



An-Najah National University
Faculty of Graduate Studies

**MOLECULAR DETECTION OF *LISTERIA*
MONOCYTOGENES IN FRESH VEGETABLES
VIA RT-PCR IN THE WEST BANK**

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**This Thesis is Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Nutrition and Food Technology, Faculty of Graduate Studies, An-Najah
National University, Nablus - Palestine.**

2024

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Dedication

To a soul through which I saw the path of my life and from whom I derived my strength and self-esteem. To a soul that taught me persistence and that nothing is impossible in life with the power of faith. To a soul who dedicated her life to performing a message she made from patience leaves, A message that teaches me how to be loyal. To a soul that was in a hurry to leave and left us early in the body and not the spirit. To your soul my beloved mother, I dedicate this study of mine with pride and pride, and I consider it worthy of God Almighty, and I ask Him to facilitate for me a path to the highest paradise with it.

To the one whose name my name is associated with and who was my role model in life. To the one who harvested the thorns on my path to pave the way for me to science. To those who strived for my comfort, happiness and success. To the greatest and most precious man in the universe. I have always promised him this success. Here I am, fulfilling my promise and dedicating it to you, my beloved father with love and pride.

To the inspiration of my success, the makers of my strength, the best of my days and the best of my times, to the candles that light the way my (my brothers and my sisters).

Dedicated to my girl's best friends, Nagham, Noura, and Sally, Kawthar.

To everyone who assisted me in completing this research while I was studying

Acknowledgements

First and foremost, I would want to express my sincere gratitude to Allah Subhana Watalla, who has granted us life, the blessing of good health, and the blessing of my ability to conduct this research.

A special thank you and love go out to my wonderful family, especially my father, who is the best person I could ever want for and who gives me courage and hope in life. I am really grateful to my amazing siblings, who have always been there for me, supported me, and encouraged me. They have taught me how to manage my time and be patient in order to accomplish my goals. Respect and gratitude go out to my friend Nora Hajja, who has been a huge help and has worked hard with me to provide me with free time to study so that I can earn a master's degree.

I have the utmost regard and admiration for all of my instructors at Najah National University for their time, tolerance, support, and leniency. They also made us feel like family during my master's level studies. I would like to express my deep heartfelt gratitude to my supervisors, Dr. Muhammad Al-Tamimi and Dr. Abdel Hafeez Dalaab, for their valuable guidance, invaluable comments, and constant encouragement. I would like to thank Dr. Issa Shtayyeh and Sister Salwa Al-Khatib for their cooperation in completing the research. I will always be grateful to the laboratory technicians for their cooperation and assistance in this research. Finally, I would like to express my thanks to my family for their constant support and encouragement. Special thanks to my father, who was my first supporter, his encouragement, and for achieving this great achievement and success.

I want to express my gratitude to everyone who helped me but was not specifically named above.

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

MOLECULAR DETECTION OF *LISTERIA MONOCYTOGENES* IN FRESH VEGETABLES VIA RT-PCR IN THE WEST BANK

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **Dania Mofeed Hassan Al-Athamneh**

Signature:

A handwritten signature in blue ink that reads "Dania" with a horizontal flourish underneath.

Date: **12/02/2024**

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Abstract

Listeria monocytogenes is an emerging foodborne pathogen that can be transmitted by a wide range of food items. This study aimed to evaluate the existence of *L. monocytogenes* in freshly selected vegetables (lettuce, parsley, watercress, spring onion, carrot juice) in five governorates in the West Bank, Palestine using both conventional and molecular methods. A total of 150 fresh vegetable samples were bought from hypermarkets and street food hawker stands. The presumed isolates were differentiated biochemically and were further confirmed by Real-Time Polymerase Chain Reaction (RT-PCR) targeting 16S rRNA gene for *Listeria* spp. and the *hly* gene, which is specific to *L. monocytogenes*. The results showed that 71% of the examined fresh vegetable were positive for *L. monocytogenes* based on the media's selectivity and the colonies' morphological characteristics. Percentages distribution of *L. monocytogenes* in the fresh vegetables were 66%, 70, 70, 73 and 73% for lettuce, watercress, spring onion, carrot juice, and parsley, respectively. On the other hand, statistical analysis of RT-PCR results indicated significant differences in *L. monocytogenes* levels among vegetable samples within the same governorate. In Tulkarm, the highest levels were found in carrot juice, while the lowest were in lettuce. Also in Nablus, the highest levels were found in carrots juice, while the lowest were in lettuce and watercress. Also, Ramallah, Bethlehem, and Hebron, each the highest in spring onion, while the lowest were lettuce. Additionally, indicated comparison of *L. monocytogenes* levels across cities for selected of vegetables significant differences that lettuce had the highest prevalence in Bethlehem, parsley in Bethlehem, also watercress, spring onion, carrot juice each the highest in Hebron. In conclusion, this study has demonstrated a high prevalence of *Listeria* in fresh vegetables using both methods however, the molecular method was more sensitive and reliable for listeria

confirmation. Contamination by *Listeria* in hypermarkets and street food as well is of great public concern and justifies more observation and strict regulations to reduce the frequent contamination of fresh vegetables with *L. monocytogenes*.

Keywords: Fresh vegetables, *Listeria monocytogenes*, Oxford agar, Real-time PCR, *hly* gene, listeriosis.

Chapter One

Introduction

1.1 Background

Foodborne illnesses affect the entire world because they cause significant morbidity, mortality, and financial expenses (Tauxe et al., 2010). Foodborne illness outbreaks and occasional instances are common occurrences around the world (Mehlhorn, 2016; Scharff, 2012).

Food contamination could occur at any stage in the food supply chain, including during storage and preparation in grocery stores, restaurants, and homes as well as in farmhouses where harvests are grown and animals are raised. Contaminated food has the potential to spread a wide range of diseases, including those caused by bacteria, viruses, parasites, chemicals, and prions (Scallan et al., 2011; Bhaskar, 2017; Bintsis, 2017).

Contamination can happen as a result of unsuitable food handling techniques, such as not washing one's hands before handling food, or due to contamination of the water, land, or air (Mishra et al., 2020). Pathogens that cause foodborne illnesses are now swiftly transmitted across international borders as a result of the globalization of the food supply in recent decades (Kirk et al., 2015). Outbreaks of foodborne illness have had a negative influence on trade and food security (Frank C et al., 2011).

Through the consumption of food infected with microbes (viruses, parasites, and bacteria) or with chemical substances, people can contract more than 200 diseases (Mead et al., 2000). Bacterial pathogens outbreaks, however, represent two-thirds of the foodborne illnesses (Bintsis, 2017). The three types of illnesses created by foodborne microorganisms are (foodborne infection, food intoxication, and foodborne toxicoinfection) (Bhunias, 2007; Vemula et al., 2012; Bintsis, 2017).

Pathogens must first enter the host in sufficient numbers to initiate an infection. Once within the host, the pathogens must endure in the constantly changeable environment to multiply and proliferate (Bhunias, 2007). Pathogens are aided in their search for a suitable habitat for colonization by factors such as adhesion, invasion, and chemotaxis (Bhunias, 2007). The main potential targeting tissue for foodborne pathogens is the alimentary canal

(Bhunia, 2007). The majority of foodborne bacteria cause tissue damage and localized infection (Bhunia, 2007). An enteric infection can only succeed if the host cooperates with a variety of factors (Bhunia, 2007). *L. monocytogenes*, one of the most emerging and common foodborne organisms, is the culprit behind both human and animal listeriosis (Vojkowska et al., 2015).

It might be difficult to relate a bacterial infection to a certain nutrient product, because of the prolonged incubation period of *L. monocytogenes* and the rarity of symptoms related to intestinal disease (Vojkowska et al., 2015). The spreading of *L. monocytogenes* from the natural environment to the human dietary chain is significantly enhanced by plants (Amagliani et al., 2007; Pochop et al., 2012; Zhu et al., 2017).

The ability to adapt of this emerging food pathogen to different environmental factors, involving rising concentrations of salinity and decreases in pH, in addition to microaerophilic and psychrotrophic properties, all been attributed to its capacity to reproduce and survive in several food items through processing and storage (Kumar et al., 2015).

For *L. monocytogenes* can endure and reproduce in several temperatures (2–40°C) any in or on food for extended periods under detrimental conditions, agricultural food products manufacturing has been highly worried about these bacteria in the past (10) years (Elmali et al., 2013). All along the line of treatment, food manufacture, holding, and distribution, the presence of *L. monocytogenes* must be monitored (Vojkowska et al., 2015).

Conventional microbiological methods are standardized and culture-reliable ways to identify *L. monocytogenes*. The difficult and time-consuming identification process is a significant drawback (Vojkowska et al., 2015).

It is necessary to have swift and sensitive technologies, including polymerase chain reaction (PCR), and real-time polymerase chain reaction(RT-PCR that can identify *L. monocytogenes* from non-pathogenic Listeria and other common bacteria accurately and quickly are needed.

A claimed sensitive and specific alternative, PCR-based approaches are distinguished by high discriminative force, quick treatment times, and high output (Vojkowska et al., 2015). Due to the paucity of research on this topic in Palestine, *L. monocytogenes* has

received a great deal of attention in recent years due to its harmful effects on human health. Additionally, even though that *L. monocytogenes* might be harmful to human health, the Palestinian Standards Institution made no compulsory inspection of its presence yet.

1.2 Problem statement

- _ It can be challenging to demonstrate a link between a specific food product and *L. monocytogenes* infection because the illness's symptoms are rare and the incubation period can last for many weeks.
- _ Traditional microbiological techniques are culture-dependable and standardized approaches for *L. monocytogenes* detection, yet they are lengthy and consist of an enormous identification and differentiation process.
- _ It is necessary to have fast and sensitive technologies that can swiftly and accurately separate *L. monocytogenes* from non-pathogenic *Listeria* and different common bacteria.

1.3 Importance of the Study

- _ *L. monocytogenes* is an emerging biological hazard that can pose a risk upon consumption of raw vegetables.
- _ Molecular methods can be employed for the detection of *L. monocytogenes* in small amounts in fresh food as a quicker substitute for culturing methods.
- _ Using Real-time polymerase chain reaction (RT-PCR) methods, *L. monocytogenes* can be easily identified, accurately measured, and distinguished.

1.4 Study Objectives

- _ To determine the prevalence of *Listeria* spp., specifically *L. monocytogenes* in selected raw vegetables in Palestine /West Bank using conventional methods such as Enrichment and selective culture.
- _ To determine the existence of *L. monocytogenes* by utilizing molecular methods.

1.5 Study Hypothesis

1.5.1 Alternative hypothesis

L. monocytogenes is found in plant environments and may cause significant contamination of raw vegetables.

1.5.2 Null hypothesis

There are not enough *L. monocytogenes* cells in raw vegetables to result in a foodborne disease.

1.6 Literature Review

One of the most pervasive issues in the modern world is foodborne infection which causes significant economic damage. Ingesting tainted food or water causes diseases, which are toxic or contagious by nature. The causing factors might be biological, such as pathogenic microbes, or chemicals, such as pesticide residues and poisonous metals.

Mycotoxins and plant toxins-related illnesses are also categorized as foodborne illnesses. When symptoms of foodborne illnesses include vomiting, nausea, and limiting diarrhea, the clinical status may be humble; but, it can also be severe if there are major nervous system abnormalities and life-threatening diarrhea (Notermans & Hoogenboom-Verdegaal, 1992). According to estimates from the World Health Organization (WHO), there were 418,608 fatalities and 600,652,361 episodes of sickness globally in 2010 as a result of foodborne infections (Keba et al., 2020).

Foodborne infections are the inducement of several diseases that have detrimental effects on individual health as well as the economy. The properties of the majority of common pathogenic bacteria (*Campylobacter jejuni*, *Bacillus cereus*, *Clostridium perfringens*, *Clostridium botulinum*, *Escherichia coli*, *L. monocytogenes*, *Cronobacter sakazakii*, *Salmonella* spp., *Vibrio* spp., *Shigella* spp., *Yersinia enterocolitica*, and *Staphylococcus aureus*), parasites (*Toxoplasma gondii*, *Trichinella spiralis*, and *Cyclospora cayatanensis*), and viruses (Noroviruses and Hepatitis A) (Bintsis, 2017).

