *Rhodotorula spp., Candida spp*. (Thanigaivel & Anandhan, 2015). In another study, a total of 165 samples from food establishments, butcher shops, and slaughterhouses

were processed and analyzed for the presence of bacterial indicator and potential food pathogens using standards methods (Tassew et al., 2010). In general, 10 different bacterial species were isolated which included, *Proteus spp* (53.9%), *E. coli* (26.6%), *Providencia spp* (13.9%) *Citrobacter spp* (9%), *Pseudomonas spp* (5.5%), *Klebsiella spp* (1.2%), *Enterobacter spp* (1.2%), *Salmonella spp* (1.2%), and *Shigella spp* (0.6%) (Tassew et al., 2010). The most common bacteria to occur on fresh meat are bacteria of the genera *Acinetobacter, Pseudomonas, Brochothrix, Flavobacterium, Psychrobacter, Moraxella, Staphylococcus and Micrococcus*, lactic acid bacteria and various genera of the *Enterobacteriaceae* family (Pennacchia, Ercolini, & Villani, 2011).

2.4. Major Factors of Meat Spoilage

The bacterial growth that causes meat spoilage is influenced by an enormous number of factors, which can be divided into four groups (Bruckner, Albrecht, Petersen, & Kreyenschmidt, 2012). First, inherent factors, which are an expression of the physical and chemical properties of the meats themselves (e.g. water activity, content of nutrients, the structure of the meats, the pH value, the initial content of psychrotrophic bacteria present on the surface of the meat) (Bruckner et al., 2012). Second, external factors, i.e. storage conditions (e.g. storage temperature and availability of oxygen) (Conforti et al., 2006). Third, processing

factors (physical or chemical methods of treating meats during processing, e.g. cooking) (Basak & Candan, 2013). Fourth, implicit factors, which are a reflection of the synergistic or antagonistic effects between bacteria (Bruckner et al., 2012).

Autoxidation of lipids is natural processes which have an effect on fatty acids and lead to oxidative deterioration of meat and off-flavors development (Conforti et al., 2006). After slaughtering of sheep, the fatty acids in tissues undergo oxidation when the blood circulation stops and metabolic processes are blocked (Addis, 2015). Oxidation of lipids in meat depends on several factors including fatty acid composition, the level of the antioxidant vitamin E and pro-oxidants such as the free iron presence in muscles (Conforti et al., 2006). Their breakage produces oxygenated compounds such as aldehydes and ketones. These secondary products can cause loss of color and nutritive value due to severe effects on lipids, pigments, proteins, carbohydrates, and vitamins (Addis, 2015).

Chapter 3

Materials and Methods

Chapter 3

3- Materials and Methods

3.1. Plant Material

Dried leaves of *Laurus nobilis* leaves were collected from the Palestinian market, specifically from the local market of Tulkarem city in March 2018. According to Caputo et al. (2017), the powder was prepared by using a blender and stored in a sterile glass bottle at room temperature.

3.2. Preparation of the Extracts

According to the Mostafa et al. (2016), extracts were prepared with some modifications, 100 g of fine powder was mixed with 500 ml of 99.9 % (v/v) methanol. On the other hand, the same weight was mixed with the same volume of 99.9 % (v/v) ethanol. Both mixtures were kept for 5 days in closed sealed vessels at room temperature, protected from light, and shaken several times daily. The two mixtures were then filtered by a double layer of filter papers to remove the solid extraction residue. In addition, the residues were squeezed to increase the volume of the liquid extract, centrifuged at 5000 rpm for 15 min, and finally filtered again through filter paper to achieve a clear filtrate. Liquid extract was transferred to vacuum rotary evaporator machine (Peaken Motor company, China), where the solvent extract was evaporated at 45°C, concentrated under reduced pressure at 140 rpm. The essential oil was

sterilized by filtration using 0.45 μm Millipore filters (Nehir El et al., 2014). The essential oil, which was obtained from the previous stage was weighted and preserved at 4 °C in a tight bottle until further use (Nehir El et al., 2014).

3.3. Samples Collection

Forty samples of sheep meat were collected at two stages in March 2018 from the local market of Tulkarem, Palestine. The first stage is a screening phase in which infected poisoned sheep meat was characterized by a high concentration of lipid, bad smell, yellowish color, and mucoid layer covered (Thanigaivel & Anandhan, 2015). A total of 25 samples of poisoned sheep meat were collected from four different regions. On the other hand, the second stage is an applying phase, about of 15 fresh, clean, and new slaughter samples were also collected from the same four regions. All of these samples were transferred in clean, dry, and sterile bottles in a refrigerator bag, and transferred to the laboratory for microbiological analysis within one hour or refrigerated at 4°C till further analysis within 24 hours after purchase (Thanigaivel & Anandhan, 2015).

3.4. Samples Processing

The working place at the laboratory was disinfected by using 0.5 % (v/v) sodium hypochlorite and flooded with 70 % ethanol to insure a complete

sterility. The samples were aseptically cut into thin smaller pieces using sterile knife. Meat samples were submerged with sterile normal saline in sterile tubes. The tubes were shaken vigorously by using the vortex several times.

3.5. Isolation of Bacteria

One microliter of each sample suspension was taken by the standard loop and cultivated on three types of culture media that include: blood agar (HiMedia M1133-500G Columbia Blood Agar Base with Hemin), Macconkey agar (HiMedia M081B-100G MacConkey Agar Medium H), and chocolate agar (HiMedia M1133-500G Columbia Blood Agar Base with Hemin). Where after , the cultivated samples were incubated at 37 °C for 24 h under aerobic and anaerobic conditions using candle jars. Characterization and identification of the isolates were achieved by initial morphological examination of the colonies on the plates (macroscopically) for colony appearance, size, elevation, form, edge, consistency, color, odour, opacity, hemolysis and pigmentation, and the results were recorded. A colony from each group of colonies that has the same properties was subcultured on its specific media.

3.6. Biochemical Identification of Bacterial Isolates

Early classification and identification methods for microorganisms relied on the phenotypic properties. Isolates were classified into groups

according to some characteristics, such as their Gram staining properties, motility, nutritional requirements, acid production, carbohydrate fermentation, pigmentation and spore formation (John Wiley & Sons Ltd, 2005).

3.6.1. Analytical Profile Index 20E

The analytical profile index 20E test (API 20E), (Biomerieux, 20 100, France) consists of 20 microtubes containing dehydrated substrates. API 20E provides a fast identification system for a number of Gram-negative Enterobacteriaceae and other non-fastidious Gram-negative rods. According to the manufacture instruction, 5 ml of distilled water was dispensed into the bottom of the tray; to create a moisture condition and prevent reaction evaporation in strips well. The API 20E strip was placed into the bottom of the moisture tray. One colony of each Gram-negative bacteria was suspended in 5 ml of sterile distilled water and directly loaded into API strips by using the sterile pipette. In addition, the anaerobic condition was created to five wells -Dihydrolase (ADH), Lysine Decarboxylase (LDC), Ornithine Decarboylase (ODC), Hydrogen sulphite production (H2S), Urease (URE)- by overlaying with mineral oil. After that, the API 20E was incubated at 37 °C for 24 h. Finally, four types of reagent were added to three wells, one drop of each Tryptophane Deaminase (TDA), Indole production (IND), Voges Proskauer 1 (VP1), and Voges Proskauer 2 (VP2) to TDA, IND, and VP

wells, respectively. For confirmation, one drop from each bacterial suspension was cultured on Macconkey agar for purity point.

3.6.2. Catalase and Coagulase Test

Other chemicals were used to identify the Gram-positive bacteria like hydrogen peroxide (H_2O_2) as catalase reagent, and human plasma for coagulase reaction (Jahan, Rahman, Parvej, Ziqrul, & Chowdhury, 2015). Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into H_2O and O_2, and the bubbling that is seen during the reaction due to the evolution of O_2 gas (Varghese & Joy, 2014). These tests were done by emulsification of one colony of each gram-positive isolate with one drop of catalase reagent and monitoring the vigorous bubbling occurring; to identify if a isolate is a *Streptococcus* or *Staphylococcus* (Reiner, 2016). Furthermore, another colony from each sample was mixed with one drop of human plasma, and the coagulation was monitored to distinguish between types of the samples that are *Staphylococcus aureus* or others (Varghese & Joy, 2014).

3.6.3. Phenotypic Identification of Coagulase-negative *Staphylococci*

Identification of coagulase-negative *Staphylococci* like, *Staphylococcus saprophyticus* was performed by the simplified manner of the biochemical test. The isolates were seeded on the blood agar with 5 % sheep blood; to monitor the presence or absence of hemolysis on the

blood media. Where after, isolates where stained by the Gram stain for the evaluation of purity and observation of their morphology and specific color (Martison, Fávero, Lia, Lourdes, & Souza, 2012). In addition, Novobiocin (5 μg) disc was used to check the resistance or susceptible of coagulase-negative *staphylococcus* bacteria on the Muller Hinton media (Pailhoriès et al., 2017). Resistance was defined as the presence of an inhibition halo ≤ 12 mm or the absence of a halo, and susceptibility was defined as the presence of an inhibition halo > 16 mm (Martison et al., 2012). The identification of *Staphylococcus saprophyticus* was performed based on Novobiocin resistance and absence of hemolysis, and susceptible strains were considered to be *Staphylococcus epidermidis* (Martison et al., 2012).

3.7. Sequencing of the 16SrRNA gene

A most precise method for identification and typing microorganisms is to determine the nucleotide sequence of a defined region of the chromosome (Malhotra, Sharma, Njk, Kumar, & Hans, 2014). The advantage of 16S rRNA gene analysis is that it can be used for the identification of all bacteria (Patel et al., 2000).

3.7.1. Extraction of Deoxyribonucleic Acid (DNA)

To extract DNA from each type of bacteria, three different types of extraction procedure were used:

1. In the first protocol, heat treatment was used as a simple method for extracting DNA from bacterial cells (Dashti, Dashti, & Jadaon, 2014). Two colonies of overnight growth bacteria were used; the colonies were placed in an eppendorf tube filled with 1ml of UltraPure DNase/RNase-Free Distilled Water and boiled for 10 minutes in a water bath, and then centrifuged for five minutes at1000 rpm.
2. In the second protocol, two colonies were dissolved in 500 µl UltraPure DNase/RNase-Free Distilled Water and were placed inside a Solo Microwave (MS23F301TAK, Malaysia) for 10 seconds, followed by centrifugation for two minutes at 1000 rpm (Dashti et al., 2014).
3. In the third protocol, the heat shock procedure of Jose and Brahmadathan (2006) was used by suspending one colony of bacteria in 50 µl of Ultrapure DNase/RNase-Free Distilled Water in a PCR tube, and placed in a PCR machine (Smart Gradient PCR B960) that adjusted to 94°C for 5 minutes, followed by cooling in ice for 3 minutes and centrifuged for 3 minutes at 1000 rpm (Jose & Brahmadathan, 2006).

3.7.2. Polymerase Chain Reaction (PCR) for Sequencing

Polymerase Chain Reaction-Ready™ (PCR-Ready™) High Specificity kit (Syntezza company, PCR-S-192, Israel) was used in this procedure. PCR-Ready™ High Specificity consists of ready-to-use strips of single-use 0.2 ml thin walled PCR reaction tubes, preloaded with a premier quality PCR master mix in a dry stable format. According to the manufacturer instructions, 25 µl-volume of diluted primers (0.5 µM of each primer) and template-DNA were added to the PCR-Ready™ tubes. The reaction mixture was composed of 11 µl of forwarding primer U968-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGA GAA GAA CCT TAC-3), 11 µl of reverse primer L1401 (5'-GCG TGT GTA CAA GAC CC-3'), and 3 µl of DNA were added. A total of 30 PCR cycles were performed in 0.2 ml tubes with the Fast Thermal Cycler by using Applied Biosystems™ Veriti™96-Well machine (cat.4375786, U.S) with the following programme (temperature profiles): 94^{o}C for 3 min followed by 94^{o}C for 30 s, 56^{o}C for 30 s and 68^{o}C for 60 sec for 30 cycles; then the PCR products were terminated at 68^{o}C for 10 min.

3.7.3. Gel Electrophoresis

Polymerase Chain Reaction products were checked by gel electrophoresis, five µl of each PCR products were applied to 2%

agarose mixed with Gel Red TM Nucleic (cat. 41003, US). The gel was run at 100 Volt for 2 hour in 0.5 X Tris-boric acid-EDTA (TBE) buffer (45 mM Tris-base, 89 mM boric acid, 2.5 mM EDTA pH 8.3), DNA molecular marker (1Kb DNA Ladder RTU, Cat. DM010-R500, Gene DireX) was used as a standard for calculation of fragments sizes. The gel was washed for 10 min, examined with a UV transilluminator and photographed (UVITEC serial No.12 102304, France).

3.7.4. Purification of PCR Products

The PCR products were purified by using the Norgen PCR Purification Kit (cat. 14400, Biotek corporation)**,** according to manufacturer's instructions. The final product was stored at -20 °C.

3.7. 5. Sample Sequencing

The PCR purified products were sent to the Molecular Genetics Laboratory in Al-Istishari Arab Hospital in Ramallah for sequencing.

3.7.6. Database Search

A database search was performed using Blast program (National Center for Biotechnology Information, Maryland, USA), and the identified sequences were deposited in the gene bank database. Accession numbers were obtained for all sequences (Appendix 1).

3.8. Antimicrobial Activity of *Laurus nobilis* Leaf Extract

3.8.1 Inoculums Preparation

The antibacterial activity of the essential oil extracted from *Laurus nobilis* leaf was tested against gram-positive and gram-negative bacteria species, which were isolated form infected and normal sheep meat samples. Each bacterial isolate was subcultured on Mueller-Hilton agar media and incubated overnight at 37 °C. The bacterial colonies were harvested using 5 ml of sterile saline, the optical densities of all cultures were adjusted to match a 0.5 McFarland standard of $1x10^8$ colony-forming units (CFU / ml). Bacterial concentration was monitored by measuring turbidity at 610 nm using GP 100 Photometer (Greiner, serial no. 401606033ET, Germany). Each bacterium was spread onto Nutrient agar plates (Balouiri, Sadiki, & Ibnsouda, 2016).

3.8.2. Agar Well Diffusion Test

Agar well diffusion technique is usually used to assess the antimicrobial activity of plants or microbial extracts (Valgas, De Souza, Smânia, & Smânia, 2007). According to Valgas et al. (2007), the bacterial inoculum was regularly spread using a sterile cotton swab on the Nutrient agar surface in the sterile Petri dish. Then, a hole with a diameter of 6 mm was punctured aseptically with a sterile Yellow tip. Seven serial dilutions of *Laurus nobilis* aqueous extracts concentrations of 20, 15, 10, 7.5, 5, 2.5, 1 % (v/v) were prepared by diluting the essential oil extract with 40

% (v/v) ethanol. Ciprofloxacin disc (Cip) used as a positive control, and using 40 % (v/v) ethanol as negative control (Ghadiri et al., 2014). After that, 100 µL of *Laurus nobilis* aqueous extract from each concentration was added to wells. Agar plates were incubated at 37 °C for 24 h. The antimicrobial activity of the different concentrations of *Laurus nobilis* extract was examined by measuring the zone of inhibition using Vernier Calliper. An antimicrobial test was repeated for three trials.

3.9. The Antimicrobials and Antioxidants Activity of *Laurus nobilis* Extract against Lipids Food Poisoning

Fifty grams of fresh sheep meat samples were divided into four parts equally, and placed in four sterile boxes. As a baseline step, one piece of fresh meat sample was taken as a control to investigate in the lab , and take all observation to build on it and compare it with the other stages. All four parts were divided into small pieces of about 10 g.

Treatment 1: the first two parts of fresh sheep meat samples were incubated at room temperature (15-30 °C), and one of them was sprayed with 10 % of *Laurus nobilis* aqueous extract.

Treatment 2: the second two parts were incubated in refrigerator (2-8 °C), also one of them was sprayed with 10 % of *Laurus nobilis* aqueous extract (Lucera et al., 2012).

According to Jamilah et al. (2008) procedure, culture, color, smell, pH, and any physical changes were observed and registered. One piece of fresh meat was investigated every day, and the differences between the results of the baseline sample and the daily sample were measured.

Chapter 4

Results

Chapter 4

4- Results

4.1. Plant material and Extracts of *Laurus nobilis*

The extract of 100 g of dried plant materials with methanol yielded essential oil extract residues ranged from 6.0 to 6.8 g. On the other hand, dried plant materials extracted with ethanol acquiesced ranged from 3.4 to 4.5 g. It was found that the time needed in the Vacuum Rotary Evaporator for the complete extraction of essential oil of *Laurus nobilis* with methanol mode was 25 ± 2 min, while 50 ± 5 min for ethanol extraction were needed. The yield of the essential oil of *Laurus nobilis* was green blackish color.

4.2. Biochemical Identification of Bacterial Isolates

The isolation of bacteria from collected samples produced many types of different morphological isolates (Figure 2). The first stage of a screening phase which was represented by collecting poisoned sheep meat samples resulted in 27 different isolates. In general, some of these isolates were found in all meat samples, which included *Staphylococcus aureus spp 4, Staphylococcus saprophyticus spp 3, Staphylococcaceae, coagulase -ve spp 3, Klebsiella pneumoniae ssp ozaenae spp 1, Cedecea lapagei spp 4, Enterobacter gergoviae spp 1, Enterobacter cancerogenus spp 2,*

Figure 2. Different bacterial isolates, which were found in poisoned sheep meats. (1) *Klebsiella pneumoniae.* (2) *Cedecea lapagei.* (3) *Staphylococcus aureus.* (4) *Proteus vulgaris.* (5) *Staphylococcus saprophyticus.* (6) *Staphylococcus haemolyticus*.

Escherichia fergusonii spp 1, Proteus vulgaris spp1, Klebsiella oxytoca1 spp 1, Enterobacter cloacae spp 2, Hafnia alvei spp 1, Salmonella choleraesuis spp1, and *2* unknown isolates. On the other hand, in the fresh sheep meat samples, only 7 different isolates were found, which included *Proteus vulgaris spp1, Klebsiella oxytoca spp1, Cedecea lapagei spp1, Enterobacter cancerogenus spp1, Klebsiella pneumoniae spp1, Staphylococcaceae, coagulase -ve spp1,* and *Pseudomonas fluorescens/putida spp1*. Bacterial isolates were identified according to the biochemical method (Table 1). Most of the gram-negative bacteria were identified by API 20E reaction strips. Depending on the numbers (7- digital Code) that were got from the changing in color of API 20E reaction strips, and translated these numbers in API 20E catalog book or API web (Table 1). Furthermore, API 20E catalog book detected the percentage identity for agreement between API and corresponding test results.

Four isolates of *Staphylococcus* were identified only at the genus level. Two types of gram-negative isolates in the first stage were not detected in the biochemical method, produced a little information were illustrated in Table 1.

Table 1. Identification of bacterial isolates by biochemical method from two stages of meat samples collection. First stage: bacteria isolated from poisoned sheep meat samples. Second stage: bacteria isolated from fresh sheep meat samples. Novo: Novobiocin disc; S: Sensitive; R: Resistance. Ident%: the percentage identity for agreement between API and corresponding test results. +ve: Positive result. –ve: Negative result.

Biochemical Method			
First Stage			
Isolates No.	Digital Code/ Reaction Result	Ident %	Identification
1	catalase +ve, coagulase+ve	99.9	*Staphylococcus aureus 1*
2	catalase +ve, coagulase+ve	99.9	*Staphylococcus aureus 2*
3	catalase +ve, coagulase-ve, Novo R	99	*Staphylococcus saprophyticus 1*
4	catalase +ve, coagulase-ve, Novo S	99	*Staphylococcaceae, coagulase -ve*
5	catalase +ve, coagulase-ve, Novo S	99	*Staphylococcaceae, coagulase -ve*
6	catalase +ve, coagulase+ve	99.9	*Staphylococcus aureus 3*
7	3204543	93.2	*Klebsiella pneumoniae ssp ozaenae*
8	catalase +ve, coagulase-ve, Novo R	99	*Staphylococcus saprophyticus 2*
9	catalase +ve, coagulase-ve, Novo R	99	*Staphylococcus saprophyticus 3*
10	5314173	99.9	*Enterobacter gergoviae*
11	3205101	99.9	*Cedecea lapagei 1*
12	3305113	99.9	*Enterobacter cancerogenus 1*
13	catalase +ve, coagulase+ve	99.9	*Staphylococcus aureus 4*
14	catalase +ve, coagulase-ve, Novo S	99	*Staphylococcaceae, coagulase -ve*
15	5144113	98.9	*Escherichia fergusonii*
16	2204101	99.7	*Cedecea lapagei 2*
17	0476021	98.9	*Proteus vulgaris 1*
18	5255573	97.4	*Klebsiella oxytoca1*
19	gram-negative, non-lactose fermenter		*Unknown*
20	3305572	95.2	*Enterobacter cloacae 1*
21	4504450	99.5	*Salmonella choleraesuis*
22	3305473	97.5	*Enterobacter cloacae 2*
23	3205103	98	*Cedecea lapagei 3*
24	3205001	98.7	*Cedecea lapagei 4*
25	5314112	99.9	*Hafnia alvei 1*
26	1305113	99.5	*Enterobacter cancerogenus 2*
27	gram-negative, non-lactose fermenter		*Unknown*
Second Stage			
28	1254773	97.9	*Klebsiella oxytoca2*
29	2201044	98.9	*Pseudomonas fluorescens/putida*
30	catalase +ve, coagulase-ve, Novo S	99	*Staphylococcaceae, coagulase -ve*
31	0474021	99.9	*Proteus vulgaris 2*
32	3005103	99.9	*Cedecea lapagei 5*
33	1305113	99.5	*Enterobacter cancerogenus3*
34	5215773	97.7	*Klebsiella pneumoniae*

4.3. Number of Colony Forming Unit (CFU/ml) of Bacterial Isolates

Of the 40 samples of sheep meat, 34 different bacterial isolates were isolated and identified. Some of these isolates were repeated in some meat samples. Bacterial counts were in a range $2x10^3$ - $1.5x10^5$ with increasing numbers in the poisoned samples. *Staphylococcus aureus* numbers were the highest in poisoned sheep meat samples (Table 2).

Table 2. The number of colony forming unit (CFU/ml) for bacterial isolates collected from two stages of meat samples. First stage: from poisoned meat samples. Second stage: fresh meat samples. Number of samples: the number of sheep meat samples that contain the bacterial isolates.

The number of colony forming unit (CFU / ml)		
Bacterial isolates	**Number of samples**	**Average number of CFU/ml**
First stage		
Staphylococcus aureus	4	1.5×10^5
Staphylococcus saprophyticus	3	2.5×10^4
Staphylococcaceae, coagulase -ve	3	4×10^4
Klebsiella pneumoniae ssp ozaenae	1	1×10^5
Enterobacter gergoviae1	1	2.1×10^4
Cedecea lapagei 1	4	5×10^4
Enterobacter cancerogenus	2	1.5×10^4
Escherichia fergusonii	1	6.5×10^4
Proteus vulgaris	1	3×10^4
Klebsiella oxytoca	1	7×10^4
Unknown	1	1×10^4
Enterobacter cloacae	2	3×10^3
Salmonella choleraesuis	1	1.5×10^4
Hafnia alvei 1	1	3×10^3
Unknown	1	3×10^4
Second Stage		
Klebsiella oxytoca	1	5×10^3
Pseudomonas fluorescens/putida	1	3×10^3
Staphylococcaceae, coagulase -ve	1	8×10^3
Proteus vulgaris	1	5×10^3
Cedecea lapagei	1	1×10^4
Enterobacter cancerogenus	1	2×10^3
Klebsiella pneumoniae	1	3×10^3

4.4. Sequencing of 16SrRNA gene

Three extraction protocols of DNA were applied on all 34 isolates. DNA of gram-positive bacteria were obtained using the three extraction protocols successfully, whereas some gram-negative bacterial DNAs were obtained by some protocols and failed in the other (Table 3). The third protocol covered most of the isolates successfully, except 4 isolates of *Cedecea lapagei* and one isolate of *Klebsiella pneumoniae*. Figure 3 shows the distribution of bacterial isolates through all protocols. Using heat shock at PCR machine was possible to extract DNA of 29 isolates. DNAs from 17 isolates were obtained by Microwave irradiation. However, using boiling method, only DNAs from 11 isolates were obtained.

Table 3. Bacterial distribution within the three extraction protocols for 34 bacterial types. Protocol 1: boiling method. Protocol 2: microwave irradiation. Protocol 3: heating shock extraction. (-): bacterial isolate failed in this protocol.