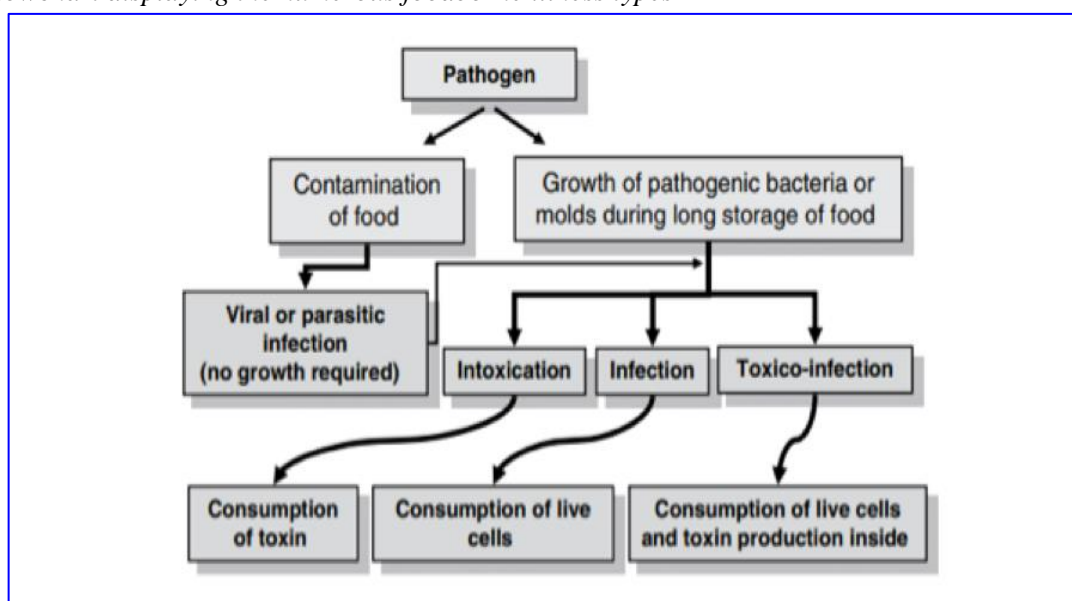
When a pathogen is used up with food and becomes fixed (and usually reproduces) into the body of a person, or when a toxigenic pathogen becomes fixed in a food product and produces a toxin, which is later used up by the body of a person, foodborne illness results

(Bintsis, 2017). Foodborne intoxication and foodborne infection are the major two categories down which foodborne illness cascades as shown below (Figure. 1). Foodborne illnesses usually take much longer than foodborne intoxications to show symptoms after consumption since they usually include an incubation period (Bintsis, 2017). There are more than 200 different food-borne illnesses known to exist (Mead et al., 2000). The most severe cases typically affect the very elderly, little young, people with compromised immune systems, and healthful people vulnerable to extraordinarily high doses of an organism (Bintsis, 2017; Gasanov et al., 2005).

The main place of activity for foodborne pathogens is the gut, and the main route of infection is the mouth. However, some penetrate to deeper tissues to cause systemic infection. The majority of foodborne bacteria produce localized infection and tissue damage. For an enteric infection to be successful, the body of a person must cooperate with a number of factors. Pathogens must initially enter the body in sufficient numbers for an infection to begin. After entering the host, the pathogens must survive in the changeable environment to reproduce and proliferate. Invasion, adhesion, and chemotaxis variables all help pathogens in find a suitable habitat for colonization. Exotoxins, endotoxins, or enzymes causing the cell's death by either necrosis or apoptosis and supporting bacterium growth and development are further methods by which pathogens harm the tissues and cells of the host (Bhunias, 2007).

Figure 1

Flowchart displaying the numerous foodborne illness types



Source: Bhunia, 2007

1.6.1 Foodborne Infection

Foodborne diseases can be brought on by living, functioning microorganisms, but they must first infect the host. After being consumed with food or drink, microorganisms go from the acidic environment of the stomach to the intestine, where they colonize and get through the intestinal barrier either by an active invasion process or by being carried by phagocytic M cells. Some bacteria produce inflammation and localized tissue damage, while others spread to extra-intestinal areas like the lymph nodes, liver, spleen, brain, and other organs (Bhunia, 2007). Food and water-borne diseases have long posed a serious hazard to human health. *L. monocytogenes*, one of the most common foodborne organisms, is the culprit behind both animal and human listeriosis (Vojkowska et al., 2015).

L. monocytogenes an infectious foodborne pathogen, is most commonly transmitted to individuals by food. This pathogen causes individuals listeriosis, a serious illness with a high death rate that can cause diseases such as meningitis, encephalitis, abortion, or septicemia. Individuals with weak immune systems, the aged, and pregnant women are among the vulnerable groups more likely to contract systemic listeriosis (Erol & taşçi, 2021). High quantities of salt, low water activity, and a range of pH values are all favorable for *L. monocytogenes* growth (Shamloo et al., 2019).

Due to its capacity to create biofilms, it can also be discovered on the processing equipment used to prepare cooked, ready-to-eat food. The use of foods that are ready to eat has been linked to listeriosis outbreaks. There were 24 confirmed listeriosis outbreaks recorded in the US between 1998 and 2008. In the European Union, listeriosis cases climbed by 19.1% in 2009 compared to 2008, and they nearly stabilized in 2012 (Abdollahzadeh et al., 2016).

1.6.2 General Characteristics of *L. monocytogenes*

L. monocytogenes is a (gram-positive) bacteria with optional anaerobic or aerobic metabolic functions (Letchumanan et al., 2018). It is a pathogenic bacteria that appears as small rods or coccobacilli that are between (0.4 - 0.5 μ m) wide and between (1.2 - 2 μ m) long and do not produce capsules or spores. It is oxidase negative, whereas catalase, esculin, and sodium hypurate hydrolysis are all positive. Although the range of (30 – 37°C) is ideal for multiplication, it can grow lowest at temperatures (0 to 45°C). Peritric

flagella are responsible for movement between (20 - 25°C), but at 37°C they stop being so. In foods with high water activity, it can grow, however, it can grow at (10%) salt, and 200 ppm NaNO₂ concentrations, and maintain its essential functions at (20-30%) salt concentrations. It tolerates NaNO₂ and NaCl. Maintains metabolic processes throughout a wide pH range that can change in response to environmental factors like acidity regulators and temperature (Erol & taşçi, 2021). (Table .1) contains a summary of the characteristics of *L. monocytogenes*.

Table 1
General Characteristics of L.monocytogenes

Characteristics	Properties
Metabolic activity	Aerobe or facultative anaerobic
Cell morphology	(0.4-0.5 µm) wide by (1-2 µm) long short rods or coccobacilli that do not produce capsules or spores.
Gram staining	(+)
Catalase Test	(+)
Esculin	(+)
Oxidase Test	(-)
Sodium hippurate hydrolysis	(+)
Motility	Provided by peritrich flagella between (20-25 °c)
Water activity	(> 0.92)
Temperature	(< 0 – 45 °c)
PH	(4.3-9.6)
NaNO ₂	Grow until 200 ppm
NaCl	Grow until (10%), Keep its vital activity at (20-30%)

1.7 *L. monocytogenes* Prevalence in Foods

Common microorganisms such as *L. monocytogenes* can be isolated from food, water, animal feces, and soil. Thus far, this pathogen has been detected in a number of prepared foods, such as salads, appetizers, and meat and dairy products. Recent increases in the consumption of ready-to-eat meals, particularly in Western nations, are linked to an increase in foodborne listeriosis infections (Erol & taşçi, 2021).

1.7.1 Fruits and Vegetables

It appears to be difficult to identify the cause of fruit and vegetable contamination throughout production. Furthermore, the soil is the place where fruits and vegetables are maintained for the most period. Fresh vegetables may become contaminated with the pathogen as a result of subpar farming practices, particularly during preharvest. Since fruits and vegetables are often consumed raw, it is unclear how frequently they are produced as other convenience foods (Lianou & Sofos, 2007). Soriano et al. found a similar conclusion when comparing the prevalence of raw and cooked lettuce in Spain (Erol & taşçi, 2021).

Seventy samples of fresh lettuce from the Afyon province in western Turkey. revealed just one incidence of *L. monocytogenes* (Erol & taşçi, 2021). Other researchers looked into the contamination rates of frozen vegetables in Portugal and found that they ranged from 14.8% to 22.6%. Seventy-two samples of vegetables were examined for the presence and severity of *L. monocytogenes* infection (Mena et al., 2004; Chen et al., 2015).

About 22% of the samples had *L. monocytogenes* results that were positive. *L. monocytogenes* was found in 3 of the Bursa frozen pepper sample analyses. Even though these products are believed to be less hazardous than those that are ready to use, the preparation instructions must be clearly stated on the label before use (Erol & taşçi, 2021).

1.8 Prevalence of *L. monocytogenes* in the World

According to the World Health Organization, 600 million individuals contract food-borne illnesses each year (EROL & TAŞÇI, 2021). Foodborne illnesses have an impact on socioeconomic development by taxing the healthcare system and negatively affecting the nation's economy, trade, and tourism. The etiologic agent of listeriosis, *L. monocytogenes*, is known to induce sporadic instances in which a specific food source is infrequently recognized.

Given that listeriosis has a protracted and variable incubation period of (3 - 70) days, it is challenging to determine the causative carrier of an outbreak. (Table. 2) shows the prevalence of *L. monocytogenes* in the world (Letchumanan et al., 2018).

Table 2
Prevalence of L. monocytogenes in the World

country	year	case number per 100.000 persons number	Death (person or %)	References
United States of America (USA)	2013	0.26	260	(Erol & taşçi, 2021).
EUROPE	2017	0.42 (over the age of 64 years).	No case.	(Erol & taşçi, 2021).
AUSTRALIA	(2011-2015)	78 cases annually, corresponding to 0.3.	No case .	(Park & Lee, 2018).
	2018	22 cases among those 70 years of age and older. Females made up about 59% of the overall cases.	31.81 %	(Park & Lee, 2018).
China	(2011_2016)	253	fatalities rate 25.7%. There were no recorded fatalities of children or pregnant women.	(Li et al., 2018)

1.9 *L. monocytogenes* Detection and Identification

1.9.1 Enrichment and Selective Culture

Numerous selective enrichment and plating media have been developed to isolate and identify *L. monocytogenes* in environmental and food samples. Most regulation organizations require isolation methods to identify at least one *Listeria* bacterium for every 25g of food. It is necessary to apply enrichment techniques to enable the living thing to multiply and achieve an observable level of (10^4 – 10^5 CFU ml⁻¹) before plating onto selective media and validating cultures (Law et al., 2015).

Since *Listeria* cells grow slowly and easily surpassed by competitors, antimicrobial agents are added to enrichment and plating media to minimize competing microflora. Three of the most regularly used selective agents are cycloheximide, a substance acriflavine and nalidixic acid (Law et al., 2015).

To prevent the growth of more Gram-positive bacteria, acroflavine is used in combination with other selective agents such as potassium thiocyanate, cycloheximide, nalidixic acid and polymyxin B-sulfate. Cycloheximide inhibits fungus, while nalidixic acid suppresses gram-negative bacteria. In addition, antimicrobial such as (lithium chloride, moxalactam, and broad-spectrum ceftazidime) are usually addition to the medium (Janzten et al., 2006).

Esculin is a significant carbohydrate that is frequently found in the media used for plating and enrichment of *Listeria*. Esculin can be hydrolyzed by *Listeria* spp. giving the medium a deep black color. This is due to the presence of ferric ions and esculin in the medium, which combine with the result of esculin breakdown by -D-glucosidase to generate a black precipitate known as 6,7-dihydroxy coumarin. The bacteria *Listeria* is common in cultures that produce black colonies.

The regulatory agencies have recommended a number of selective enrichment mediums for *L. monocytogenes*, including buffered *Listeria* enrichment broth, Fraser broth, and University of Vermont Medium *Listeria* enrichment broth (Law et al., 2015).

The US Food and Drug Administration recommends utilizing buffered *Listeria* enrichment broth (BLEB), a bacteriological and analytical approach, for isolating and detecting *L. monocytogenes*. The addition of (disodium phosphate) enhances the

medium's enrichment qualities and increases its ability to act as a buffer. Selective agents such as cycloheximide, acriflavine, and nalidixic acid are added to the medium after the initial four hours of non-selective pre-enrichment, according to Vermont University. To isolate and identify *L. monocytogenes*, the US Department of Agriculture's Food and Safety method recommends using the medium *Listeria* enrichment broth. This enrichment broth should be used as the primary broth when mending heat-damaged *L. monocytogenes*. Two selective agents, acriflavine and nalidixic acid, are added to the medium (Law et al., 2015).

Fraser broth has been recommended as a component of the International Organization for Standardization (ISO) 11290 method for the selective enrichment of *L. monocytogenes* in food and environmental samples.

Fraser broth is manufactured by mixing lithium chloride and ferric ammonium citrate with USDA secondary enrichment broth, according to a method developed by Fraser and Sperber (Law et al., 2015). Two carefully selected ingredients, nalidixic acid and acriflavine, are added to Fraser broth as supplements. Fraser broth can be used in 48 hours to identify presumed *L. monocytogenes* (Fraser & Sperber, 1988).

Selective differential plating media such as Oxford, Polymyxin Acriflavin Lithium-Chloride Ceftazidime Aesculin Mannitol (PALCAM), and ISO are typically recommended by the Food and Drug Administration's Bacteriological and Analytical Method (FDA-BAM), the USDA, and ISO for the isolation of *Listeria* spp. (Zunabovic et al., 2011).

Oxford and Polymyxin acriflavine lithium-chloride ceftazidime esculin mannitol (PALCAM) are useful for isolating *Listeria* spp. because they can heal injured *Listeria* cells and/or have a diverse range of competing bacteria (El Marrakchi et al., 2005).

1.9.2 Polymyxin Acriflavin Lithium-Chloride Ceftazidime Esculin Mannitol (PALCAM) Agar

PALCAM agar was first created to identify and count *L. monocytogenes* and other *Listeria* spp. (van Netten et al., 1989). Blood Agar from Columbia the primary components of polymyxin acriflavin lithium-chloride ceftazidime aesculin mannitol (PALCAM) agar are (0.5 g/L) glucose, (23.0 g/L) protease peptones, (3.0 g/L) yeast extract, (1.0 g/L) starch, and (5.0 g/L) sodium chloride.