Sample No.	Protocol 1	Protocol 2	Protocol 3
1	*Staphylococcus aureus1*	*Staphylococcus aureus 1*	*Staphylococcus aureus 1*
2	*Staphylococcus aureus 2*	*Staphylococcus aureus 2*	*Staphylococcus aureus 2*
3	*Staphylococcus saprophyticus 1*	*Staphylococcus saprophyticus 1*	*Staphylococcus saprophyticus 1*
4	*Staphylococcaceae, coagulase -ve*	*Staphylococcaceae, coagulase -ve*	*Staphylococcaceae, coagulase -ve*
5	*Staphylococcaceae, coagulase -ve*	*Staphylococcaceae, coagulase -ve*	*Staphylococcaceae, coagulase -ve*
6	*Staphylococcus aureus 3*	*Staphylococcus aureus3*	*Staphylococcus aureus3*
7	-	-	*Klebsiella pneumoniae ssp ozaenae*
8	*Staphylococcus saprophyticus2*	*Staphylococcus saprophyticus2*	*Staphylococcus saprophyticus2*
9	*Staphylococcus saprophyticus3*	*Staphylococcus saprophyticus3*	*Staphylococcus saprophyticus3*
10	-		*Enterobacter gergoviae*
11	-	*Cedecea lapagei1*	-
12	-	-	*Enterobacter cancerogenus1*
13	*Staphylococcus aureus4*	*Staphylococcus aureus4*	*Staphylococcus aureus4*
14	*Staphylococcaceae, coagulase -ve*	*Staphylococcaceae, coagulase -ve*	*Staphylococcaceae, coagulase -ve*
15	-	-	*Escherichia fergusonii*
16	-	*Cedecea lapagei 2*	-
17	-	-	*Proteus vulgaris 1*
18	-	-	*Klebsiella oxytoca 1*
19	-	-	*Unknown(gram-negative, non-lactose fermenter)*
20	-	-	*Enterobacter cloacae1*
21	-	-	*Salmonella choleraesuis*
22	-	-	*Enterobacter cloacae2*
23	-	*Cedecea lapagei 3*	-
24	-	*Cedecea lapagei 4*	-
25	-	-	*Hafnia alvei 1*
26	-	-	*Enterobacter cancerogenus 2*
27	-	-	*Unknown (gram-negative, non-lactose fermenter)*
28	-	-	*Klebsiella oxytoca 2*
29	-	-	*Pseudomonas fluorescens/putida*
30	*Staphylococcaceae, coagulase -ve*	*Staphylococcaceae, coagulase -ve*	*Staphylococcaceae, coagulase -ve*
31	-	-	*Proteus vulgaris 2*
32	-	*Cedecea lapagei 5*	*Cedecea lapagei 5*
33	-	-	*Enterobacter cancerogenus 3*
34	-	*Klebsiella pneumoniae*	-

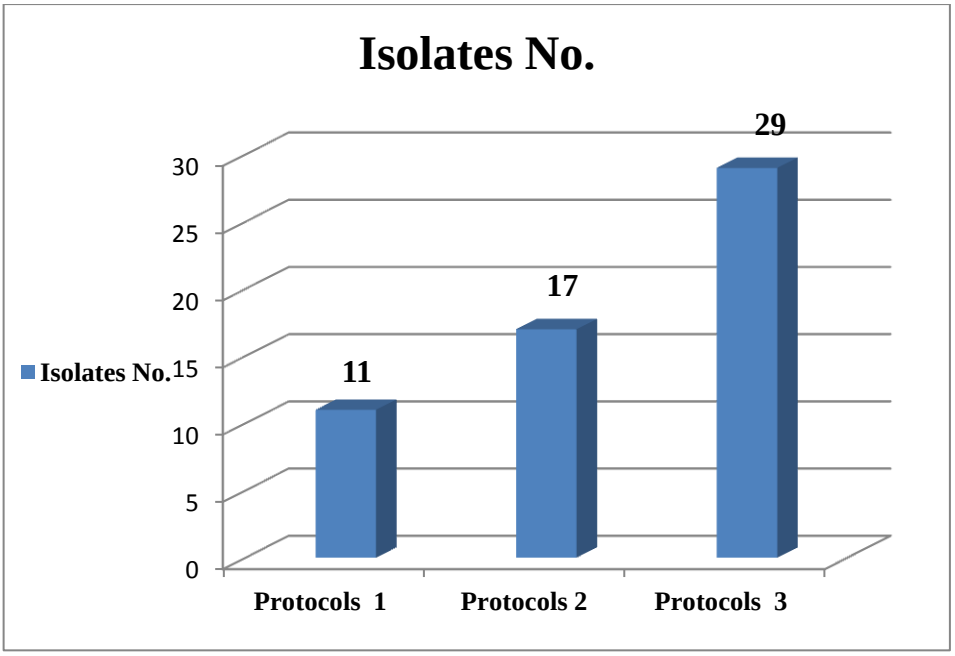

Figure 3. The distribution of bacterial isolates through all protocols. Protocol 1: boiling method. Protocol 2: microwave irradiation. Protocol 3: heating shock extraction.

PCR amplicons were produced successfully in all DNA samples integrated into this study. The amplified products were obtained using primers specific for the 16SrRNA gene (U968 and L1401 primers), which produced about 450 bp, which were the expected product size of the amplified gene with the set of primers used. Figure 4 shows a photograph of agarose gel electrophoresis of PCR amplicons.

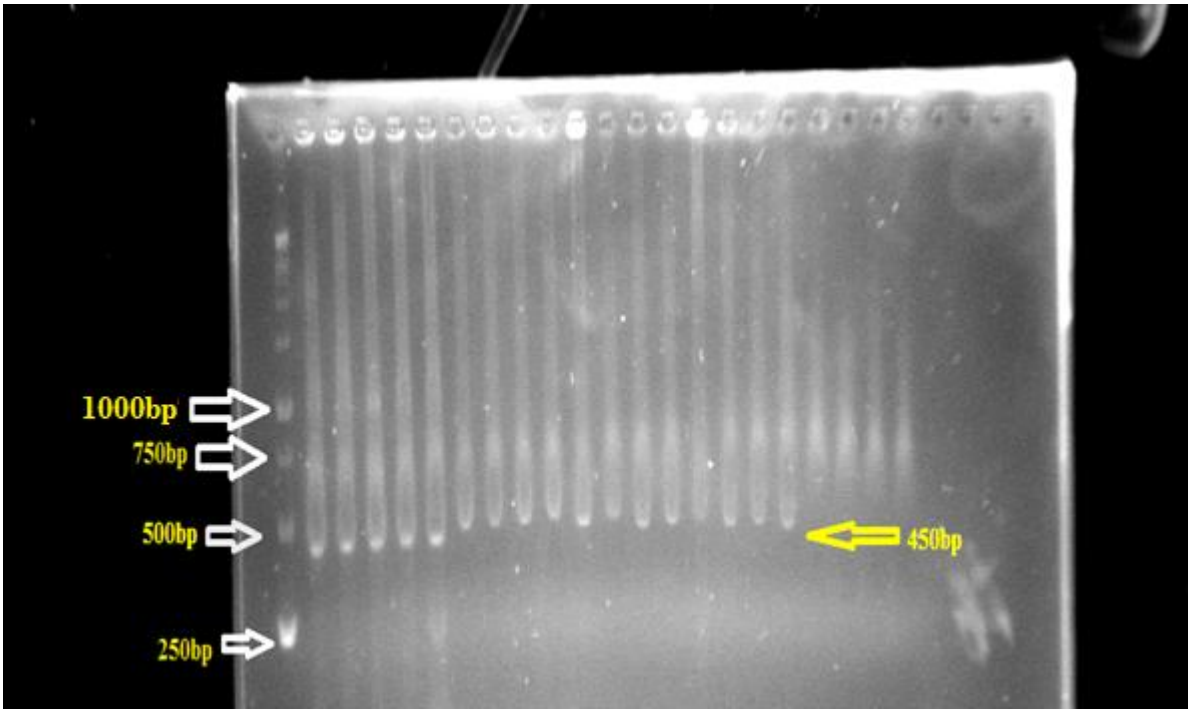

Figure 4. Agarose gel electrophoresis showing the 450-bp PCR amplicons for segment of 16SrRNA gene in samples.

After purification and sequencing steps, all 34 amplicons sequences result were received from Molecular Genetics Laboratory in Al-Istishari Arab Hospital. All 34 sequences obtained were deposited in the Gene bank database where they were identified using Blast algorithm. An accession numbers for all samples were illustrated in Table 4. All 34 sequences are illustrated in the appendices part (1).

Table 4. The sequences results for bacterial isolates. Identical %: the percentage identity for agreement between Blast program and corresponding test results. Accession.

Sequencing of 16SrRNA gene			
Isolates No.	Sequences Result	Identical %	Accession Number
1	*Staphylococcus aureus*	99.43	MK695866
2	*Staphylococcus aureus*	98.36	MK695940
3	*Staphylococcus saprophyticus*	99.41	MK695941
4	*Staphylococcus edaphicus*	100	MK695942
5	*Staphylococcus haemolyticus*	99.71	MK713339
6	*Staphylococcus aureus*	98.93	MK713337
7	*Enterobacter cancerogenus*	99.74	MK713336
8	*Staphylococcus saprophyticus*	100	MK713332
9	*Staphylococcus saprophyticus*	97.05	MK696049
10	*Pluralibacter gergoviae*	98.1	MK696050
11	*Cedecea lapagei*	99.74	MK696051
12	*Enterobacter cancerogenus*	99.72	MK713323
13	*Staphylococcus aureus*	99.51	MK713325
14	*Staphylococcus edaphicus*	99.75	MK713324
15	*Enterobacter tabaci*	98.04	MK713331
16	*Enterobacter xiangfangensis*	99.68	MK713330
17	*Proteus vulgaris*	100	MK713329
18	*Enterobacter cancerogenus*	99.74	MK713335
19	*Acinetobacter lwoffii*	99.75	MK689408
20	*Enterobacter hormaechei*	99.76	MK690048
21	*Salmonella enterica/choleraesuis*	98.81	MK690186
22	*Enterobacter hormaechei*	99.76	MK689181
23	*Cedecea lapagei*	99.22	MK689855
24	*Cedecea lapagei*	98.84	MK713334
25	*Hafnia paralvei*	99.76	MK684353
26	*Enterobacter cancerogenus*	99.48	MK695980
27	*Acinetobacter lwoffii*	99.5	MK713321
28	*Enterobacter hormaechei*	99.75	MK704397
29	*Pseudomonas helmanticensis*	99.75	MK695699
30	*Macrococcus epidermidis*	99.75	MK690643
31	*Proteus vulgaris*	98.69	MK685208
32	*Cedecea lapagei*	98.93	MK713322
33	*Pluralibacter gergoviae*	99.72	MK684347
34	*Klebsiella pneumoniae*	99.4	MK684237

Many information were provided by molecular sequencing, which could not be obtained by biochemical phenotyping. Two unknown samples (19 and 27) were identified by sequencing only as *Acinetobacter lwoffii* for both samples.

Gram-positive samples (4, 5, 14, and 30) which was only identified at the genus level as *Staphylococcaceae* coagulase –ve by biochemical method was identified by 16S rRNA gene sequencing as *Staphylococcus edaphicus*, *Staphylococcus haemolyticus*, *Staphylococcus edaphicus*, *Macrococcus epidermidis*, respectively. Samples 20 and 22 were identified by the biochemical method as *Enterobacter cloacae*, but using sequencing method, it was identified as *Enterobacter hormaechei*..

4.5. Difference between biochemical identification and sequencing 16S rRNA gene results of bacterial isolates

The comparison process between biochemical identification and Sequencing of 16S rRNA gene shows differences in 6 samples out of 34 isolates. Biochemical identification for samples 7, 15, 16, 28, 29, and 33 shows *Klebsiella pneumoniae ssp ozaenae*, *Escherichia fergusonii*, *Cedecea lapagei*, *Klebsiella oxytoca*, *Pseudomonas fluorescens/putida*, and *Enterobacter cancerogenus*, respectively. On the other hand, sequencing of 16S rRNA gene for the same samples shows *Enterobacter cancerogenus*, *Enterobacter tabaci*, *Enterobacter xiangfangensis*, *Enterobacter hormaechei*, *Pseudomonas helmanticensis*, and *Pluralibacter gergoviae*, respectively (Table 5).

Table 5. Difference between biochemical identification and sequencing 16S rRNA gene results of bacterial isolates.

Sample No.	Biochemical identification	Ident %	Sequencing 16SrRNA	Ident%
7	*Klebsiella pneumoniae ssp ozaenae*	93.2	*Enterobacter cancerogenus*	99.74
15	*Escherichia fergusonii*	98.9	*Enterobacter tabaci*	98.04
16	*Cedecea lapagei*	99.7	*Enterobacter xiangfangensis*	99.68
28	*Klebsiella oxytoca*	97.9	*Enterobacter hormaechei*	99.75
29	*Pseudomonas fluorescens/putida*	98.9	*Pseudomonas helmanticensis*	99.75
33	*Enterobacter cancerogenus*	99.5	*Pluralibacter gergoviae*	99.72

4.6. Antimicrobial Activity of *Laurus nobilis* Leaf Extract

The antimicrobial activity of *Laurus nobilis* leaves extract were applied to all the 34 bacterial isolates, which were isolated from two stages of meat samplings (poisoned and fresh samples). The results of the activity, namely the inhibition of the diameters calculated as mm. Ciprofloxacin disc (Cip) used as a positive control, and 40 % (v/v) ethanol was used as a negative control. The results of the current research showed that hydroalcoholic solution of *Laurus nobilis* extract has antibacterial activity against 16 isolates out of 34 isolates. It was reported that essential oils of *Laurus nobilis* have showed a greater antimicrobial activity against gram-positive bacteria than gram-negative (Figure 5, 6, and 7), in terms of bacteria numbers that have a sensitivity to essential oil and effectiveness which measured by zone inhibition (Mostafa et al., 2016).

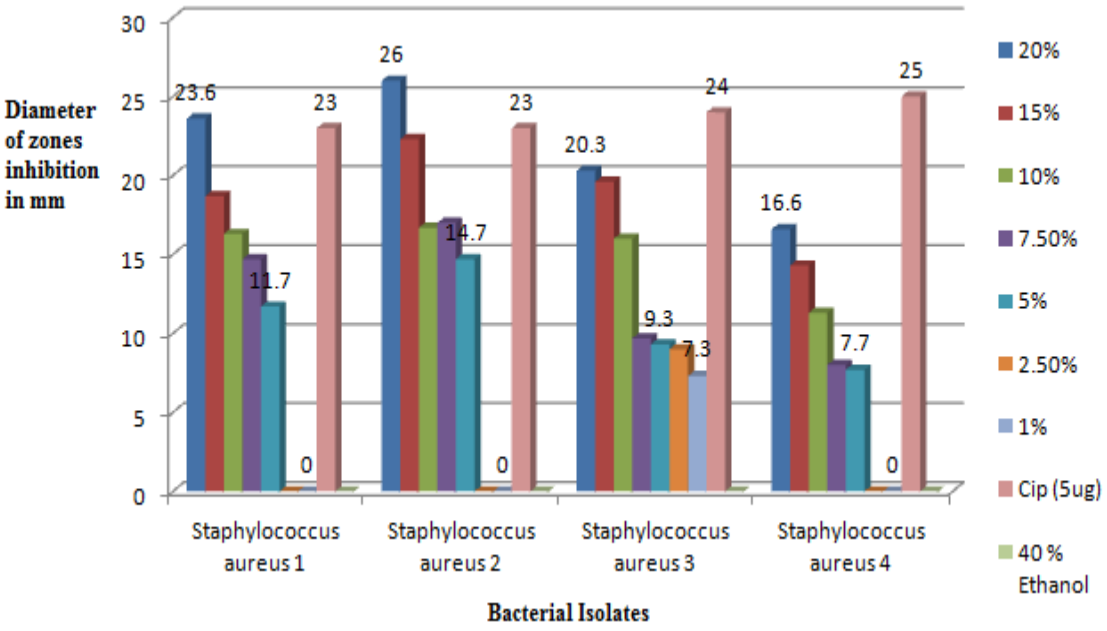

Figure 5. Antimicrobial activity of *Laurus nobilis* essential oil against four isolates of *Staphylococcus aureus*. 7 different colour represent the different concentrations of essential oil of *Laurus nobilis*. Ciprofloxacin is a positive control (Cip 5ug). 40% Ethanol: is a negative control. The highest of columns represent the diameter of zones inhibition in mm.

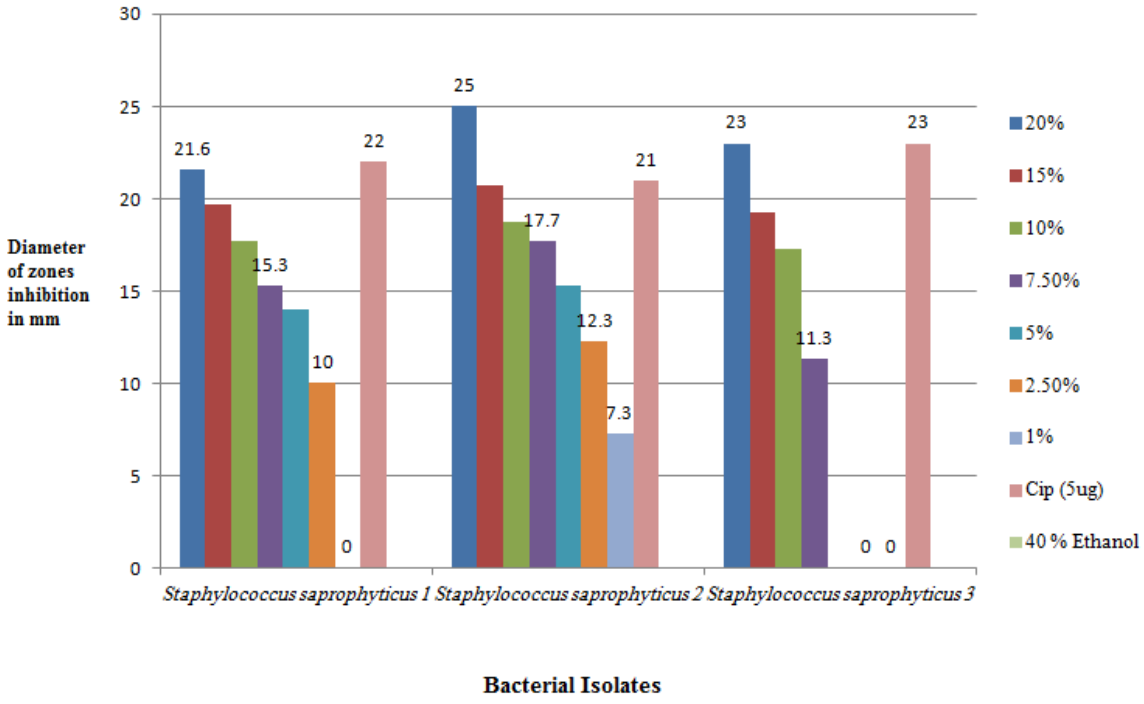

Figure 6. Antimicrobial activity of *Laurus nobilis* essential oil against three isolates of *Staphylococcus saprophyticus*. 7 different colour represent the different concentrations of essential oil of *Laurus nobilis*. Ciprofloxacin is a positive control (Cip 5ug). 40% Ethanol: is a negative control. The highest of columns represent the diameter of zones inhibition in mm.

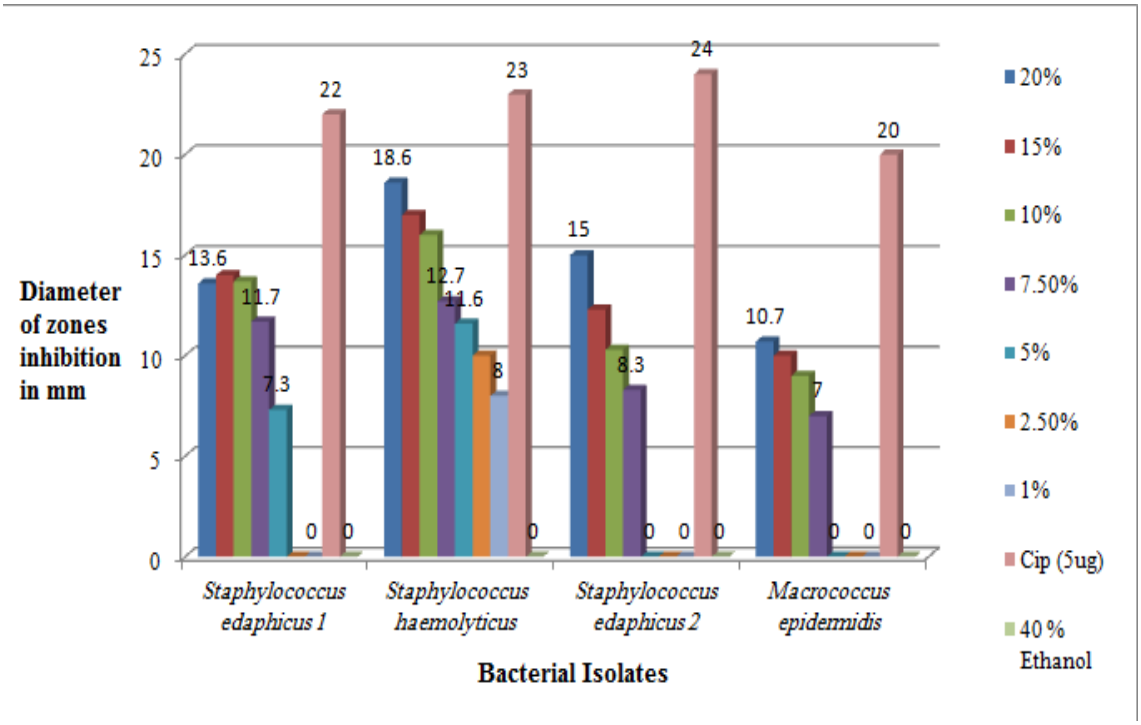

Figure 7. Antimicrobial activity of *Laurus nobilis* essential oil against two isolates of *Staphylococcus edaphicus*, *Staphylococcus haemolyticus*, and *Macrococcus epidermidis*. 7 different colour represent the different concentrations of essential oil of *Laurus nobilis*. Ciprofloxacin is a positive control (Cip 5ug). 40% Ethanol: is a negative control. The highest of columns represent the diameter of zones inhibition in mm.

In addition, some gram-negative types were affected by acting of the aqueous extract like, *Proteus vulgaris* 1, *Proteus vulgaris* 2, *Salmonella enterica/choleraesuis*, *Hafnia paralvei*, *Pseudomonas helmanticensis* (Figure 8). The antimicrobial effect is illustrated in Figure 9, and others are shown in appendices part (2).

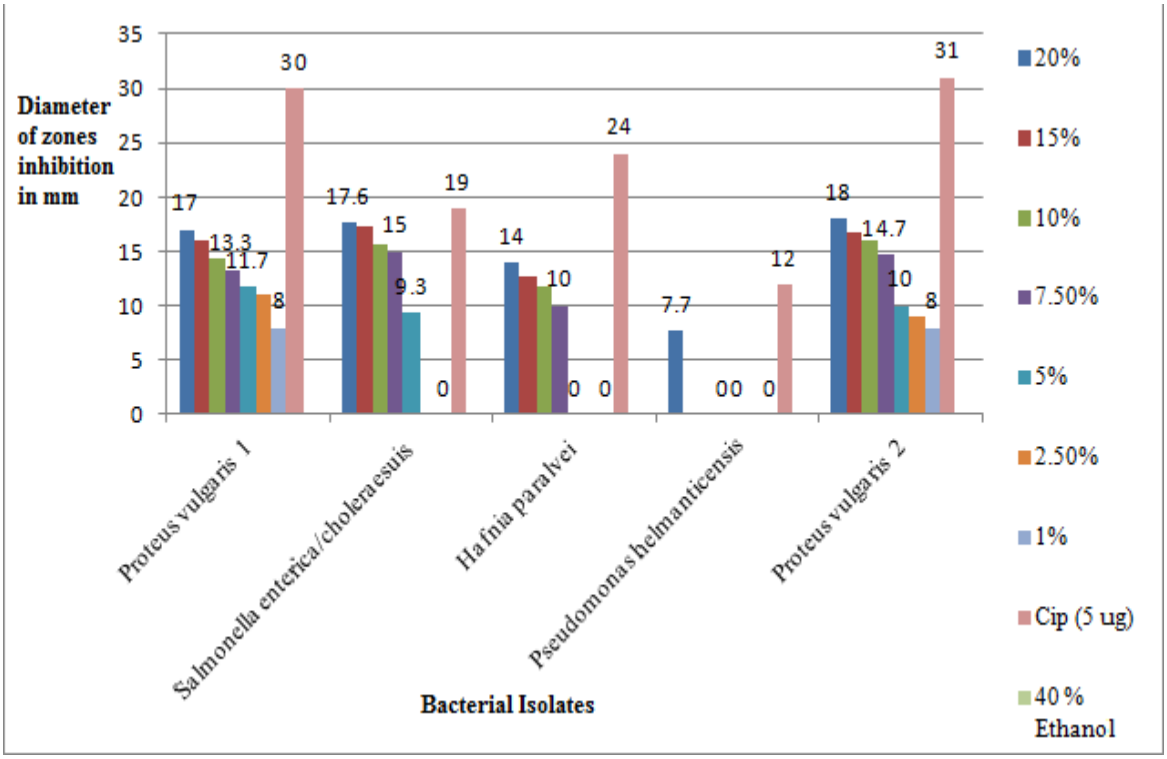

Figure 8. Antimicrobial activity of *Laurus nobilis* essential oil against two isolates of *Proteus vulgaris*, *Salmonella enterica/choleraesuis*, *Hafnia paralvei*, and *Pseudomonas helmanticensis*. 7 different colour represent the different concentrations of essential oil of *Laurus nobilis*. Ciprofloxacin is a positive control (Cip 5ug). 40% Ethanol: is a negative control. The highest of columns represent the diameter of zones inhibition in mm.