The selectivity of this medium is increased by adding (0.01 g/L) polymyxin B, (15 g/L) lithium chloride, and (0.02 g/L) ceftazidime, (0.005 g/L) acriflavine. The bases for distinction on PALCAM agar are esculin hydrolysis, which involves adding (0.5 g/L) ferric salt, and (0.8 g/L) esculin, and mannitol fermentation, which involves adding (0.08 g/L) phenol red and (10 g/L) mannitol (van Netten et al., 1989; Law et al., 2015).

PALCAM agar with bacteria plated on it is typically incubated at 37°C for (24-48 hours) (Jia & Jukes, 2013; Law et al., 2015). Considering that all *Listeria* spp. exhibit a black the medium, their colonies measure approximately 2 mm in diameter and have a gray-green color with a black sunken center and a black halo. Esculin can be hydrolyzed by them. Sometimes, *Enterococcus* or *Staphylococcus* spp. can grow on PALCAM agar (Law et al., 2015).

They can be separated from *Listeria* spp. by fermenting mannitol. When mannitol ferments, acids are produced, which is why the colony's color changes from red or gray to yellow along with the surrounding medium. These organisms that digest mannitol produce colonies that are either yellow with a yellow halo or gray with a brownish-green halo (van Netten et al., 1989; Law et al., 2015).

1.9.3 Oxford Agar

The first *L. monocytogenes* isolate from clinical samples was obtained using Oxford agar. *L. monocytogenes* has been extracted and identified from the variety of food items using Oxford agar in numerous studies. Columbia Oxford is made up of blood agar, (5.0 g/L) sodium chloride, (23 g/L) protease peptones, and (1.0 g/L) starch.

To improve Oxford's selectivity, add (0.005 g/L) acriflavine, (15 g/L) lithium chloride, (0.4 g/L) cycloheximide, (0.02 g/L) colistin sulfate, and (0.01 g/L) fosfomycin, (0.002 g/L) cefotetan. Oxford agar is based on the analysis of esculin, which is made easier by adding (1 g/L) esculin and (0.5 g/L) ferric ammonium citrate to the agar (Law et al., 2015).

After plating, Oxford agar is stored for (24-48 hours) at 37°C. *L. monocytogenes* colonies have an olive green core with a black halo following incubation for 24 hours. Following incubation for 48 hours, *L. monocytogenes* colonies have a sunken black center around black zones, measuring roughly 2-3 mm in diameter.

Colonies resemble those of *L. monocytogenes*. Some species of *Listeria* produce colonies that are black with a black halo after 24 hours and continue to be black but with a sunken core after 48 hours. On Oxford agar, *Staphylococcus* species can sporadically thrive and produce irregularly shaped and occasionally yellow colonies. Oxford agar has been modified to make Modified Oxford Agar (MOX).

MOX is advised for isolating and identifying of *L. monocytogenes* from poultry products and processed meat. In contrast, Oxford agar is advised for the isolation of *L. monocytogenes* from enriched broth cultures (Law et al., 2015).

The main disadvantage of PALCAM and Oxford is their incapacity to identify between *Listeria* spp. that are pathogenic and non-pathogenic (El Marrakchi et al., 2005). These plate media do not allow for the rapid detection of *L. monocytogenes* in food. Because of this, chromogenic media that may differentiate between pathogenic *Listeria* spp. or/and *L. monocytogenes* have been developed, aiding in the improvement of *L. monocytogenes* isolation. The chromogenic medium is used to detect important pathogenicity factors for *Listeria* spp. Stores sell most of these media as ready-to-use plates (Zunabovic et al.,

2011; Law et al., 2015). Using the chromogenic media, presumed *L. monocytogenes* can be identified after a 24 hours.

Research has shown that chromogenic media, like Agar Listeria according to Ottaviani and Agosti (ALOA) agar and CHROMagar™ Listeria, are more specific, sensitive, money- and time-saving, and better at detecting *L. monocytogenes* than nonchromogenic media like polymyxin acriflavin lithium-chloride ceftazidime esculin mannitol (PALCAM) and Oxford (Law et al., 2015).

1.9.4 Chromogenic Media

The development of chromogenic media allowed for a new degree of success in *L. monocytogenes* isolation. Agar Listeria was among the first of these agars, according to Ottaviani and Agosti (ALOA) agar. This medium contains 5-bromo-4-chloro-3-indolyl-D-glucopyranoside, which serves as a substrate for the detection of β -glucosidase, an enzyme that catalyzes the hydrolysis of glycosidic linkages to terminal nonreducing residues in β -D-glucosides and oligosaccharides with producing glucose (Osek et al., 2022; Law et al., 2015). β -glucosidase is produced by all types of *Listeria*, which appear as green-blue colonies on the Agar *Listeria* as defined by Ottaviani and Agosti (ALOA) agar plates.

To improve selectivity, lithium chloride is added to the medium along with anti-microbial agents including cycloheximide, polymyxin B, ceftazidime, and nalidixic acid. Based on Ottaviani and Agosti's (ALOA agar) method, *L. monocytogenes* produces a phospholipase C (PI-PLC) that is specific to phosphatidylinositol. This enzyme hydrolyzes L- α -phosphatidylinositol, resulting in green-blue colonies encircled by an opaque halo. This feature further distinguishes *L. monocytogenes* from other varieties of *Listeria* (Osek et al., 2022).

Some types of *Listeria*, on the other hand, develop as green-blue colonies without the halo. These species lack the enzyme phospholipase C. Rapid 'L. mono agar, an enhanced ALOA medium, employs PI-PLC to hydrolyze a novel substrate, 5-bromo-4-chloro-3-indolyl-myo-inositol-1-phosphate, according to Ottaviani and Agosti (ALOA Agar), although it still requires the same set of enzymes as Agar *Listeria* (Osek et al., 2022).

Because the Rapid 'L.mono agar medium contains xylose, a sugar that *L. ivanovii* can metabolize and which results in blue colonies with a yellowish halo, blue colonies of *L. monocytogenes* are easily identifiable and can be told from other *Listeria* spp. *L. monocytogenes*' colonies appear blue instead of haloed because it is unable to digest this sugar (Zunabovic et al., 2011).

One of the ALOA variants of chromogenic medium that is widely used to identify *L. monocytogenes* is CHROMagar™ *Listeria*. While other *Listeria* spp. colonies display a blue hue without a halo, *L. monocytogenes* colonies display a blue color with a white halo on this medium. It should be mentioned that on CHROMagar™ *Listeria*, certain strains of *L. ivanovii* can also form blue colonies with a white halo.

Any chromogenic medium works well for the rapid isolation and division of *L. monocytogenes* from other nonpathogenic *Listeria* spp. These media's specificity may vary depending on the food samples examined (Osek et al., 2022; Law et al., 2015).

Table 3*Features of Typical Isolation Media for L. monocytogenes and Listeria spp. Colonies*

	cultures media type	Colony properties of <i>Listeria</i> spp.	Colony properties of <i>L. monocytogenes</i>
According to the activity of phosphatidylinositol phospholipase C	ALOA	<i>(L. ivanovii)</i> : spherical, blue-green colonies that have a halo. <i>(Other Listeria)</i> : Regular spherical colonies in shades of blue-green without or with a halo.	colonies with an opaque halo and a blue-green color.
	Rapid' L. mono	<i>(L. ivanovii)</i> : colonies that are blue-green with a yellow halo. <i>(Other Listeria)</i> : white ,Whether or not it has a yellow halo.	Blue colonies, ranging from light blue to dark blue.
	CHROM™ agar Listeria	<i>(L. ivanovii)</i> : blue ,with a white halo. <i>(Other Listeria)</i> : blue without halo.	Blue with white halo
According to the aesculin's hydrolysis	OXA/MOX	Black with black halo at 24 hours Remain black after 48 hours with a black halo but a sunken center.	Olive-green with a black halo at 24 hours develop darker after 48 hours, with a hollow black center surrounding by black zones.
	PALCAM	Grey-green with a black halo.	Grey-green with a black zone.

Source: Magalhães et al., 2014

1.10 Biological and Cultural Confirmation

Biochemical and phenotypic indications were crucial to early identification techniques, and they are being used extensively today. The majority of selective agars use the esculinase reaction, which is based on β -D-glucosidase activity, to separate and identify *Listeria* from other bacteria. *Listeria* colonies typically have a black color and a black zone surrounding them in the medium. Moreover, the selection agents contained in these media inhibit the development of the majority of other species (Gasarov et al., 2005).

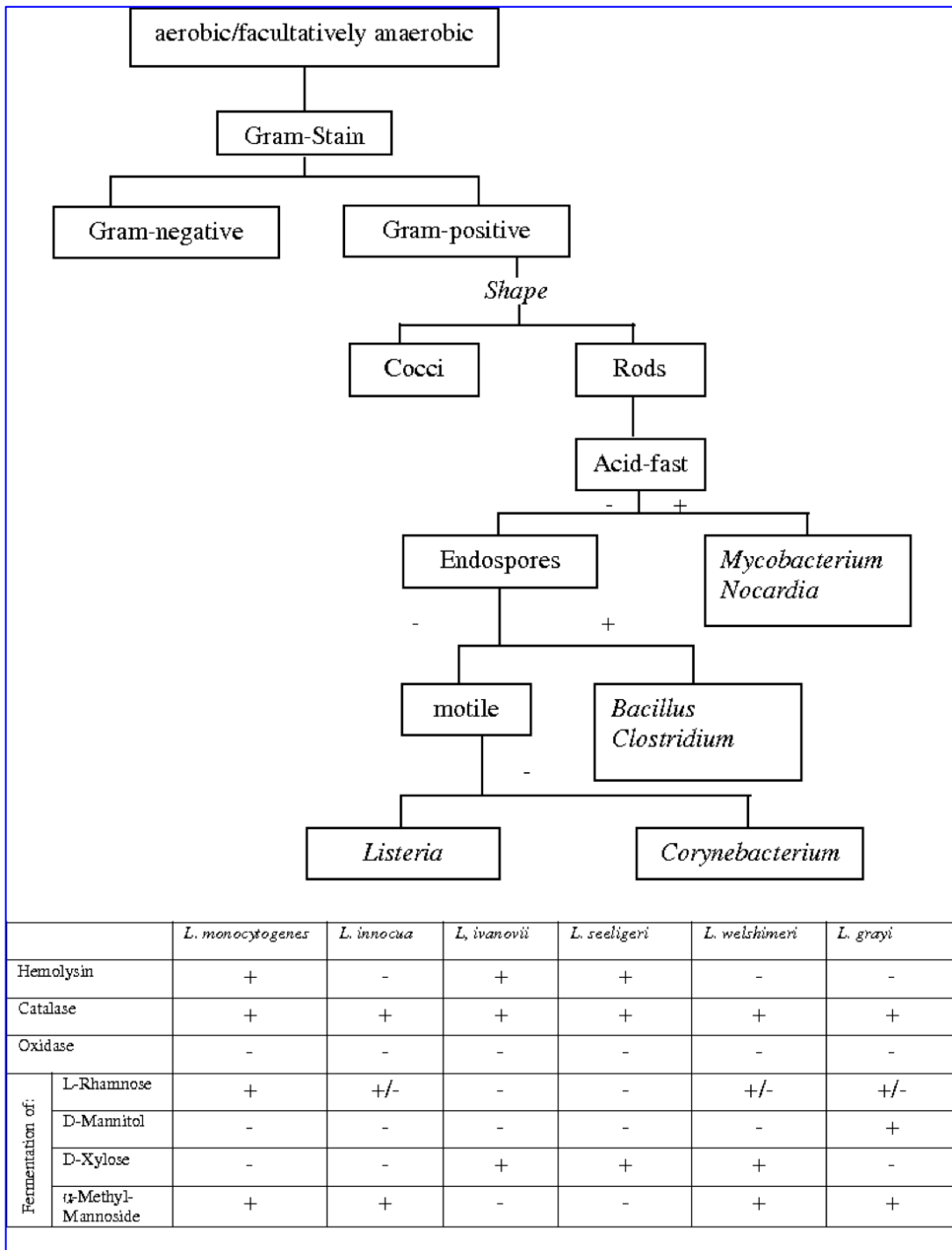
Certain species, such as *Enterococcus* and *Bacillus* spp., will grow on specific plates. Use esculin as well, and they may appear to be the same. Doubtful selective agar colonies must first be checked for purity before *Listeria* colonies can be positively identified through further testing. Traditionally, tryptose soy agar plates containing dubious cultures were used to view *Listeria* colonies under oblique lighting. This technique gives the colonies a distinctive blue-green color and makes them appear reticulated (Gasarov et al., 2005).

Currently, dubious bacteria are frequently classified as *Listeria* if they exhibit the properties listed in (Figure.2) Rods with a gram-positive color that ferment carbohydrates and release acid without gas.

They are also non-spore-forming, facultatively anaerobic, aerobic, oxidase negative, and catalase positive. Most strains are motile at 28 °C and at 37 °C, they are not. Traditional biochemical testing, which uses assays that evaluate how much sugar is utilized to identify between species, takes a week. Commercial identification kits are a common substitute for this method. Test strips like as Micro-IDTM and API *Listeria* have undergone extensive validation and are now considered standard procedure (Gasarov et al., 2005).

Figure 2

Methodological framework for *Listeria* phenotypic identification.