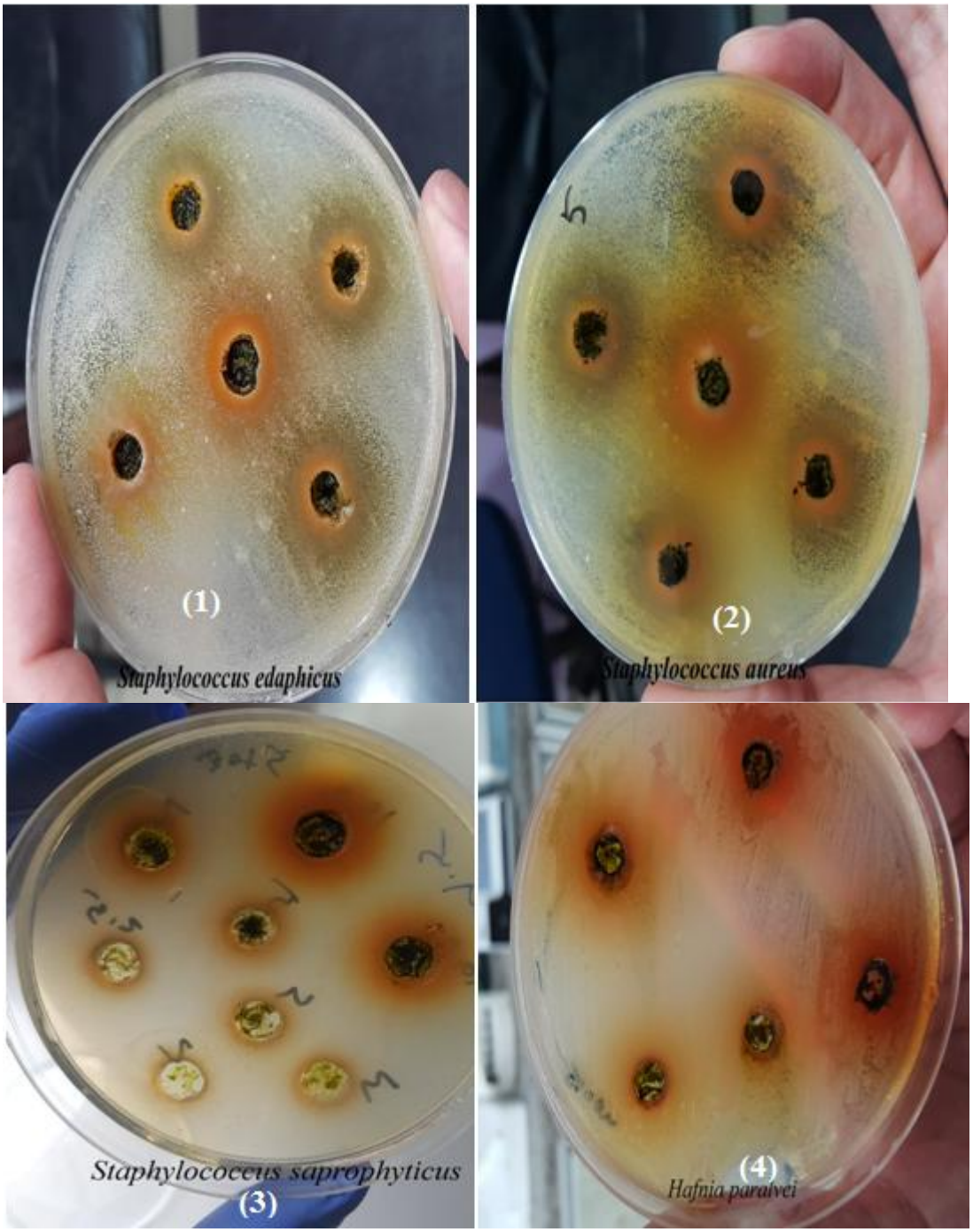

Figure 9. Represent the antimicrobial activity of *Laurus nobilis* essential oil against (1) *Staphylococcus edaphicus.* (2) *Staphylococcus aureus*. (3) *Staphylococcus saprophyticus*. (4) *Hafnia paralvei*. The antimicrobial activity represented by the zone of inhibition around the wells, which means inhibition of the growth of bacterial isolates near the aqueous extract wells.

4.7. The activity of *Laurus nobilis* extract against Lipids Food Poisoning

Spraying of 10% *Laurus nobilis* essential oil showed a significant change on microbiological and physicochemical shelf-life of sheep meat. The results in this section were divided into four categories of

application fields; to detect the activity of *Laurus nobilis* leaves aqueous extract in increasing the shelf-life of sheep meat with suitable and improper condition. Spoilage of sheep meat leads to deterioration of texture and change in flavor, pH, smell, and color. The normal sample which was sent to the lab directly was used as a control. Normal red sheep lean color, pH 5.7, normal smell, no any physical changes, and the culture results illustrated in Table 6. were recorded from the control sample.

Table 6. Bacterial culture from normal fresh samples .

Bacterial Isolates	CFU/ml
Klebsiella oxytoca	$5x10^3$
Pseudomonas fluorescens/putida	$3x10^3$
Macrococcus epidermidis	$8x10^3$
Proteus vulgaris	$5x10^3$
Cedecea lapagei	$1x10^4$
Enterobacter cancerogenus	$2x10^3$
Klebsiella pneumoniae	$3x10^3$

The first category (Room temperature at 15-30 °C without any addition of extract): showed many changes after 24 h. Yellowish color, pH = 5.1, bad smell (mild), mucoid layer on samples, and increasing in the growth of bacteria which already exist (Table 7). Poisoning of samples became clear after 48 h; yellow color with green dots have appeared, pH = 5.0, very bad smell (strong), a large amount of mucus and gas bubbles were noticed on the samples.

The second category (Room temperature at 15-30 °C with the spraying of extract): represented by spraying the sheep meat samples with 10 % of *Laurus nobilis* essential oil for one time only. This action provided samples with the laurel smell and greenish spices color without any physical characteristic changes. The first physical changes were noticed in this stage after 72h, started by small white dots, mixed of bad and laurel smells, and appearing of bacterial growth in this time (Table 7).

The third category (Refrigerator at 2-8° C without addition of extract): showed significant changes after the sixth day, which represented by appearing small bubbles of gas, thin mucoid layer on the samples surface, pH 5.1, small white dots, and slightly elevated in bacterial colony growth (Table 7).

The fourth category (Refrigerator at 2-8°C with the spraying of 10 % of *Laurus nobilis* essential oil extract for one time only): showed no changes in meat characteristics in the first 13 days of treatment. After 13 days, the first poisoning mark was noticed, started by disappearing of laurel smell and very little white dots on the surface of samples.

Table 7. Bacterial culture for meat samples at the poisoning point. The bacterial culture results of meat samples for four categories at the poisoning point, which means the day that registered an increasing in colony forming unit. This day was 1, 3, 6, and 13 for the first, second, third, and fourth category; respectively. Before that day, the culture results were similar to the fresh sample. Fresh sample: control (normal) sample.

Bacterial Isolates	Count of CFU/ml in the four categories at poisoning point				
	Fresh sample	First category	Second category	Third category	Fourth category
Klebsiella oxytoca	$5x10^3$	$11x10^3$	$7x10^3$	$6x10^3$	$6x10^3$
Pseudomonas fluorescens/putida	$3x10^3$	$5x10^3$	$3x10^3$	$4x10^3$	$2x10^3$
Macrococcus epidermidis	$8x10^3$	$1.5x10^4$	0	$1x10^4$	0
Proteus vulgaris	$5x10^3$	$8x10^3$	0	$7x10^3$	0
Cedecea lapagei	$1x10^4$	$1.5x10^4$	$1.1x10^4$	$1x10^4$	$1.1x10^4$
Enterobacter cancerogenus	$2x10^3$	$4x10^3$	$3x10^3$	$3x10^3$	$2x10^3$
Klebsiella pneumoniae	$3x10^3$	$8x10^3$	$4x10^3$	$5x10^3$	$5x10^3$

Proteus vulgaris and *Macrococcus epidermidis* have disappeared in the second and fourth categories, and deceleration the growth of others for a period of time (Poisoning point). The comparison process between the four categories showed that shelf life was longest in category four (Figure 9).

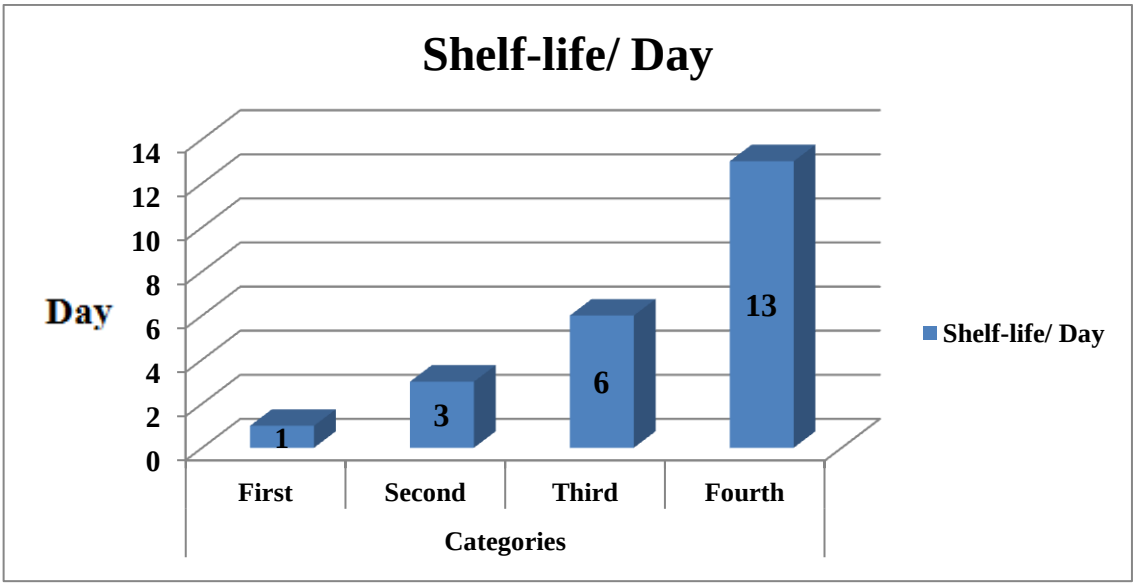

Figure 10. The shelf life for the four categories per day. First, meat samples were preserved at room temperature at 15-30°C without any additions. Second, meat samples were preserved at room temperature at 15-30°C with addition of sprayed extract. Third, meat samples were preserved at refrigerator at 2-8°C without any additions. Fourth, meat samples were preserved at refrigerator at 2-8°C with addition of sprayed extract.

Chapter 5

Discussion and Conclusions

Chapter 5

5- Discussion and Conclusion

5.1. Discussion

Food poisoning is considered as one of the universal reasons for sickness and death in developing countries and represents a major problem in industrialized countries (Mostafa et al., 2016). This study demonstrated that *laurel* essential oil can be used as a natural preservative, for the prevention of lipped foods poisoning. Prevention of food spoilage and food poisoning pathogens is usually achieved by the use of chemical preservatives which have negative effects on human health. The antimicrobial activity of the *Laurus nobilis* essential oils has taken great importance as an alternative for synthetic antimicrobials because they are a part of the human diet and their biodegradability suggest low poisonous residue problems. An aqueous extract of *Laurus nobilis* leaf and chitosan was used as a natural edible coating to increase the shelf life of cashew (Azimzadeh & Jahadi, 2018). This natural edible coating successfully delayed lipid oxidation in comparison with the uncoated cashew (Azimzadeh & Jahadi, 2018). To our knowledge, no studies have been conducted to examine the antimicrobial activity of *Laurus nobilis* leaf against bacteria present in fresh sheep meat causing lipid poisoning, or to evaluate the antimicrobial effects of *Laurus nobilis* leaf extract as a fresh meat preservative without any additions**.** The results of this

research showed that Laurel extract has the potential to be used as natural alternative preventive to control food poisoning diseases and preserve foodstuff avoiding health hazards of chemically antimicrobial agent applications.

In the present study, the comparison between methanol and ethanol extraction method showed that the methanol yield amount of essential oil, and the time taken in producing the extract was shorter than ethanol extraction; which indicate that methanol is an ideal material for oil extraction. Methanol has been reported to be a high-quality solvent for extraction and it was used in biology because of its polarity, and low molecular weight (Karimi & Moradi, 2015).

There was an agreement between biochemical and molecular methods in identification of bacteria isolated from meat samples. The major advantage of the API 20E system is that it is more convenient, rapid, and easier to identify gram-negative bacteria than the conventional tests (Juang & Morgan, 2001). In addition, the gram-positive bacteria were identified by the phenotypic reactions of catalase reaction, coagulase reaction, and Novobiocin disc test. Phenotypic information provided many facilities in identification of gram positive bacteria (Martison et al., 2012). A most precise method for identification and typing of microorganisms is to determine the nucleotide sequence of a defined

region of the chromosome (Malhotra et al., 2014). The RNA genes are necessary for the continued existence of all microorganisms and highly conserved in the bacterial kingdom (Yoon et al., 2017). The highly conserved structure and sequence of the rRNA genes facilitate the use of PCR amplification and sequencing of those genes (Cody, Bennett, & Maiden, 2014). The advantage of 16S rRNA gene analysis is that it can be used for the identification of all bacteria (Patel et al., 2000). Phenotypic identification of all species belonging to enzymes activity, or other proteins production is usually difficult and not always reliable; therefore, molecular methods are often used (Mezzatesta et al., 2012). In this point, a good proof to depend on the result of sequencing 16S rRNA; due to increase the accuracy of the identity percentage. Biochemical identification test could be used as a trial test, but the molecular methods are more accurate and should be used as confirmatory tests for hard-to-identify isolates (Moraes, Perin, Júnior, & Nero, 2013). According to Moraes et al. (2013), twenty-nine lactic acid bacteria (LAB) isolates were submitted for identification using Biolog, API50CHL, 16S rDNA sequencing, and species-specific PCR reactions. The different methods of evaluation provided different patterns of genera and species identification for the LAB isolates; the identification results were compared, and it was concluded that the molecular analyzes were the most reliable (Moraes et al., 2013). All differences results of bacterial

isolates were from the same family, which is Enterobacteriaceae. Characteristics of this family include being motile, catalase positive, oxidase negative, reduction of nitrate to nitrite; and acid production from glucose fermentation (Janda & Abbott, 2015). Enterobacteriaceae family contains a large number of genera that are biochemically and genetically related to one another; for this reason, many additional morphological, biochemical, and physiological tests are always required (Juang & Morgan, 2001). According to Juang and Morgan. (2001) study, API identification systems mostly can identify the gram-negative microorganisms in activated sludge only at the genus level, many additional morphological, biochemical, and physiological tests are always required to further identification.

According to Ahmed and Sabiel. (2016), the members of the family Enterobacteriaceae are usually associated with the contamination of meat products, their incidence in meat was considered as a public health problem (Ahmed & Sabiel, 2016). This finding was in agreement with the results of this study, that has most screening results was from Enterobacteriaceae family.

Staphylococcus aureus has the ability to colonize on the raw meat, and spread into meat products during the different processing stages of the meat supply chain (Velasco, Quezada-Aguiluz, & Bello-Toledo, 2019).

The pathogenicity of *Staphylococcus aureus* is given by bacterial structures and extracellular products, among which are toxins, could cause staphylococcal diseases transmitted by contaminated meat (Velasco et al., 2019). In the present work, *Staphylococcus aureus* was the most bacterium present in meat samples tested.

According to El Malti and Amarouch. (2009), the laurel extract has a significant role in moderating antimicrobial activities against *Staphylococcus aureus* 25923, *Proteus vulgaricus,* and *Salmonella enteridis.* These findings were in agreement with the results of this study. However, a disagreement in *Klebsiella pneumoniae* resistance, which was found to have only 7 mm zone of inhibition (El Malti & Amarouch, 2009). The higher resistance of gram-negative bacteria than gram-positive bacteria could be due to the differences in the structure of cell walls of these bacterial groups.

The differences in results between studies in the antimicrobial test may refer to the variance in terms of its nature, origin (country of origin, altitude at which it grows, harvest season), production process, level of purity and preservation. Among the problems worth mentioning are those that arise from the material used, all of which help to determine the presence of variable concentrations of antimicrobials in the final product.

The sheep meat samples were divided into many parts; all of these partitions were made to give abundances of a chance for testing the samples with time. Spraying is one of the successful ways of active agents applications as a coating system to fresh-cut food (Lucera et al., 2012).

Proteus vulgaris and *Macrococcus epidermidis* disappeared in the second and fourth categories, which prove the ability of *Laurus nobilis* essential oil to kill them, and inhibit the growth of others for a period of time. The comparison process between the four categories showed that the fourth category has the longest in shelf-life compared to other categories (Figure 8). The features of the *Laurus nobilis* played an important role in delaying the poisoning point at the second and fourth categories. It can be seen from the present work, that Laurus nobilis essential oil is a good natural preservative, which can be used in fresh lamp meat to extend its shelf-life.

5.2. Conclusions

The antimicrobial activity of essential oils has taken great importance as an alternative for synthetic antimicrobials because they are a part of the human diet and their biodegradability suggest low poisonous residue problems. This study demonstrated that *laurel* essential oil can be used as a natural preservative, for the prevention of deterioration of lipped foods during storage. A higher yield of essential oil was obtained using methanol than ethanol as well as less time was taken using methanol as an extractor. Methanol seems to be a good material for extraction of essential oil from *laurel nobilis.* The results of this study indicate that the antibacterial action of the *Laurus nobilis* extract has specificity for the treatment of gram-positive bacterial infection like *Staphylococcus* family. On the other hand, a little effect of laurel oil on the gram-negative was observed. Sequencing of the 16S rRNA gene was shown to be a good and accurate method for identification of bacterial isolates at the species level.

5.3. Recommendations

1. Essential oil of *Laurus nobilis* has a strong potential to be used as antimicrobial against gram-positive bacteria, and some type of gram-negative bacteria, which worth further studies to be applied in food industry.
2. The resistance problem from some types of bacterial isolates might be solved by the use of combination between the essential oils and some additives like organic acids, or natural salts; to inhibit microbial growth of recalcitrance bacteria and proliferation of pathogens.
3. Further research is needed to find new sources of antimicrobial substances, including plant metabolites. This pushes forward the search of food authorities and researchers for gentle preservation techniques to improve microbial quality and safety without causing nutritional and organoleptic losses, and to utilize the natural active agents which promote the accepted criteria of food sustainability.
4. The choice of identification method for bacterial isolates must be carefully analyzed. Some factors must be considered, such as the origin of the samples (food or clinical isolates), the number of isolates to be identified, and staff qualifications.

5. It is highly recommended to use 16S rRNA gene sequencing for identification of bacteria isolated from food samples as it appears to more reliable and faster method than phenotyping methods.

5.4. Limitation

This study has number of limitations. This includes:

1- Three protocols were used in the bacterial DNA extraction part; due to the differences in the structure of the cell wall, and lack of the material resources. Heat treatment and Microwave irradiation were used as simple methods to heat the bacterial colonies, and exploit the wave to penetrate the cell wall of bacteria.

2- Using of 40 % ethanol as a solvent material instead of others; due to the lack of suitable solvent extraction. In this context, the 40 % ethanol was used as a negative control to insure that has not any effect on the experiment.

3- Counting the colony forming units (CFU / ml) of bacterial isolated from collected sheep meat samples especially poisoned samples was very difficult; because of the presence of different colonies on the same petri dish plate. Therefore, the colonies may normally interfere with each other. This problem was solved by making an average counting of colony number between samples; to cover all result that located on all petri dish plates.

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Appendices

Appendices

Appendex (1): Sequncess for all 34 samples with the percentage identity and an accesion number

Sample 1

TTGCACACTACAATCCGAACTGAGAACAACTTTATGGGATTTG
CTTGACCTCGCGGTTTCGCTGCCCTTTGTATTGTCCATTGTAGC
ACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCAACTTAGA
GTGCCCAACTTAATGATGGCAACTAAGCTTAAGGGTTGCGCTC
GTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA
CAACCATGCACCACCTGTCACTTTGTCCCCCGAAGGGGAAGG
CTCTATCTCTAGAGTTGTCAAAGGATGTCAAGATTTGGTAAGG
TTCTTCTCGTTCCCCCCGTGCCCCCGCCCCGCCCGGGGCGCGC
CCCGGGC

Staphylococcus aureus strain ATCC 12600 16S ribosomal RNA, complete sequence, 99.43% , MK695866

Sample 2

CCTCGCGGTTTCGCTGCCCTTTGTATTGTCCATTGTAGCACGTG
TGTAGCCCAAATCATAAGGGGCATGATGATTTGACGTCATCCC
CACCTTCCTCCGGTTTGTCACCGGCAGTCAACTTAGAGTGCCC
AACTTAATGATGGCAACTAAGCTTAAGGGTTGCGCTCGTTGCG
GGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA
TGCACCACCTGTCACTTTGTCCCCTGAATGGGAATTCTCTATCT
CTAGAGTTGTCAAAGGATGTCAAGATTTGGTAAGGTTCTTCTC
GTTCCCCCCGTGCCCCCGCCCCGCCCGGGGCGCGCCCCGGGCG
GG

Staphylococcus aureus strain NBRC 100910 16S ribosomal RNA gene, partial sequence , 98.36%, MK695940

Sample 3

TTCCGAACTGAGAACAACTTTATGGGATTTGCATGACCTCGCG
GTTTAGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAGCC
CAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTC

CTCCGGTTTGTCACCGGCAGTCAACCTAGAGTGCCCAACTTAA
TGATGGCAACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCA
CCTGTCACTTTGTCCCCCGAAGGGGAAGGCTCTATCTCTAGAG
TTTTCAAAGGATGTCAAGATTTGGTAAGGCTCTTCTCGTTCCC
MCCGTGCMCCCGCCCCGMCCGGRGCGCGCCACGGRCGWAT

Staphylococcus saprophyticus subsp. saprophyticus strain ATCC 15305 16S ribosomal RNA, complete sequence, 99.41% , MK695941

Sample 4

CCGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGAC
TACAATCCGAACTGAGAACAACTTTATGGGATTTGCATGACCT
CGCGGTTTAGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGT
AGCCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCA
CCTTCCTCCGGTTTGTCACCGGCAGTCAACCTAGAGTGCCCAA
CTTAATG

Staphylococcus edaphicus strain CCM 8730 16S ribosomal RNA, partial sequence, 100% , MK695942

Sample 5

TCCGAACTGAGAACAACTTTATGGGATTTGCTTGACCTCGCGG
TTTCGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAGCCC
AAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCC
TCCGGTTTGTCACCGGCAGTCAACTTAGAGTGCCCAACTTAAT
GATGGCAACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCA
CCTGTCACTTTGTCCCCCGAAGGGGAARGCTCTATCTCTAGAG
TTGTCAAAGGATGTCAAGATTTGGTAAGGTTCTTCTCGTTCCC
CCCGTGCCCCCGCCCCGCCCGGGGCGCGCCCCGGGCG

Staphylococcus haemolyticus strain JCM 2416 16S ribosomal RNA gene, partial sequence, 99.71%, MK713339

Sample 6

ATTCCAGCTTAATGTAGTCGAGTTGCAGACTACAATCCGAACT
GAGGACAACTTTATGGGATTTGCTTGACCTCGCGGTTTCGCTG
CCCTTTGTATTGTCCATTGTAGCACGTGTGTATCCCAAATCAT

AAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTT
TGTCACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCA
ACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACA
TCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACT
TTGTCCCCCGAAGGGGAAGGCTCTATCTCTAGAGTTGTCAAAG
GATGTCAAGATTTGGTAAGGTTCTTCTCGTTCCCCCCGTGCCC
CCGCCCCGTCCGGGGCGCGCCCCGGGCGG

Staphylococcus aureus strain ATCC 12600 16S ribosomal RNA, complete sequence , 98.93%, MK713337

Sample 7

TTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCA
ATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCG
AGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGC
CCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTT
CCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCKG
ACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCA
GCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGCT
AAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCTCGTTCC
CCCCGTGCCCCCGCCCCGCCCGGGGCGCGCCCCGGGCG

Enterobacter cancerogenus strain LMG 2693 16S ribosomal RNA gene, partial sequence, 99.74%, MK713336

Sample 8

AATCTTGACATCCTTTGAAAACTCTAGAGATAGAGCCTTCCCC
TTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAGGTT
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
ATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAAT
GGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATC
CCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGAC
TACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCT
ACGGTGAATACGTTCCCGGGTCTTGTACACACGCA

Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 16S ribosomal RNA, complete sequence, 100 %, MK713332

Sample 9

CGAGTTGCAGACTACAATCCGAACTGAGAACAAAAATATGGG
ATTTGCATGACCTCGCGGTTTAGCTGCCCTTTGTATTGTCCATT
GTAGCACGTGTGAAGCCCAAATCATAAGGGGCATGATGATTT
GACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCAAC
CTAGAGTGCCCAACTTAATGATGGCAACTAAGCTTAAGGGTC
GCTCTCGTTGCGGGACGTAACCCAGAGAT

Staphylococcus saprophyticus subsp. saprophyticus strain ATCC 15305 16S ribosomal RNA, complete sequence, 97.05%, MK696049