1.11 Molecular Detection Techniques for *Listeria Monocytogenes* Detection

1.11.1 Polymerase Chain Reaction (PCR)

Molecular techniques are a well-established substitute for traditional culture methods in the identification of bacteria in foods and food production (Gasarov et al., 2005). To find target-specific DNA sequences, this technique can be applied using (PCR), (multiplex PCR), (multiplex q-PCR), (real-time PCR), (next-generation sequencing), (quantitative PCR), and DNA microarray (Osek et al., 2022; Law et al., 2015). Molecular techniques are quite delicate, however, they are sometimes hampered by various dietary additives. DNA amplification techniques are unable to distinguish between the target microorganism's living and dead cells.

Furthermore, enrichment is necessary to boost the number of live microorganisms and lessen potential inhibitors (Janzten et al., 2006). Many PCR techniques have been utilized to detect pathogens, like *L. monocytogenes*, which are microorganisms found in food (Gasarov et al., 2005; Law et al., 2015).

Application of a thermodynamic polymerase reaction and 2 single-stranded synthetic oligonucleotides, this technique magnifies a specific goal DNA chain in 3 steps utilizing a thermal cycle (Law et al., 2015; Osek et al., 2022). After being separated by electrophoresis on agarose gels, the PCR amplification result is dyed differentially using a DNA dye. PCR is widely used to identify *L. monocytogenes* following a 24- to 48-hour selective enrichment period (Gasarov et al., 2005). Many targeted sequences exist for the identification of particular *Listeria* spp. All strains of *Listeria* include the highly conserved 16S rRNA sequence, which frequently serves as the basis for (PCR) primers (Law et al., 2015).

Finding sequence variations in the amplified 16S rRNA gene and identifying the virulence genes unique to *L. monocytogenes* provide for the possibility of differentiating this species from other *Listeria* spp. These pathogenic molecular markers were identified in *L. monocytogenes* and are intended to be detected by PCR. They include the *hlyA* gene, which produces the surface protein *ActA* and is implicated in cell invasion, the *iap* gene, which produces the invasion-associated protein p60, and the *actA* gene (Osek et al., 2022).

One of the more targeted sequences for *L. monocytogenes* PCR detection is the *hlyA* gene. Listeriolysin O (LLO), a protein linked to pore-forming activity, is encoded by it. The *hlyA* gene is an important genetic marker for differentiating pathogenic strains of *L. monocytogenes* since it encodes a hemolytic activity that is found in all clinical isolates of the bacteria (Osek et al., 2022).

1.11.2 Multiplex PCR

Using multiplex PCR, several target genes are amplified at the same time (Law et al., 2015). Although the principles of mPCR are fairly similar to those of classical PCR, a number of important considerations must be taken into account when designing a particular and accurate multiplex assay. A reliable amplification product can only be produced by a combination of factors, one of which is the primer design, primer concentration in the reaction mixture, and primer annealing temperature (Zhao et al., 2014).

Other important factors are the quantity of the PCR buffer, deoxynucleotide, magnesium chloride, and templates, as well as the temperatures encountered throughout the amplification steps (Law et al., 2015).

It is possible to identify multiplex PCR or to simultaneously differentiate between several *L. monocytogenes* virulence-associated genes. by focusing on different genes specific to each species using a single PCR tube (Ryu et al., 2013). mPCR was developed using primers that targeted three virulence-related genes in *L. monocytogenes*, and it was effectively used to identify the pathogen in milk. developed a new mPCR that targets the *inlA*, *inlC*, and *inlJ* genes in order to classify *L. monocytogenes* based on pathogenicity and species (Osek et al., 2022).

1.11.3 Real-Time Polymerase Chain Reaction (RT-PCR)

The primary distinction between the two techniques is that, in contrast to conventional PCR, gel electrophoresis is not required for quantitative or real-time PCR in order to identify the outcomes of DNA amplification. Certain intercalating pigments or dual-labeled sensors are employed in the process to generate a fluorescence emission signal that may be used to measure the number of PCR products produced throughout the reaction (Law et al., 2015).

Because the amount of amplicons for PCR is closely associated with the fluorescence intensity, real-time amplification monitoring may be achieved without the tedious and time-consuming gel electrophoresis (Zhao et al., 2014). Real-time PCR can make use of SYBR® Green, the most widely used fluorescent dye specific to double-strand DNA that binds to the minor groove of the DNA double helix.

Compared to other fluorescent dyes, SYBR® Green has a significantly larger sign fluorescent, but it too suppresses PCR and has a lower reproducibility, range dynamic narrower, and less repeatability. Because SYBR® Green-based real-time PCR is automated, extremely sensitive, and capable of detecting even minuscule quantities of target DNA, it diminishes the potential of (false positive) outcomes created by the potential of cross-contamination among amplification products.

Additionally, PCR products are not manipulated after amplification. There are various rivals to SYBR® Green dye in real-time techniques, including the TaqMan® probes made by Applied Biosystems (Osek et al., 2022). The probes are oligonucleotides with a reporter dye covalently coupled to the 5' end and a quenching dye attached to the 3' end, both of which are fluorophores. A fluorophore-based detection signal is produced when Taq DNA polymerase's exonuclease activity cleaves a dual-labeled probe during hybridization to the corresponding target sequence.

A certain nucleotide sequence in one of the strands of the amplicon that is internal to both primers is complementary to the TaqMan® probe. As an alternative to SYBR® Green dye or TaqMan® probe in real-time PCR, a molecular beacon, or probe with a hairpin/stem-and-loop arrangement, is available (Osek et al., 2022).

The probe's loop segment contains the complementary sequence to a target sequence. The TaqMan® probe needs the polymerase's exonuclease activity, even though molecular beacons can hybridize with template DNA during annealing and go through a spontaneous conformational shift that splits the two dyes and produces the fluorescent signal directly (Osek et al., 2022).

To identify *L. monocytogenes* in various food items, real-time PCR was utilized. To accurately identify *L. monocytogenes* and to differentiate it from other non-pathogenic species of *Listeria*, different target genes were used. ActA is a significant virulence determinant that is expressed by one of them, the actA gene. Actin polymerization, intracellular motility, and cell-to-cell dispersion of the bacteria all depend on ActA. Using

5'-nuclease real-time PCR, this target gene was used to detect and quantify *L. monocytogenes* in food (Osek et al., 2022). *L. monocytogenes* was discovered in fresh vegetables using a molecular beacon-q-PCR approach to target the *hlyA* gene, which is in charge of the synthesis of listeriolysin O (LLO) (Osek et al., 2022).

1.11.4 Nucleic acid sequenced-based amplification (NASBA)

The selective replication of nucleic acid sequences under isothermal circumstances is accomplished by nucleic acid sequence-based amplification, a transcription-based amplification technique (Guatelli et al., 1990). After RNA is amplified via NASBA, reverse transcriptase transforms the resulting single-stranded RNA into complementary DNA (cDNA) (Osek et al., 2022).

Nucleic acid sequence-based amplification is a very quick diagnostic technique that doesn't require any specific equipment and only takes four to five cycles to produce up to roughly 10^6 – 10^9 amplified RNA copies in 1.5 hours (Osek et al., 2022).

Through mRNA amplification and the identification of RNA targets, the later detection approach may identify living microorganisms and differentiate between viable and nonviable bacterial cells, both of which are crucial in food testing (Dwivedi & Jaykus, 2011). Blais et al. (1997) developed a particular NASBA technique for the detection of *L. monocytogenes* based on the *hlyA* mRNA sequences.

Following 48 hours of enrichment, the assay demonstrated 92.6% specificity in detecting low concentrations of the bacteria (<10 CFU/g) in artificially contaminated dairy and egg products. The detection of the *L. monocytogenes hlyA* gene in smoked salmon was also accomplished using a different NASBA test in conjunction with molecular beacon-based real-time (Osek et al., 2022).

NASBA using 16S rRNA sequences as the goal also identified *L. monocytogenes*. Utilizing 25 g food samples and enrichment for 48 hours before NASBA detection, the technique was also contrasted with a modified FDA culture method for the detection of *L. monocytogenes* in foods, and the outcomes were the same for both approaches (Uyttendaele et al., 1995). BioMerieux has produced a NASBA kit for the detection of *L. monocytogenes* (NucliSENS EASYQ®). It is an automated method that uses molecular beacons for real-time detection and NASBA amplification (Osek et al., 2022).

Chapter Two

Methodology

2.1 Food Samples

One hundred fifty samples of raw vegetables from several governorates in the West Bank (south, middle, and north) were collected between March to June 2023. Table 4 represents the number of vegetable samples (Lettuce, Parsley, Spring Onion, Watercress, and Carrot Juice) taken from supermarkets and street food in different locations.

Table 4
Distribution of food samples throughout the several governorates

governorate's location	The northern				The middle		southern			
	Tulkarem		Nablus		Ramallah		Hebron		Bethlehem	
Type of food sample	HM	SF	HM	SF	HM	SF	HM	SF	HM	SF
Lettuce	–	5	2	3	4	6	4	1	5	–
Parsley	–	5	–	5	4	6	4	1	5	–
Watercress	3	2	2	3	4	6	3	2	5	–
Spring Onion	5	–	5	–	4	6	5	–	5	–
Carrot Juice	3	2	5	–	10	–	5	–	5	–
Total number from each governorate	50				50		50			
Total number of food samples					150					

Where: HM means hypermarket. SF means street food.

2.2 Detection and Isolation *L. monocytogenes*

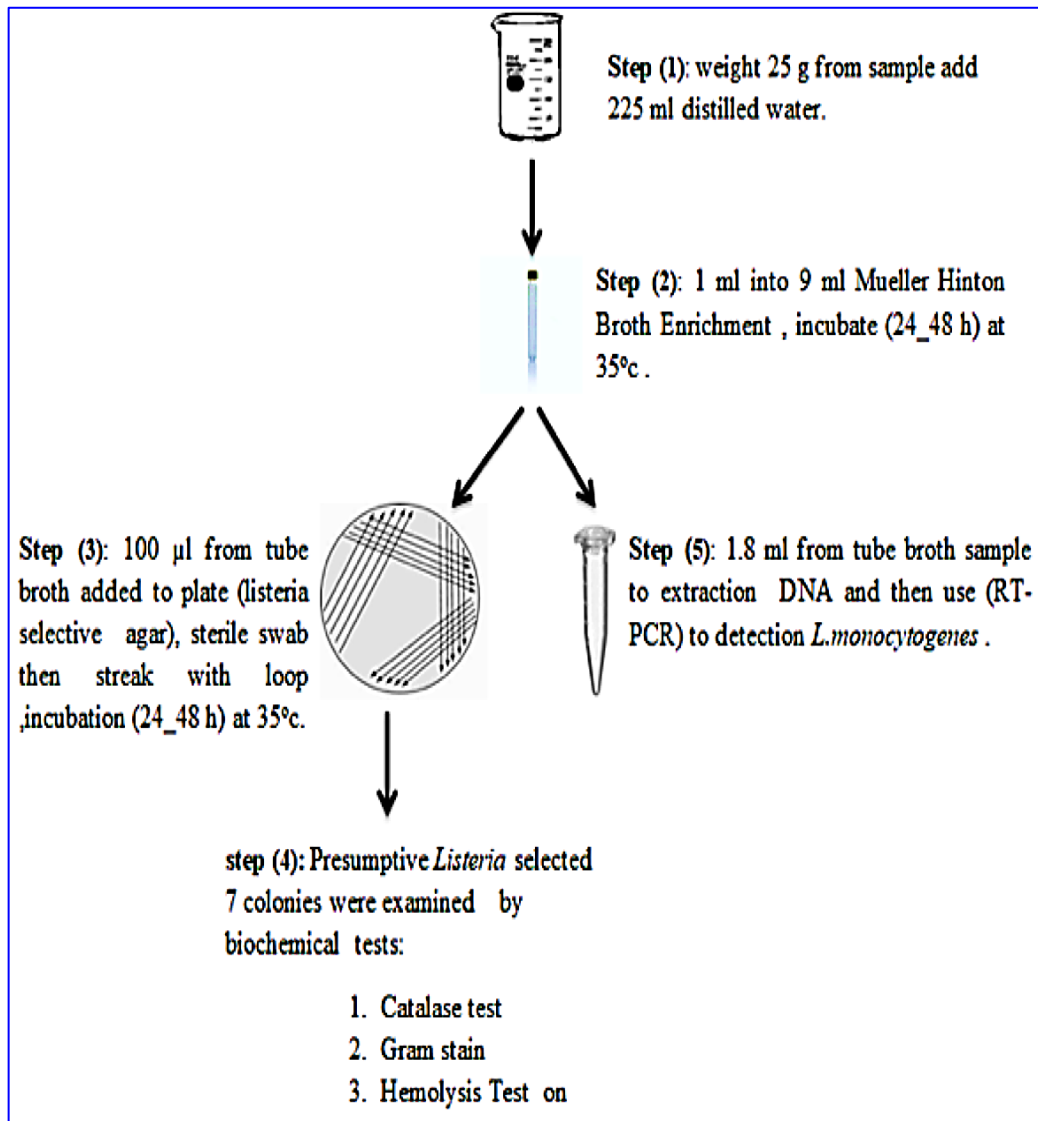
L. monocytogenes was isolated and identified in this investigation using the ISO 11290 method. To facilitate further processing while maintaining aseptic handling, all experimental tests and circumstances, including sampling, were conducted in the scientific research lab of the Faculty of Agriculture and Veterinary Medicine at An-Najah National University, Tulkarem, Palestine. If necessary, samples will be carefully cleaned to get rid of any apparent soil and organic matter. Briefly, 225 ml of distilled water and 25 g of samples were combined in a beaker. After that, (1 ml) was micropipetted into a tube of Muller-Hinton broth (9ml) as the first enrichment culture and incubated at 35 °C for (24 - 48 h). *Listeria* selective agar (Oxford formulation) was streaked with a loopful of broth after it had been treated with antibiotics and incubated for 24 to 48 hours at 35 °C. Re-streaked presumed *Listeria* colonies were cultured for 24 to 48 hours at 35 °C on *Listeria* selective agar look at (Figure 3) below shows the steps detection and isolation of *L. monocytogenes*.