Sample 10

TTGACTTCATGGCCTCGAGTTGCAGACTCCAATCCTTACTACG
ACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTC
TTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAC
GGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTAT
CACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAAC
AAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT
TTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCA
CAGTTCCCGAAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGA
TGTCAAGAGTAGGTAAGGTTCTTCTCGTTCCCCCCGTGCCCCC
GCCCYGCCCGGGGCACGCCCCGGKCGKRT

Pluralibacter gergoviae strain JCM1234 16S ribosomal RNA gene, partial sequence, 98.1%, MK696050

Sample 11

TATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTC
CAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCG
CGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTA
GCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACC
TTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCG
AACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGA
CTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGC
AGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGC
TAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCTCGTTC
CCCCCGTGCCCCCGCCCCGCCCGGGGCGCGCCCCGGGCGG
Cedecea lapagei strain DSM 4587 16S ribosomal RNA gene, partial sequence , 99.74%, MK696051

Sample 12

ATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACT
TTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATG
CGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGA
TGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCA
GTCTCCTTTGAGTTCCCGGCCKGACCGCTGGCAACAAAGGATA
AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACA
CGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCG
AAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGATGTCAAGA
GTAGGTAAGGTTCTTCTCGTTCCCCCCGTGCCCCCGCCCCGCC
CGGGGCGCGCCCCGGGCG

Enterobacter cancerogenus strain LMG 2693 16S ribosomal RNA gene, partial sequence, 99.72 %, MK713323

Sample 13

AGCSWAATACCGTAGCATGCTGCTCTACGATTACTAGCGATTC
CAGCTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTGAG
AACAACTTTATGGGATTTGCTTGACCTCGCGGTTTCGCTGCCC
TTTGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAG
GGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGT
CACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAAC
TAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATC
TCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTT
GTCCCCCGAAGGGGAAGGCTCTATCTCTAGAGTTGTCAAAGG
ATGTCAAGATTTGGTAAGGTTCTTCTCGTTCCCCCCGTGCCCC
CGCCCCGCCCGGGGCGCGCCCCGGGCG

Staphylococcus aureus strain ATCC 12600 16S ribosomal RNA gene, partial sequence, 99.51%, MK713325

Sample 14

TACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTC
ATGTAGTCGAGTTGCAGACTACAATCCGAACTGAGAACAACT
TTATGGGATTTGCATGACCTCGCGGTTTAGCTGCCCTTTGTATT
GTCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATG

ATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGC
AGTCAACCTAGAGTGCCCAACTTAATGATGGCAACTAAGCTT
AAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAC
ACGAGCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCC
GAAGGGGAAGGCTCTATCTCTAGAGTTTTCAAAGGATGTCAA
GATTTGGTAAGGTTCTTCTCGTTCCCCCCGTGCCCCCGCCCCG
CCCGGGGCGCGCCCCGGGCG

Staphylococcus edaphicus strain CCM 8730 16S ribosomal RNA, partial sequence, 99.75%, MK713324

Sample 15

GAATACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGC
CCACTCCAATCCGGACTACGACTCACAATATGAGGTCCGCTTG
CTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACG
TGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATC
CCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCC
CGGCCGAACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTT

Enterobacter tabaci strain YIM Hb-3 16S ribosomal RNA, partial sequence, 98.04%, MK713331

Sample 16

AATGTGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGC
ACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGT
ATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCA
TGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTG
GCAGTCTCCTTTGAGTTCCCGGCCTAACCGCTGGCAACAAAGG
ATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACA
ACACGAGCTGACGACAGCCATGCATCACCTGTCTCAGAGTTCC
CGAAGGCACCAAAGCAT

Enterobacter xiangfangensis strain 10-17 16S ribosomal RNA gene, partial sequence, 99.68%, MK713330

Sample 17

CGGCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTG
CAGACTCCAATCCGGACTACGACAGACTTTATGAGTTCCGCTT
GCTCTCGCGAGGTCGCTTCTCTTTGTATCTGCCATTGTAGCAC

GTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCAT
CCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTC
CCGCCATTACGCGCTGGCAACAAAGGATAAGGGTTGCGCTCG
TTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGAC
AGCCATGCAGCACCTGTCTCAGCGTTCCCGAAGGCACTCCTCT
ATCTCTAAAGGATTCGCTGGATGTCAAGAGTAGGTAAGGTTCT
T

Proteus vulgaris strain ATCC 29905 16S ribosomal RNA, partial sequence, 100%, MK713329

Sample 18

CACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGA
CTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTC
TCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGT
GTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCC
ACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGG
CCKGACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCG
GGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCA
TGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTC
TGCTAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCTCG
TTCCCCCCGTGCCCCCGCCCCGCCCGGGGCGCGCCCCGGGCG

Enterobacter cancerogenus strain LMG 2693 16S ribosomal RNA, partial sequence, 99.74%, MK713335

Sample 19

CCTGGGTCTTCGWMMWMACTRAGAACTTTCCAGAGATGGAT
TGGTGCCTTCGGGAACTTACATACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTTTCCTTATTTGCCAGCGGGTTAAGCCGGGAACTT
TAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACG
ACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGT
GCTACAATGGTCGGTACAAAGGGTTGCTACCTCGCGAGAGGA
TGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGC
AACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATC
AGAATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACGC

Acinetobacter lwoffii strain JCM 6840 16S ribosomal RNA, partial sequence, 99.75%, MK689408

Sample 20

TACCTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTGC
CTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGA
GACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAA
GTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAAT
GGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAC
CTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
CTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGC
CACGGTGAATACGTTCCCGGGTCTTGTACACACGCA

Enterobacter hormaechei subsp. xiangfangensis strain 10-17 16S ribosomal RNA, partial sequence, 99.76%, MK690048

Sample 21

AAACTAGCGATTCCGACTTCATGGAGTCGAGTTGCACACTCCA
ATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCG
AGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTATC
CCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTT
CCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGACCTA
ATCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCA
GCACCTGTCTCACAGTTCCCGAAGGCACCAATCCATCTCT

Salmonella enterica subsp. enterica serovar Typhimurium strain ATCC 13311 16S ribosomal RNA, partial sequence, 98.81% , MK690186

Sample 22

TACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTGCC
TTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAG
ACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAG
TCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATG
GCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCT
CATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCA
CGGTGAATACGTTCCCGGGTCTTGTACACACGCA

Enterobacter hormaechei subsp. xiangfangensis strain 10-17 16S ribosomal RNA, partial sequence, 99.76%, MK689181

Sample 23

TACTTACTTGCGATTCCGACTTCATGGTGTCGAGTTGCAGACT
CCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTC
GCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGT
AGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCAC
CTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCC
GAACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGG
ACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATG
CAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTG
CTAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCTCGTT
CCCCCCGTGCCCCCGCCCCGCCCGGGGCGCGCCCCGGGCGG

Cedecea lapagei strain DSM 4587 16S ribosomal RNA, partial sequence, 99.22%, MK689855

Sample 24

CACGATTACTAGCGATTCCGACTTCAGGGAGTCGAGTTGCAG
ACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCT
CTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTG
TGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCC
CACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCG
GCCGAACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGC
GGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCC
ATGCAGCACCTGTCTCAGAGTTCCCGAAGGCGCTATAGCATCT
CTGC

Cedecea lapagei strain DSM 4587 16S ribosomal RNA, partial sequence, 98.84%, MK713334

Sample 25

CTTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGAC
TTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACATA
CTTTATGAGGTCCGCTTGCTCTCGCGAGTTCGCTTCTCTTTGTA
TATGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCAT
GATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGG
CAGTCTCCTTTGAGTTCCCGACATTACTCGCTGGCAACAAAGG

ATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACA
ACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTC
CCGAAGGCACTAAGCTATCTCTAGCAAATTCTCTGGATGTCAA
GAGTAGGTAAGGTTCTTCTCGTTCCCCCCGTGCCCCCACCCCG
CCCGGGGCGCGCCCCGGGCGRCG

Hafnia paralvei strain ATCC 29927 16S ribosomal RNA, partial sequence , 99.76 % , MK684353

Sample 26

ATACTAGCGATTCCGACTTCATGGACTCGAGTTGCAGACTCCA
ATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCG
AGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGC
CCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTT
CCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCKG
ACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCA
GCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGCT
AAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCTCGTTCC
CCCCGTGCCCCCGCCCCGCCCGGGGCGCGCCCCGGGCG

Enterobacter cancerogenus strain LMG 2693 16S ribosomal RNA, partial sequence, 99.48%, MK695980

Sample 27

CCGGCATTCTGAACCGCGATTACTAGCGATTCCGACTTCATGG
AGTCGAGTTGCAGACTCCAATCCGGACTACGATCGGCTTTTTG
AGATTAGCATCCTCTCGCGAGGTAGCAACCCTTTGTACCGACC
ATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGAC
TTGACGTCGTCCCCGCCTTCCTCCAGTTTGTCACTGGCAGTATC
CTTAAAGTTCCCGGCTTAACCCGCTGGCAAATAAGGAAAAGG
GTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA
GCTGACGACAGCCATGCAGCACCTGTATGTAAGTTCCCGAAG
GCACCAATCCATCTCTGGAAAGTTCTTACTATGTCAAGACCAG
GTAAGGTTCTTCTCGTTCCCCCCGTGCCCCCGCCCCGCCCGGG
GCGCGCCCCGGGC

Acinetobacter lwoffii strain JCM 6840 16S ribosomal RNA, partial sequence , 99.5 %, MK713321

Sample 28

ATACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTT
CATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCAC
TTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTAT
GCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATG
ATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGC
AGTCTCCTTTGAGTTCCCGGCCTAACCGCTGGCAACAAAGGAT
AAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAAC
ACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCC
GAAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGATGTCAAG
AGTAGGTARGGTTCTTCTAGTTCCCCCCGTGCCGCCGCCCCGC
CCGGGGGGCCGCCCCGGGGGCGGGGKAAAAACGWAACAAA
MAT

Enterobacter hormaechei subsp. xiangfangensis strain 10-17 16S ribosomal RNA, partial sequence, 99.75 %, MK704397

Sample 29

TACCGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTC
ACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTT
TATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACC
GACCATTGTAGCACGTGTGTAGCCCAGGCCGTAAGGGCCATG
ATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGC
AGTCTCCTTAGAGTGCCCACCATTACGTGCTGGTAACTAAGGA
CAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGA
CACGAGCTGACGACAGCCATGCAGCACCTGTCTCAATGTTCCC
GAAGGCACCAATCCATCTCTGGAAAGTTCATTGGATGTCAAG
GCCTGGTAAGGTTCTTCTCGTTCCCCCCGTGCCCCCGCCCCGC
CCGGGGCSCGCCCCGGGCGA

Pseudomonas helmanticensis strain OHA11 16S ribosomal RNA, partial sequence, 99.75%, MK695699

Sample 30

TACCGTACCATGCTGATCTACGATTACTAGCGATTCCAGCTTC
ATGTAGTCGAGTTGCAGACTACAATCCGAACTGAGAATGGTTT
TATGGGATTTGCTTGACCTCGCGGTTTTGCTGCCCTTTGTACCA
TCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGAT

GATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAG
TCTCTCTAGAGTGCCCAACTTAATGATGGCAACTAAAGATAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
AGCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCCGAA
GGGGAAAGCTCTATCTCTAGAGTTGTCAAAGGATGTCAAGAT
TTGGTAAGGTTCTTCTYSTTCCCCCCGTGCCGCCCCCCCSCCCC
CGGGGCGCCGCCCCGGCGGRGAMAAAMARRAAAAAAAKAAC
ATWACYAAATAGAMAAATAGAAAATAAAAAAAAAGATTGCT
CCTCYGGGGGAAAARAAAAAGAGAAGC

Macrococcus epidermidis strain CCM 7099 16S ribosomal RNA, complete sequence , 99.75% , MK690643

Sample 31

CACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGA
CTCCAATCCGGACTACGACAGACTTTATGAGTTCCGCTTGCTC
TCGCGAGGTCGCTTCTCTTTGTATCTGCCATTGTAGCACGTGT
GTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCC
ACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCGC
CATTACGCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGC
GGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCC
ATGCAGCACCTGTCTCAACGTTCCCGAATGCACTCCTCTATCT
CTAAAGGATTCGCTGGATGTAAAGAGTGGGTAAAGTTCTTCTG
TAAGGTYCTTCGCGGCCGCCCCGCCCCCCCCCCCCSCCG

Proteus vulgaris strain ATCC 29905 16S ribosomal RNA, partial sequence, 98.69%, MK685208