2.3 Confirmation of presumptive *Listeria* colonies by biochemical tests

Presumptive *Listeria* selected 7 colonies were examined biochemically using the following tests: catalase test, gram staining, hemolysis test on blood agar, and confirmed by real-time polymerase chain reaction (RT-PCR). RT-PCR was used to confirm the colonies of *Listeria* and to compare with the results of conventional culturing.

Figure 3

Flowchart of *L. monocytogenes* isolation and detection using ISO 11290 technique



Source: Lynne, 2005

2.4 DNA Isolation

In order to isolate high-quality genomic DNA from bacteria grown from food, the powerfood® microbial DNA Isolation kit was developed in accordance with FDA requirements (Bacteriological Analytical Handbook, Edition 8, Revision A / 1998). With the use of our exclusive Inhibitor Removal Technology® (IRT) and reformulated buffers, the kit produces high-quality, inhibitor-free DNA for use in PCR and qPCR, among other downstream applications. Real-time quantitative PCR has been used to recover and identify DNA from a variety of known food pathogens using the PowerFood Microbial DNA Isolation Kit.

2.5 Required Equipment

1. BagMixer® 400 VW
2. BagMixer® 400 Bags
3. Microcentrifuge (13,000*g)
4. Pipettors
5. Vortex-Genie® 2 Vortex (MO BIO Catalog# 13111-V or 13111-V-220)
6. Vortex Adapter for 2 ml Tubes (MO BIO Catalog# 13000-V1-24)

2.6 PowerFood® Microbial DNA Isolation Protocol

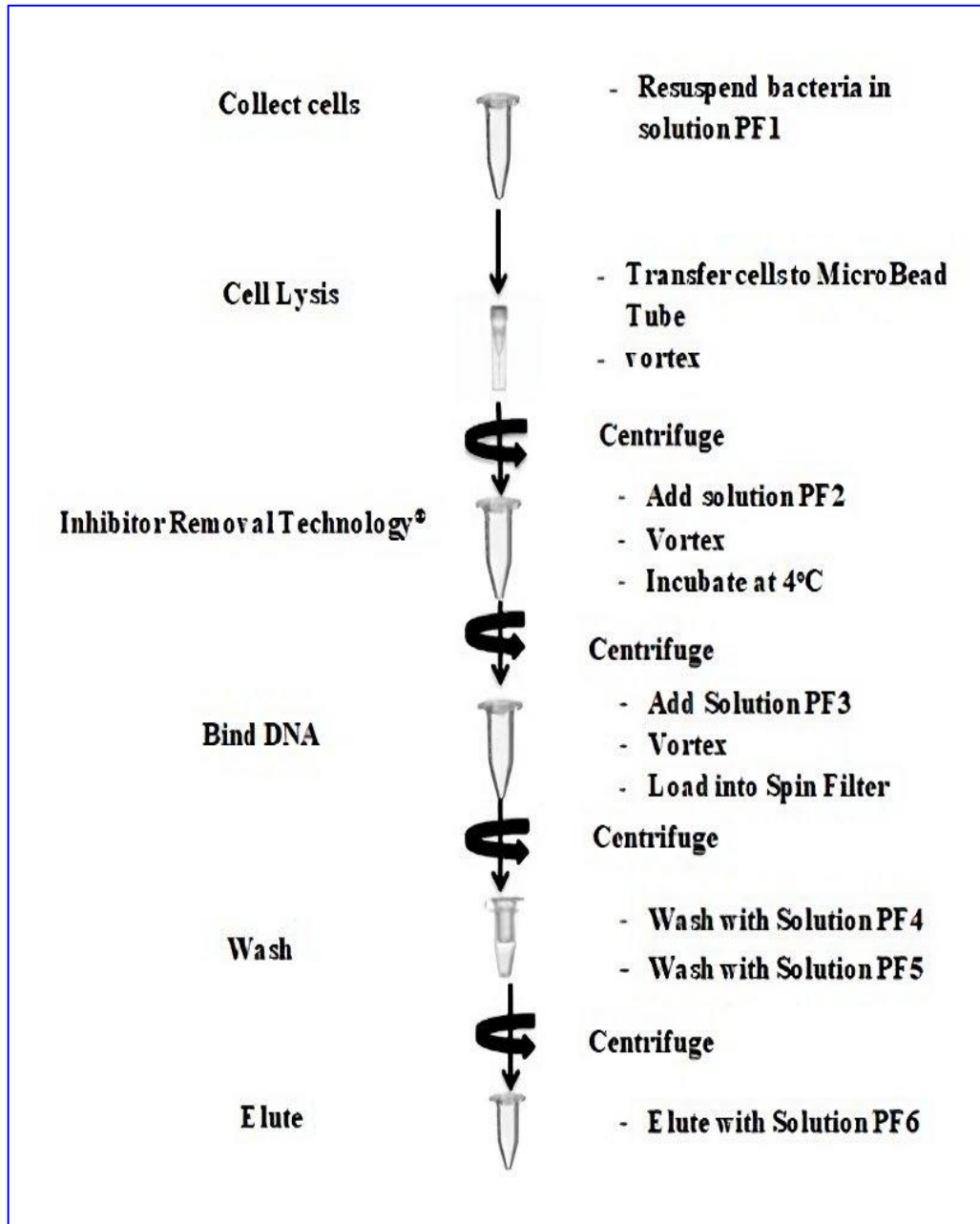
Stage one includes heating of solution buffers. Solution buffer 1 (PF1) was heated at 55°C for (5-10 minutes), and checked was the heated of Solution buffer 3 (PF3) and heated at 55°C for 5-10 minutes. In stage two, the food sample was homogenized using a lab blender, following FDA guidelines. In stage three, the microbial food culture was prepared by adding 1.8 ml of microbial food culture to a 2 ml collection tube. The supernatant then was removed using a centrifuge at 13,000 x g for 1 minute. In stage four, the cell pellet is resuspended in 450 µl of warmed Solution PF1. In stage five, the process is performed in a MicroBead Tube. Cells were transferred to a MicroBead Tube, vortexed at maximum speed for 10 minutes, and then centrifuged at 13,000 x g for 1 minute. The supernatant is then shifted to a clean collection tube. In stage sixth, treatment with solution buffer 2 (PF2) and solution buffer 3 (PF3). 100 µl of the solution PF2 was added and vortex at 1 minute. Incubate at 4°C for 5 minutes followed. Then the centrifuge at 13,000 x g for 1 minute.

The supernatant was transferred to a clean Collection Tube. 900 µl of Solution PF3 is added, and vortex to mix. Stage seven includes the Spin Filter Loading and Washing. 650 µl of supernatant is loaded onto a Spin Filter and centrifuged at 13,000 x g for 1 minute. The flow-through was removed, and the process was repeated until all supernatant was loaded onto the spin filter. The Spin Filter basket was placed into a clean Collection Tube. 650 µl of Solution buffer 4 (PF4) is added, and centrifuged at 13,000 x g for 1 minute. Then, the flow-through is removed. Then 650 µl of Solution buffer 5 (PF5) is added and centrifuged at 13,000 x g for 1 minute. Then, the flow-through is removed, and to confirm the removal of residual wash, the centrifugation was repeated. In stage eight, DNA elution. the Spin Filter basket is placed into a clean Collection Tube. Then 100 µl of

Solution buffer 6 (PF6) is added to the center of the white filter membrane. A centrifuge was used. In the final stage, the Spin Filter basket was discarded, and the DNA was ready for downstream applications. Eventually, DNA is stored frozen at -20°C look below (Figure. 4) represents a summary of the procedures for using the Powerfood® microorganism DNA Isolation kit (MO BIO, Carlsbad, CA, USA).

Figure 4

The procedures for using the Powerfood® microorganism DNA Isolation kit



Source: MO BIO, Carlsbad, CA, USA

Solution buffer 1 (PF1) . Solution buffer 2 (PF2) . Solution buffer 3 (PF3)

Solution buffer 4 (PF4) . Solution buffer 5 (PF5) . Solution buffer 6 (PF6) .

2.7 Real-Time Polymerase Chain Reaction (RT-PCR)

Comparatively speaking The GoTaq® qPCR Master Mix kit, which was utilized for Real-Time qPCR analysis (BIO-RAD, Hercules, CA, USA), was provided by CFX96 Touch™ Madison, Promega, USA, CA. The 20µl reaction mix was made with 2 µl of each of the forward and reverse primers pm/µl, 2 µl of sample DNA, 4 µl of nuclease-free water, and 10 µl of the GoTaq® qPCR Master Mix (2X). The cycling conditions were 1 minute at (95°C), 40 cycles at (95°C) for 10 seconds, 30 seconds at (60°C), and 10 seconds at (72°C) with a final melting at (95°C) for 20 seconds

.

Table 5*Primers Selected For Detection of L. monocytogenes*

No.	Target gene	Primers	Primer sequence	Size of amplified product (bp)	References
1	16S rRNA	LM3	5'GGACCGGGGCTAATACCGAATGATAA3'	1200	(Wiedmann et al., 1995).
		LM5	5'TTCATGTAGGCGAGTTGCAGCCTA3'		
2	Hemolysin	HLY 1	5'-ATTTCCCTTCACTGATTGC-3'	276	(Cooray et al., 1994).
		HLY2	5'-CACTCAGCATTGATTTGCCA-3'		
3	Hemolysin	ILMHLYF	5'-GCAATTTTCGAGCCTAACCTA-3'	188	(Klein & Juneja, 1997).
		ILMHLYR	5'-ACTGCGTTGTTAACGTTTGA-3'		

The preferred approach for identifying and discriminating microorganisms due to the many copies (10^4) of rRNA present in a cell is the use of the 16S rRNA gene as a molecular marker for microbial identification and classification (Kumar et al., 2015).

2.8 Statistical Analysis

The gene expression data was presented as means \pm standard error. Using IBM SPSS Statistics 20 software, a chi-squared test was used to compare the various parameters in each group (IBM, Chicago, USA). At ($p < 0.05$), differences were deemed significant.

Chapter Three

Results

3.1 Isolation and detection of *L. monocytogenes* on selective agar and biochemical tests

In this study, we used listeria-selective Oxford agar media to isolate and differentiate *L. monocytogenes*. Out of 150 fresh vegetable samples examined, 71% tested positive for *L. monocytogenes* based on the media's selectivity and the colonies' morphological characteristics. Look at (Figure. 5) shows the growth of *L. monocytogenes* on Listeria selective agar. The *L. monocytogenes* percentage distribution in the fresh vegetables was (66%) from lettuce, watercress (70%), and spring Onion (70%), carrot juice (73%), parsley (73%).

3.2 Confirmation of selected colonies using RT-PCR methods

7 presumptive colonies underwent confirmation through biochemical tests, revealing positive results for gram staining as positive gram short rods under the microscope. Look at (Figure .6) shows the gram stain results of *L. monocytogenes* from vegetable samples and also positive results the catalase test, and β -hemolysis; look at (Figure .7) shows the hemolysis on blood agar media by selected *Listeria* colonies.

However, an interesting outcome emerged when we progressed to molecular detection using RT-PCR for identifying *L. monocytogenes* pathogenic strains. Specifically, we used two pairs of primers targeting specific variants within the Listeriolysin O gene (LLO), known as a virulence factor associated with the bacterium's pathogenicity. Despite the positive results from biochemical tests, none of the seven isolates that were chosen had the Listeriolysin O (LLO) gene detected as seen in Table 6.

Figure 5

Growth of L. monocytogenes after 24 hours of incubation on Listeria selective agar

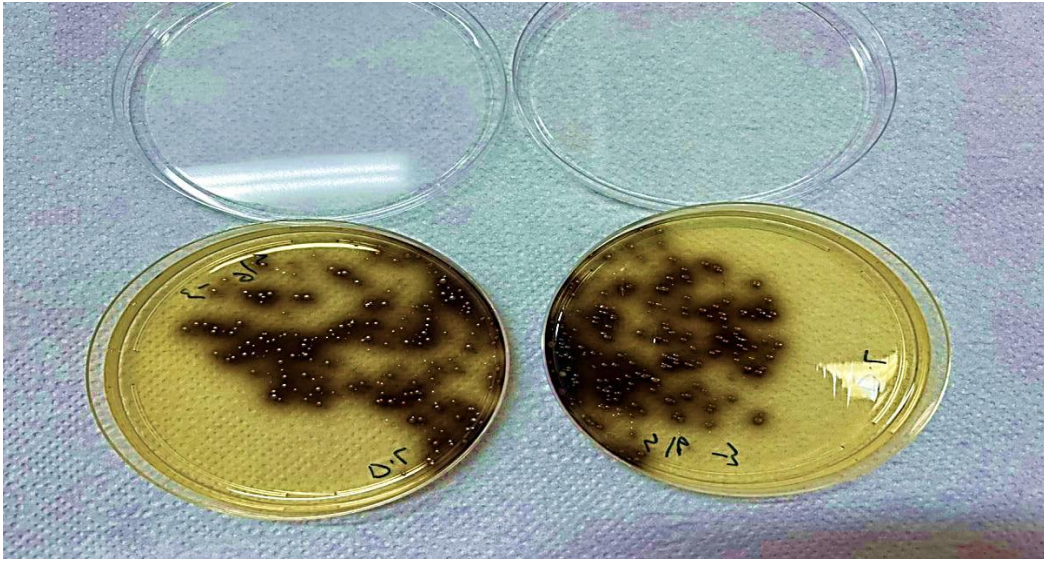


Figure 6

Gram Stain Results of L. monocytogenes From Vegetable Samples

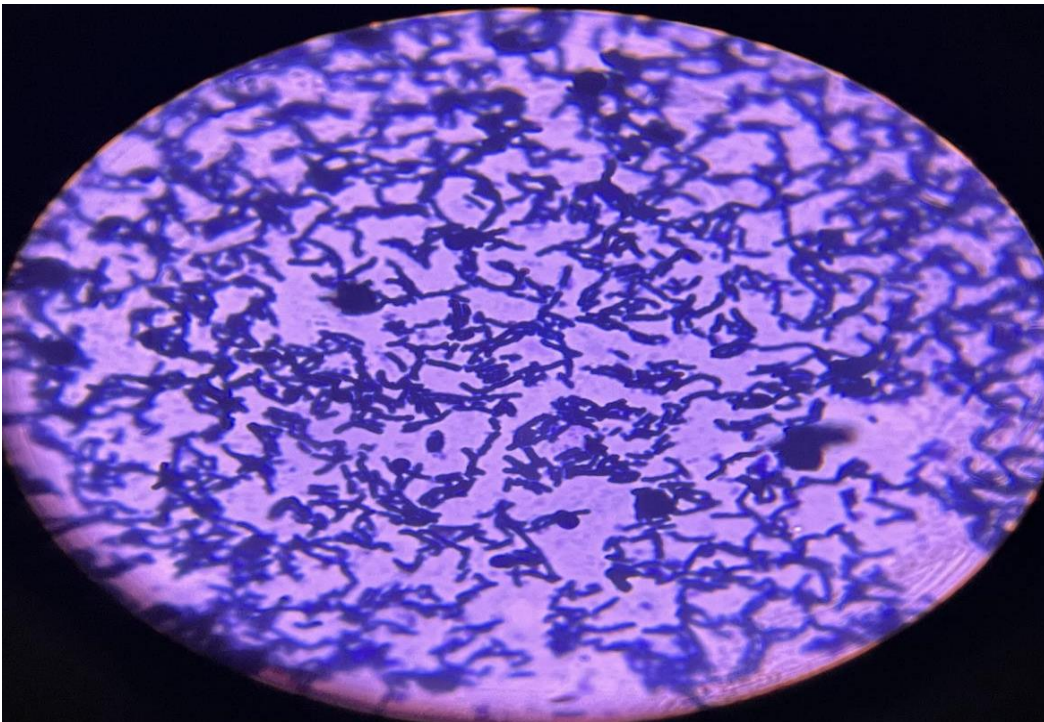


Figure 7*Hemolysis on Blood Agar Media by Selected Listeria Colonies***Table 6***Biochemical Tests for L. monocytogenes and Confirmation by Real-Time Polymerase Chain Reaction (RT-PCR)*

Colonies	Type sample	Catalase test	Gram staining	Hemolysis test	HLV1/2ILMHLYF/R	HF9/H R9
Colony (1)	Lettuce	+	Short rod	β -hemolysis	-	-
Colony (2)	Parsley	+	Short rod	β -hemolysis	-	-
Colony (3)	Lettuce	+	Short rod	α hemolysis	-	-
Colony (4)	Carrot juice	+	Short rod	α hemolysis	-	-
Colony (5)	watercress	+	Short rod	β -hemolysis	-	-
Colony (6)	watercress	+	Short rod	β -hemolysis	-	-
Colony (7)	spring onion	+	Short rod	α hemolysis	-	-
Colony (H)	Animal product	+	Short rod	β -hemolysis	+	-

Key: (+) positive; (-) negative; (α) partial hemolysis; (β) complete hemolysis.

Where colonies from (1-7) are selected presumptive colonies on selective media while colony H was used as a reference.

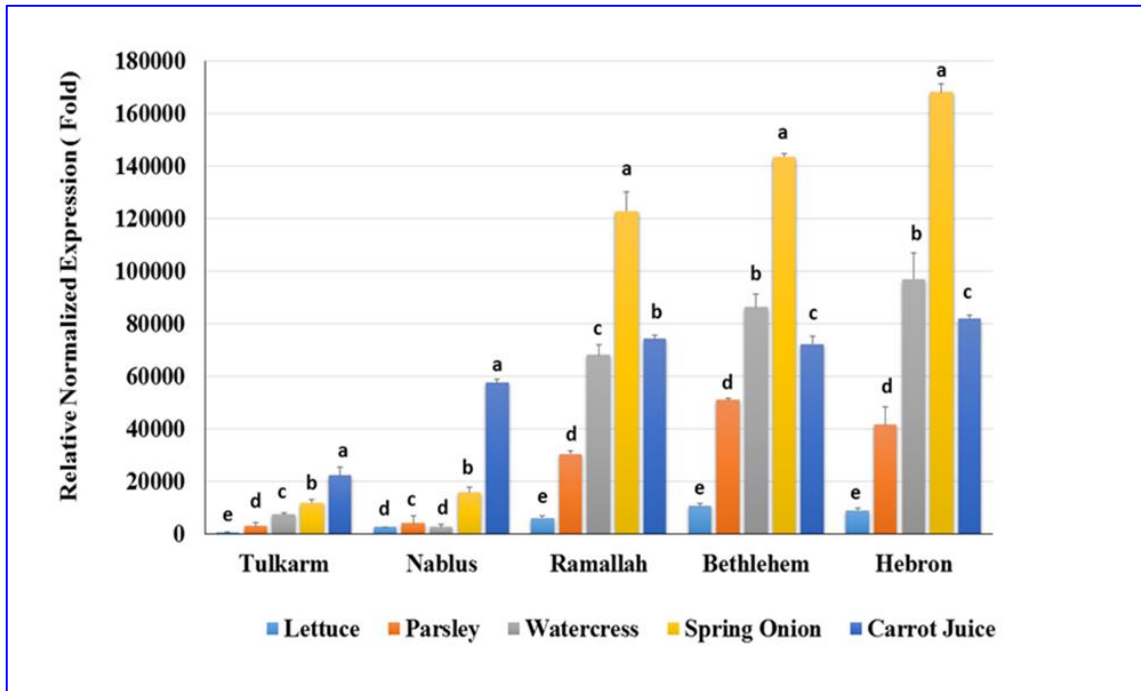
3.3 Comparison of *L. monocytogenes* gene expression levels in vegetable samples within the same governorate

The statistical analysis in results of this study revealed significant differences in *L. monocytogenes* levels across different vegetable samples within the same governorate. Tulkarm governorate had the highest proportion of *L. monocytogenes* in carrot juice, followed by spring onions, watercress, and parsley, and the lowest percent in lettuce, according to real-time PCR) results. Additionally, Nablus governorate registered had the highest proportion in carrot juice, followed by spring onion, parsley, and lowest level in same both watercress and Lettuce.

Additionally, Ramallah governorate registered had the highest proportion in Spring onion, followed by carrot juice, watercress, parsley, and lowest level in Lettuce. Then, Bethlehem governorate registered had the highest proportion in Spring onion, followed by watercress, carrot juice, parsley, and lowest level in Lettuce. Additionally, Hebron governorate registered had the highest proportion in Spring onion, followed by watercress, carrot juice, parsley, and lowest level in Lettuce. Look at below (Figure 8) shows Comparison of *L. monocytogenes* gene expression levels in different samples within each city.

Figure 8

Comparison of *L. monocytogenes* gene expression levels in different samples in each city



Note: Folds (a-e) with distinct superscripts exhibit a significant difference at ($P < 0.05$)

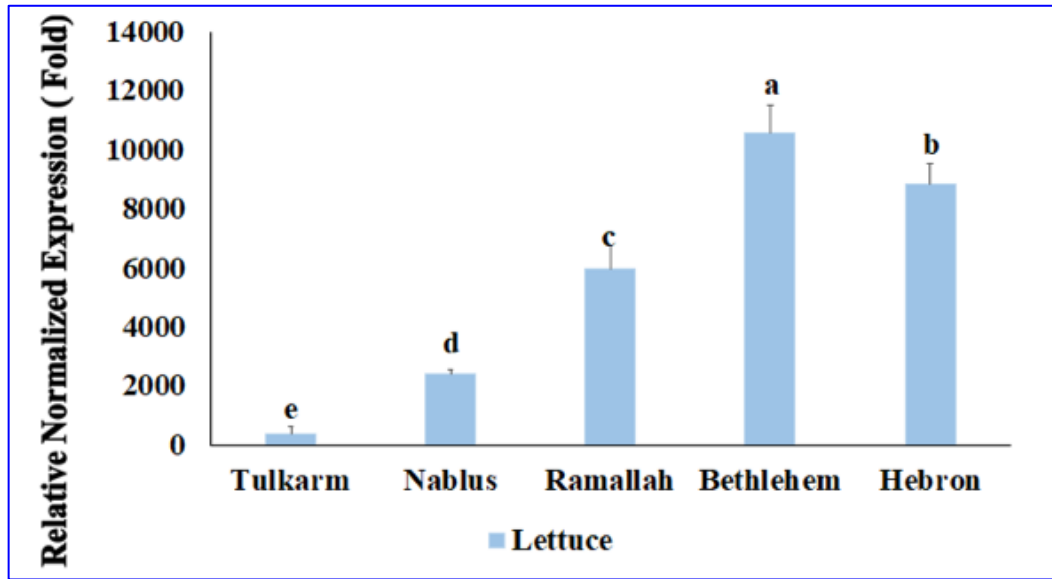
3.4 Comparison of *L. monocytogenes* gene expression levels between cities according to the food item.

a) Lettuce

Lettuce registered of the highest percent of *L. monocytogenes* in Bethlehem Governorate, followed by Hebron, Ramallah, and Nablus, and the lowest percent in Tulkarm (Bethlehem > Hebron > Ramallah > Nablus > Tulkarm) with significant differences ($p < 0.05$) between cities look at (Figure 9) shows gene expression analysis of *L. monocytogenes* in lettuce in different governorate of Palestine.

Figure 9

Gene expression analysis of *L. monocytogenes* in lettuce in different governorate of Palestine



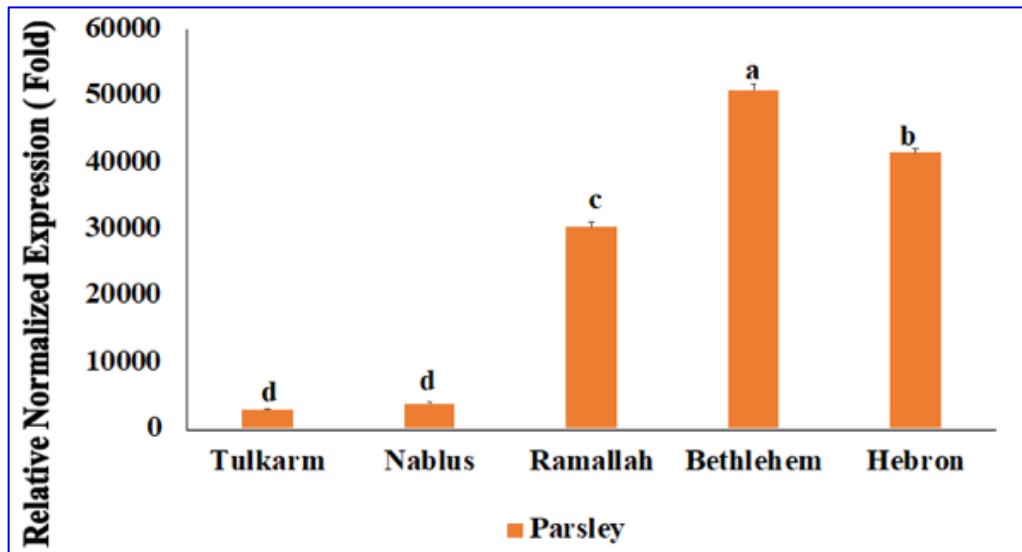
Note: Folds (a-e) with distinct superscripts exhibit a significant difference at ($P < 0.05$)

b) Parsley

parsley registered of the highest percent of *L. monocytogenes* in Bethlehem Governorate, followed by Hebron, Ramallah, and the same level least in both Nablus and Tulkarm. (Bethlehem > Hebron > Ramallah > Nablus and Tulkarm) with significant differences ($p < 0.05$) between cities look at (Figure 10) shows gene expression analysis of *L. monocytogenes* in parsley in different governorate of Palestine.