Sample 32

ATTTCCGACTTAACGGAGTCGAGTTGCAGACTCCAATCCGGAC
TACGATGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCT
TCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGT
AAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTT
TATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGC
AACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAA
CATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCT
CAGAGTTCCCGAAGGCACCAAAGCATCTCTGCTAAGTTCTCTG

GATGTCAAGAGTAGGTAAGGTTCTTCTCGTTCCCCCCGTGCCC
CCGCCCCGCCCGGGGCGCGCCCCGGGCG

Cedecea lapagei strain DSM 4587 16S ribosomal RNA, partial sequence, 98.93 % , MK713322

Sample 33

TACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTC
ATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACT
TTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATG
CGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGA
TGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCA
GTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGGATA
AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACA
CGAGCTGACGACAGCCATGCAGCACCTGTCTCAAAGTTCCCG
AAGGCACCAG

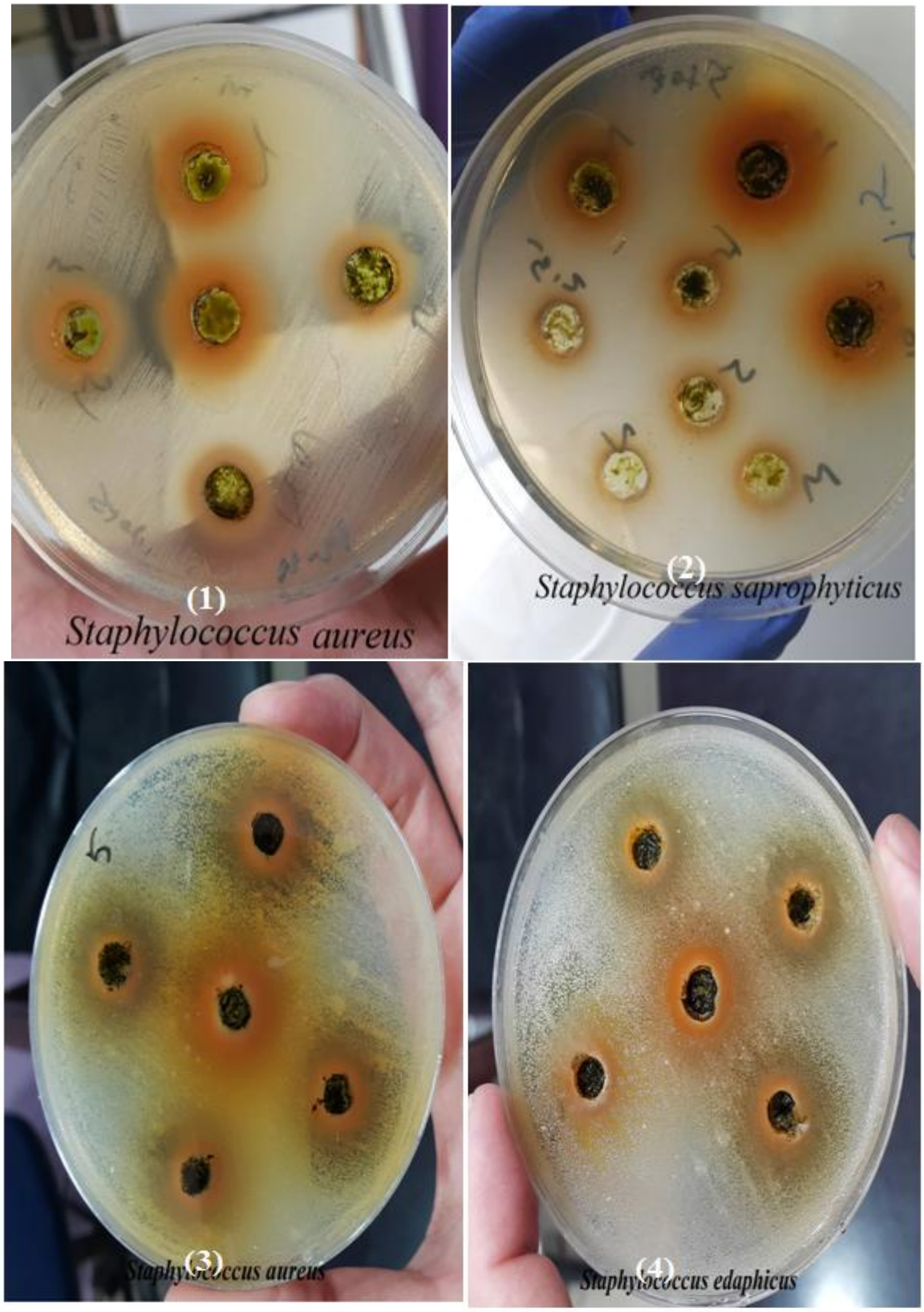
Pluralibacter gergoviae ATCC 33028 = NBRC 105706 strain JCM 1234 16S ribosomal RNA, partial sequence , 99.72%, MK684347

Sample 34

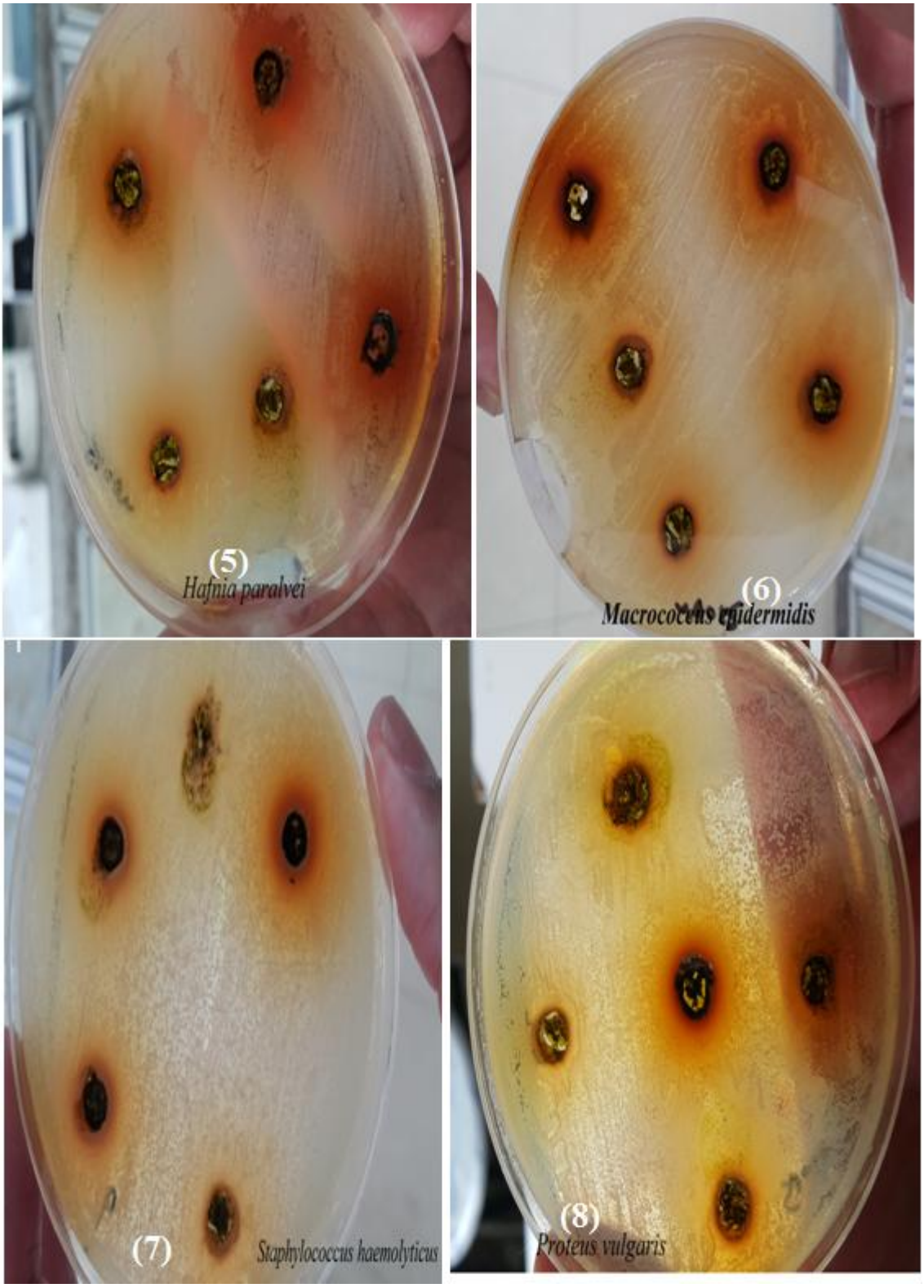
CAGTTGCAGACTCCAATCCGGATTACGACATACTTTATGAGGT
CCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATATGCCATTGT
AGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGA
CGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTT
GAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGA
CGACAGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGCACC
AATCCATCTCTGGAAAGTTCTGTGGATGTCAA

Klebsiella pneumoniae strain DSM 30104 16S ribosomal RNA, partial sequence, 99.4 %, MK684237

Appendex (2): The zone inhibition figures for *Laurus nobilis* aqueous extract against bacterial isolates



The zone inhibition figures for *Laurus nobilis* aqueous extract against bacterial isolates; (1) *Staphylococcus aureus*. (2) *Staphylococcus saprophyticus.* (3) *Staphylococcus aureus*. (4) *Staphylococcus edaphicus.*



The zone inhibition figures for *Laurus nobilis* aqueous extract against bacterial isolates; (5) *Hafnia paralvei.* (6) *Macrococcus epidermidis.* (7) *Staphylococcus haemolyticus.* (8) *Proteus vulgaris.*

The zone inhibition figures for *Laurus nobilis* aqueous extract against bacterial isolates; (9) *Proteus vulgaris*. (10) *Salmonella enterica/choleraesuis. (11) Pseudomonas helmanticcensis. (12) Staphylococcus saprophyticus.*

Abstract (in the Arabic language)

تقييم مدى تأثير المضادات الجرثومية الموجودة في مستخلص ورق الغار على حفظ اللحوم الطازجة

محمود سبتي حمدان

د. وفاء مسعود

الملخص

تعتبر اللحوم الحمراء وخاصة لحوم الخراف، بطبيعتها مصدر مغذي ولديها قابلية للتحلل الى مواد اولية بسيطة من خلال عمليات الايض بسهولة، وبالتالي انتاج مواد مناسبة تعتبر ركائز اساسية لنموالكائنات الحية الدقيقة وعمليات الايض الخاصة بها. التلوث الغذائي هو عبارة عن مجموعة من الجراثيم او السموم التي لديها القدرة على احداث العديد من الامراض المعوية الجرثومية المعدية من خلال استهلاك الاغذية الملوثة بالجراثيم او السموم الناتجة عن تلك الجراثيم. ان عملية الوقاية من التسمم الغذائي والمسببات له تتم عادة من خلال استخدام المواد الحافظة الكيميائية التي لها تاثير سلبي على صحة الانسان. الهدف من هذه الاطروحة هو ايجاد مواد حافظة طبيعية صحية بديلة ذات قيمة اكبر، وأكثرامانا. تتركز خطوات العمل في هذه الرسالة على فحص عينات لحوم الخراف والبحث عن انواع البكتيريا التي تسبب تلوث الاغذية الدهنية، والتحقق من قدرة مستخلص ورق الغار على تثبيط نمو البكتيريا، و الكشف عن المدة التي يمكن حفظ الأغذية الدهنية فيها من خلال عمل مضادات الأكسدة ومضادات الميكروبات الموجودة في ورق الغار. تم عزل 34 عزلة بكتيرية وتحديد نوعها باستخدام تقنيات الكيمياء الحيوية المعروفة، وتحديد التسلسل الخاص بالجين 16SrRNA لجميع هذه العزلات. كان الميثانول اختيارًا جيدًا لإعداد المستخلص كمادة مذيبة وانتاج كمية اكبر من الزيت العطري، اكثر من الايثانول. تم اختبار قدرة نشاط المضادات الجرثومية الموجودة في مستخلص اوراق الغار ضد جميع العزلات الـ 34 باستخدام تقنية الحفر في الوسط الغذائي للبكتيريا وملئها بالمستخلص. نتائج ايجابية لتثبيط النموالبكتيري حول منطقة الحفر ضد العزلات الموجبة الجرام ، وبعض النتائج القليلة على العزلات سالبة الجرام. يتمتع مستخلص الغار بالقدرة على زيادة العمر الافتراضي للحوم الأغنام إلى 13 يومًا مع ميزات مقبولة في الثلاجة مقارنة بالوضع الطبيعي، باستخدام طريقة الرش ، ولثلاثة أيام في درجة حرارة الغرفة. يمكن استخدام مستخلص الغار الذي ثبت فعاليته كبديل وقائي طبيعي للسيطرة على أمراض التسمم الغذائي والحفاظ على المواد الغذائية مع تجنب المخاطر الصحية للتطبيقات الكيميائية المضادة للميكروبات.