Figure10

Gene expression analysis of *L. monocytogenes* in parsley in different governorate of Palestine



Note: Folds (a-d) with distinct superscripts exhibit a significant difference at ($P < 0.05$)

c) Watercress

watercress registered of the highest percent of *L. monocytogenes* in Hebron Governorate, followed by Bethlehem, Ramallah, Tulkarm and the lowest level in Nablus. (Hebron > Bethlehem > Ramallah > Tulkarm > Nablus) with significant differences ($p < 0.05$) between cities look at (Figure 11) in appendix c shows gene expression analysis of *L. monocytogenes* in watercress in different governorate of Palestine.

d) Spring onion

Spring onion registered a higher percent of *L. monocytogenes* in Hebron Governorate, followed by Bethlehem, Ramallah, Nablus and the lowest level in Tulkarm. (Hebron > Bethlehem > Ramallah > Nablus > Tulkarm) with significant differences ($p < 0.05$) between cities look at (Figure 12) in appendix c shows gene expression analysis of *L. monocytogenes* in spring onion in different governorate of Palestine.

e) Carrot juice

Carrot juice registered of the highest percent of *L. monocytogenes* in Hebron Governorate, followed by Ramallah, Bethlehem, Nablus and the lowest level in Tulkarm. (Hebron > Ramallah > Bethlehem > Nablus > Tulkarm) with significant differences ($p < 0.05$) between cities look at (Figure 13) in appendix c shows gene expression analysis of *L. monocytogenes* in carrot juice in different governorate of Palestine.

3.5 Matching results of culture confirmation Assay and real-time polymerase chain reaction

In this study, evaluated matching the outcomes of culture confirmation assays and real-time polymerase chain reaction (RT-PCR) on 106 samples. The findings indicated that between the results of the real-time PCR and the culture confirmation, there was a 53% matching rate (56 out of 106) and a 47% non-matching percentage (50 out of 106) respectively as seen in (figure .14) in appendix c shows matching and non-matching results between two methods culture and (RT-PCR).

Chapter Four

Discussion, Conclusion & Recommendations

4.1 Discussion

The present study is the initial one to utilize molecular techniques, specifically real-time polymerase chain reactions (RT-PCR), to detect and confirm the existence of the pathogenic bacteria *Listeria monocytogenes* in vegetables and determine how common it is in governorates West Bank /Palestine.

The World Health Organization (WHO) advises individuals to eat 400 grams or more of fruits and non-starchy vegetables every day, and because vegetables are so high in nutrients, they should be a significant part of a person's diet (Dardona et al., 2021), particularly in light of the rising rates of obesity and coronary heart disease in developing nations. It is advised to eat vegetables due to their low-calorie content and easy to prepare and serve (Elmali et al., 2013). It is believed that vegetables may increase one's chance of contracting foodborne illnesses, specifically (listeriosis) (Jamali et al., 2013), primarily while eating these vegetables raw or very mildly cooked (Dardona et al., 2021). Due to poor agricultural practices, like the usage of contaminated irrigation water, improper organic fertilizers, failures during harvesting, treatment, and delivery, and improper treatments during washing, transporting, and storing of vegetables, vegetables are extremely vulnerable to microbiologic contamination (Cordano & Jacquet, 2009). Vegetables have short shelf life when eaten raw, fresh vegetables could then pose a significant risk for human health (Dardona et al., 2021).

It's a popular misconception that fresh vegetables sold in supermarkets are safer to eat than those sold by street sellers. Because they frequently offer vegetables in less-than-ideal circumstances that seem cleaner than street food. Although using refrigerators and air conditioners may seem beneficial for preserving vegetables, the level of cleanliness in the supermarket to prevent contamination of vegetables is questionable (Jamali et al., 2013).

This study focuses on investigating the presence of *L. monocytogenes* in various types of vegetables selected from supermarkets and street food in several governorates in West Bank /Palestine.

In this study, a total of 150 samples of selected fresh vegetables were analyzed for prevalence of *L. monocytogenes*. The method used ISO 11290 on listeria-selective Oxford agar media to isolate and differentiate *L. monocytogenes* of vegetable samples. The culture results showed a positive of 71% *L. monocytogenes* based on the media's selectivity and the colonies' morphological characteristics. However, selection of presumptive colonies for the biochemical test showed results positive all for isolates, but when confirmed by RT-PCR for all isolates shown results were negative. Indicating a discrepancy in the detection of pathogens between molecular and culture-based techniques. This indicates that the culture findings of samples that were believed to have been positive for *L. monocytogenes* may be "false positive".

This outcome spotlights the significance of considering the limitations of depending only on conventional culture and biochemical methods for identifying *L. monocytogenes*. It centers on the requirement to incorporate molecular techniques for accurate pathogen confirmation.

Previous research investigated the detection efficiencies of three plating media for *L. monocytogenes*, utilizing this ISO 11290 approach to isolate and identify the bacteria from food samples. To distinguish *L. monocytogenes* from different *Listeria* spp., additional determination via biochemical tests or PCR was required. Nevertheless, the usage of CHROMagar™ Listeria enabled the direct detection and distinction of *L. monocytogenes* in samples with high microbial backgrounds. However, the use of Listeria-selective Oxford agar and PALCAM agar media prevent allow one-step detection of *L. monocytogenes* (Jamali et al., 2013). Our findings agree strongly with this study on the effectiveness of listeria-selective Oxford agar media.

In this study, the results of 106 samples' real-time polymerase chain reaction (RT-PCR) and culture confirmation assays were compared in this investigation. Based on the results of the real-time PCR and the culture confirmation, there was a 53% matching rate (56 out of 106) and a 47% non-matching percentage (50 out of 106) according to the hemolysis test positive and the RT-PCR was negative, or vice versa.

A false positive can be explained by the difference between the negative RT-PCR result and the positive hemolysis result. This could occur because external variables that influence microbial growth could affect the biochemical test results (Kubrat A. Oyinlola,

2022). Furthermore, isolates that were not detected by PCR might have been the result of genetically altered strains of *Listeria* that are undetectable to particular primers. (Shojaei et al., 2016).

A false negative could be explained by the difference between the RT-PCR results, which were positive, and the hemolysis test results, which were negative and revealed no hemolytic activity on blood agar. The absence of hemolytic action may be due to inadequate culture conditions or hemolysin expression (Shojaei et al., 2016).

This study used real-time polymerase chain reaction (RT-PCR) to determine the prevalence of *L. monocytogenes* fresh-chosen vegetable samples. The molecular technique selectively targeted the (*hly* gene) for *L. monocytogenes* and the (16S rRNA gene) for *Listeria* spp. Out of the chosen vegetable samples confirmed by RT-PCR examine, of samples were significant differences positive for *L. monocytogenes* of the bacterium in fresh vegetables chosen from governorates in the West Bank/ Palestine.

Among the five types of freshly selected vegetables were significant differences of present *L. monocytogenes* in lettuce, watercress, and carrot juice, spring onion, parsley, this may be due to contamination from soil or pollution of irrigation water.

In a previous study, Moutong Chen et al. an analysis 419 commercial obtainable fresh vegetables in (43) typical Chinese cities/ areas where the prevalence of 23 (5.49%) had a positive of *L. monocytogenes*. Samples of lettuce had a higher contamination rate (7.78%). This may be because of the lettuce's relatively broad leaf surface, which is near the soil's surface and hence quickly contaminated by environmental water and soil, a finding that is consistent with our findings (Chen et al., 2019).

This study in the governorates of the West Bank / Palestine found a significant differences of level *L. monocytogenes* in leafy vegetables (lettuce, watercress, parsley, and spring onion). This finding was relatively comparable to a previous study carried out in Malaysia that found a 22% incidence of *L. monocytogenes* in leafy vegetables. It was proposed that these vegetables could harbor detectable quantities of *L. monocytogenes* and may be allowed to grow if they are kept in a warm, humid environment (Steinbruegge et al., 1988; Ponniah et al., 2010).

According to our research, there are significant differences in the incidence of *L. monocytogenes* in carrots. This contradicts the results of a previous study conducted by Ajayeoba et al. (2016), which looked at 555 ready-to-eat (RTE) vegetable samples, including carrots and other vegetables, from six states in South Western Nigeria. Carrots showed the lowest incidence % of *L. monocytogenes*, presumably because their tissues contain antimicrobial compounds including falcarinol and isocoumarin that may have anti-*listeria* effects (Ajayeoba et al., 2016).

Compared to other vegetable types used for our study, parsley had a moderate incidence of *L. monocytogenes*. This could be because parsley contains essential oils that prevent the growth of pathogenic bacteria. These findings align with the earlier research by Öktem et al. (2006), which looked at (100) parsley samples chosen from several Ankara marketplaces. In parsley, *L. monocytogenes* was comparatively uncommon. This may be the case because essential oils and other bactericidal substances found in parsley make it an unsuitable medium for the growth of germs. It has been demonstrated by Elgayyar et al. (2001) that the essential oils in parsley reduce *L. monocytogenes* (Öktem et al., 2006).

The degree of *L. monocytogenes* contamination of the vegetables differs significantly throughout the governorates of Ramallah, Bethlehem, Nablus, Tulkarm, and Hebron in this study. The degree of environmental contamination, the processing and handling methods, and city-by-city differences in agricultural techniques could all play a role in this.

Ajayeoba et al. (2016), this study investigated six states in South Western Nigeria and examined the prevalence of *L. monocytogenes* in ready-to-eat (RTE) vegetables. The highest incidence of *L. monocytogenes* contamination was found in Lagos state (55%) and was followed by Ekiti (33.33%), Osun (34.38%), Ogun (44.09%), Oyo (48.75%), and Ondo (48.89%), states. These differences in contamination levels may be caused by the various agriculture patterns and post-harvest handling techniques used by farmers, the degree of knowledge among retail traders, exposition to pollution environmental and RTE vegetable storage temperatures (Ajayeoba et al., 2016).

In another study, Pospo et al., (2023) verified the investigation, of *L. monocytogenes* infection of salad vegetables collected from 6 various markets of 5 Upazilas of Mymensingh district. This study was conducted and salad vegetables were shown to have

a high frequency of *L. monocytogenes* (10.6%), which is widespread in the environment, including surface water and soil, and thus salad veggies probability. There was contamination when being grown, harvested, handled, or distributed (Mir et al., 2018; Pospo et al., 2023).

Another earlier study, by Jamali et al. (2013), was conducted in Malaysia. To investigate for the existence of *L. monocytogenes*, (396) RTE food samples were bought from street food vendors and hypermarkets. Vegetables and salads exhibited the greatest prevalence of *L. monocytogenes* (14.7%) among the RTE foods that were investigated. Thus salads and vegetables accounted for the majority of the high prevalence of *L. monocytogenes* in ready-to-eat foods. This could be because none of these vegetables was subjected to heat processes. In comparison to a study conducted by Wong et al. (2011), *L. monocytogenes* was discovered when chicken burger patties were cooked for 4 minutes as opposed to 6 minutes. This explains the significant contamination levels of *L. monocytogenes* seen in vegetables and salads (Jamali et al., 2013).

Another previous investigation was conducted out by Elmali et al. (2013). In the southeast Turkish district of Antakya/Hatay, *L. monocytogenes* was detected in 3 (2.58%) of 116 salad samples that were used to test for its presence in 239 RTE food samples. This may be due to the possibility that the contamination with *L. monocytogenes* was brought on by a high microbial load of the materials, cross-contamination, subpar production, improper handling and processing conditions, or water irrigation of vegetables with tainted water (Elmali et al., 2013).

In a previous study, Aytac et al. analyzed 164 samples of various leafy green vegetables (spring onion, watercress, parsley, and lettuce) that were randomly gathered from agricultural areas near Ankara Stream over the course of eight months, from April to November 2007. The incidence of *L. monocytogenes* on leafy green vegetables cultivated in the Ankara, Turkey, area was 8.5%. The contamination of ambient soil and water may be the reason of this, which is in line with our findings (Aytac et al., 2010).

4.2 Conclusion

- Vegetables are typically sold moist in supermarkets and produced in unsanitary conditions.
- The outcomes of our study demonstrate that *L. monocytogenes* is widely prevalent (69% as per PCR results) in fresh vegetables that have been chosen from various supermarkets and street food in the governorates of West Bank / Palestine.
- Our outcomes study showed *L. monocytogenes* levels significantly differ in vegetable samples within the same governorate.
- Our outcomes study showed *L. monocytogenes* levels significantly differ between cities according to the food item (lettuce, carrot juice, parsley, spring onion, watercress).
- The outcomes of our study demonstrated the degree of matching between the two methods RT-PCR and culture confirmation were 53% matching and 47% non-matching.
- There is a source of contamination possibility from irrigation water for the vegetables.
- From the standpoint of food safety, these fresh vegetables became a possible cause of *L. monocytogenes* and a possible risk to public health.
- Local farmers, retailers and consumers are likely unaware of the risks involved in consuming contaminated vegetables.

4.3 Recommendations

- It is significant to apply stringent hygiene and manage procedures to avoid contamination that is happening throughout the production method and leading up to consumption, such as Good Agricultural Practices (GAPs) involving field sanitation, worker hygiene, and equipment cleanliness.
- Good Manufacturing Practices (GMPs) containing temperature Control and keeping proper refrigerated temperatures (below 4°C) to inhibit the growing of *L. monocytogenes* throughout transport and storage.
- Sanitizing surfaces is part of post-harvest handling. Ensure that the tools and surfaces used for post-harvest processing and handling are routinely cleaned and sanitized.
- Boost surveillance and monitoring programs to learn more about the prevalence and associated risks of these food-borne illnesses.

- Educate people on the need to maintain a commitment to hygiene and safe food handling procedures.
- Enhance the inspection processes, such as routine sampling from various local marketplaces and periodic inspections, and implement the molecular methods routinely.
- It is imperative to provide education to the farmers on the potential risks associated with using tainted surface water for agricultural irrigation and the use of organic fertilizers.
- Public awareness initiatives increase consumer knowledge of proper vegetable preparation and handling.
- Provide consumers with advice on how to store and refrigerate vegetables properly to reduce the formation of bacteria.
- Education of important stakeholders on the biology of food-borne illnesses and the critical requirement to maintain the cold chain order to prevent food-borne outbreaks, including product suppliers, store management, cleaning workers, and hygiene consultants.
- Public health measures against *L. monocytogenes* in fresh vegetables should be put in place going forward, particularly for children, expectant mothers, and those with immunosuppressive conditions.
- Good procedures, education, and routine monitoring are all crucial parts of a successful food safety plan.
- Epidemiological research and other investigations into the causes of contamination are advised.

4.4 Limitation

- *L. monocytogenes* is difficult to identify and detect using simply traditional methods; the process is time-consuming and yields unreliable results.
- Even though RT-PCR is usually specific, it can occasionally produce false positives by cross-reacting with other *Listeria* species that are not pathogenic.
- Polymerase chain reaction (PCR) methods are more sensitive to contamination by very low number of cells.
- The specialized equipment and reagents needed for RT-PCR can be prohibitively expensive for many labs, particularly those with low resources.
- DNA extraction from vegetables can be difficult because of the high levels of polysaccharides and polyphenols, which can co-extract and impede the process.
- Vegetables are not available in all seasons.

List of abbreviations

Abbreviation	Meaning
(WHO)	World Health Organization.
(ISO)	International Organization for Standardization.
(USA)	United States of America.
(PALCAM)	Polymyxin Acriflavin Lithium-Chloride Ceftazidime Aesculin Mannitol.
(ALOA)	Agar Listeria according to Ottaviani and Agosti.
(PI-PLC)	phospholipase C.
(MOX)	Modified Oxford Agar.
(PCR)	Polymerase Chain Reaction.
(m-PCR)	Multiplex Polymerase Chain Reaction.
(q-PCR)	Quantitative Polymerase Chain Reaction.
(NASBA)	Nucleic acid sequenced-based amplification.
(RT-PCR)	Real-Time Polymerase Chain Reaction.
(DNA)	Deoxyribonucleic acid.
(cDNA)	Complementary DNA
(NNDSS)	National Notifiable Diseases Surveillance System.
(HM)	Hypermarket.
(SF)	Street Food.
(BLEB)	Buffered Listeria enrichment broth.
(FDA -BAM)	Food and Drug Administration - bacteriological and analytical method.
(<i>L. Monocytogenes</i>)	<i>Listeria monocytogenes</i> .
(LLO)	Listeriolysin O.
(CFU/ml)	Colony-Forming Unit per milliliter.
(g)	Gram.
(g/L)	Gram per liter.
(μ m)	Micrometer.
(°C)	Degree Celsius.
(%)	Percentage.
(β -hemolysis)	Beta-hemolysis.

(α -hemolysis)	Alpha-hemolysis.
(+)	Positive.
(-)	Negative.
(N/A)	Not Applicable.
(RTE)	Ready To Eat.
(GAPs)	Good Agricultural Practices.
(GMPs)	Good Manufacturing Practices.
(IRT)	Inhibitor Removal Technology®.

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Appendices

Appendix A

The number of *Listeria monocytogenes* bacteria detected using different primers real-time polymerase chain reaction primers in all samples

sample food	governorates	HLY1/2	ILMHL YF/ILM HLYR	HF9/HR9
Lettuce(s1)	Tulkarm	0.00351	N/A	N/A
Lettuce(s2)	Tulkarm	0.0000	N/A	N/A
Lettuce(s3)	Tulkarm	0.00005	N/A	N/A
Lettuce(s4)	Tulkarm	0.00002	N/A	N/A
Lettuce(s5)	Tulkarm	0.00000	N/A	N/A
Lettuce(6)	Nablus	0.00000	N/A	N/A
Lettuce(7)	Nablus	0.00007	N/A	N/A
Lettuce(8)	Nablus	45077211.4	N/A	N/A
Lettuce(9)	Nablus	0.01365	N/A	N/A
Lettuce(10)	Nablus	10486179.3	N/A	N/A
Lettuce(s66)	Hebron	42406152.2	N/A	N/A
Lettuce(s67)	Hebron	174543733.85	N/A	N/A
Lettuce(s68)	Hebron	N/A	N/A	N/A
Lettuce(s69)	Hebron	25574008.5	0.00013	N/A
Lettuce(s70)	Hebron	N/A	N/A	N/A
Lettuce(s81)	Ramallah	N/A	N/A	N/A
Lettuce(s82)	Ramallah	N/A	N/A	N/A
Lettuce(s83)	Ramallah	0.80936	193835.89	N/A
Lettuce(s84)	Ramallah	0.00002	N/A	N/A
Lettuce(s85)	Ramallah	518615.852	N/A	N/A
Lettuce(s111)	Ramallah	920418.91430	0.00001	N/A
Lettuce(s112)	Ramallah	1.68893	0.00001	N/A
Lettuce(s113)	Ramallah	47.45228	9199.25	N/A
Lettuce(s114)	Ramallah	2.60704	N/A	N/A
Lettuce(s115)	Ramallah	0.00129	N/A	N/A
Lettuce(s141)	Bethlehem	2597118970.20443	N/A	N/A
Lettuce(s142)	Bethlehem	12515815280.9202	N/A	N/A
Lettuce(s143)	Bethlehem	1678982153.1	N/A	N/A
Lettuce(s144)	Bethlehem	27268784691.10	N/A	N/A
Lettuce(s145)	Bethlehem	8979797820.21	N/A	N/A
Parsley(s11)	Tulkarm	724970.001	N/A	N/A
Parsley(s12)	Tulkarm	0.72394	N/A	N/A
Parsley(s13)	Tulkarm	0.00000	N/A	N/A
Parsley(s14)	Tulkarm	1461.981	N/A	N/A
Parsley(s15)	Tulkarm	17.4185	N/A	N/A
Parsley(s16)	Nablus	N/A	N/A	N/A
Parsley(s17)	Nablus	34389976.4	N/A	N/A
Parsley(s18)	Nablus	N/A	N/A	N/A

Parsley(s19)	Nablus	N/A	N/A	N/A
Parsley(s20)	Nablus	N/A	N/A	N/A
Parsley(s61)	Ramallah	2476163.10	N/A	N/A
Parsley(s62)	Ramallah	N/A	N/A	N/A
Parsley(s63)	Ramallah	N/A	333837.44	N/A
Parsley(s64)	Ramallah	N/A	N/A	N/A
Parsley(s65)	Ramallah	N/A	96202.2	N/A
Parsley(s86)	Ramallah	1887.8	N/A	N/A
Parsley(s87)	Ramallah	8454.6	N/A	N/A
Parsley(s88)	Ramallah	132714.1	N/A	N/A
Parsley(s89)	Ramallah	6.93443	N/A	N/A
Parsley(s90)	Ramallah	1741573596.0032	N/A	N/A
Parsley(s76)	Hebron	N/A	145710.7	N/A
Parsley(s77)	Hebron	N/A	0.00006	N/A
Parsley(s78)	Hebron	N/A	N/A	N/A
Parsley(s79)	Hebron	N/A	N/A	N/A
Parsley(s80)	Hebron	N/A	N/A	N/A
Parsley(s136)	Bethlehem	17700112421.4	N/A	N/A
Parsley(s137)	Bethlehem	1580958303.02	N/A	N/A
Parsley(s138)	Bethlehem	2672901172.2141	N/A	N/A
Parsley(s139)	Bethlehem	1787615127.7	N/A	N/A
Parsley(s140)	Bethlehem	N/A	N/A	N/A
Watercress(s21)	Nablus	N/A	N/A	N/A
Watercress(s22)	Nablus	N/A	N/A	N/A
Watercress(s23)	Nablus	N/A	N/A	N/A
Watercress(s24)	Nablus	N/A	N/A	N/A
Watercress(s25)	Nablus	0.0156	N/A	N/A
Watercress(s26)	Tulkarm	N/A	N/A	N/A
Watercress(s27)	Tulkarm	N/A	N/A	N/A
Watercress(s28)	Tulkarm	N/A	N/A	N/A
Watercress(s29)	Tulkarm	5551637.3133	N/A	N/A
Watercress(s30)	Tulkarm	8477438.144	N/A	N/A
Watercress(s56)	Ramallah	147108723.7	N/A	N/A
Watercress(s57)	Ramallah	510223.002	N/A	N/A
Watercress(s58)	Ramallah	37.76002	133426.79	N/A
Watercress(s59)	Ramallah	44937526.6	N/A	N/A
Watercress(s60)	Ramallah	N/A	305236.9431	N/A
Watercress(s96)	Ramallah	2781504.55	3538.95	N/A
Watercress(s97)	Ramallah	1349315.10	3112.085	N/A
Watercress(s98)	Ramallah	60.3510	891.095	N/A
Watercress(s99)	Ramallah	0.54161	471492656.3	N/A
Watercress(s100)	Ramallah	6.44715	0.03033	N/A
Watercress(s71)	Hebron	56171350.4	1572.27	N/A
Watercress(s72)	Hebron	690853712.6	N/A	N/A
Watercress(s73)	Hebron	70203590.4	N/A	N/A
Watercress(s74)	Hebron	N/A	N/A	N/A
Watercress(s75)	Hebron	N/A	N/A	N/A
Watercress(s131)	Bethlehem	2337535764.5	N/A	N/A
Watercress(s132)	Bethlehem	268575330.1	N/A	N/A
Watercress(s133)	Bethlehem	10149444588.85	N/A	N/A
Watercress(s134)	Bethlehem	9855687203.742	N/A	N/A
Watercress(s135)	Bethlehem	5144692620.8323	N/A	N/A

Spring Onion (s31)	Nablus	55556227.64	N/A	N/A
Spring Onion (s32)	Nablus	5327.02	N/A	N/A
Spring Onion (s33)	Nablus	N/A	N/A	N/A
Spring Onion (s34)	Nablus	55952944.51	N/A	N/A
Spring Onion (s35)	Nablus	N/A	N/A	N/A
Spring Onion (s36)	Tulkarm	N/A	N/A	N/A
Spring Onion (s37)	Tulkarm	N/A	N/A	N/A
Spring Onion (s38)	Tulkarm	N/A	N/A	N/A
Spring Onion (s39)	Tulkarm	190796246.704	N/A	N/A
Spring Onion (s40)	Tulkarm	N/A	N/A	N/A
Spring Onion (s51)	Ramallah	N/A	555556.836	N/A
Spring Onion (s52)	Ramallah	37003226.8	227034.47	N/A
Spring Onion (s53)	Ramallah	N/A	N/A	N/A
Spring Onion (s54)	Ramallah	3.10003	0.00831	N/A
Spring Onion (s55)	Ramallah	0.02369	N/A	N/A
Spring Onion (s91)	Ramallah	2177.4	N/A	N/A
Spring Onion (s92)	Ramallah	0.77573	N/A	N/A
Spring Onion (s93)	Ramallah	0.00017	N/A	N/A
Spring Onion (s94)	Ramallah	3.69336	0.00000	N/A
Spring Onion (s95)	Ramallah	51.56334	41140.834	N/A
Spring Onion (s121)	Hebron	37533632607.3	N/A	N/A
Spring Onion (s122)	Hebron	1.11066	N/A	N/A
Spring Onion (s123)	Hebron	0.10605	N/A	N/A
Spring Onion (s124)	Hebron	5298473345.704	N/A	N/A
Spring Onion (s125)	Hebron	N/A	N/A	N/A
Spring Onion (s146)	Bethlehem	6779915947.6201	N/A	N/A
Spring Onion (s147)	Bethlehem	15700968094.74	N/A	N/A
Spring Onion (s148)	Bethlehem	N/A	N/A	N/A
Spring Onion (s149)	Bethlehem	N/A	N/A	N/A
Spring Onion (s150)	Bethlehem	4729642658.143	N/A	N/A
Carrot juice(s41)	Nablus	N/A	N/A	N/A
Carrot juice(s42)	Nablus	578240602.12	N/A	N/A
Carrot juice(s43)	Nablus	1590680614.6	N/A	N/A
Carrot juice(s44)	Nablus	N/A	N/A	N/A
Carrot juice(s45)	Nablus	563221264.7	N/A	N/A
Carrot juice(s46)	Tulkarm	314601113.51	N/A	N/A
Carrot juice(s47)	Tulkarm	208066332.31	N/A	N/A
Carrot juice(s48)	Tulkarm	N/A	N/A	N/A
Carrot juice(s49)	Tulkarm	N/A	N/A	N/A
Carrot juice(s50)	Tulkarm	3974623.2241	N/A	N/A
Carrot juice(s101)	Ramallah	3.21551	133324.9	N/A
Carrot juice(s102)	Ramallah	0.08137	0.00001	N/A
Carrot juice(s103)	Ramallah	3.27006	257106313.42	N/A
Carrot juice(s104)	Ramallah	73.52714	5985.664	N/A
Carrot juice(s105)	Ramallah	0.00534	3632.04	N/A
Carrot juice(s106)	Ramallah	0.00002	13959.5	N/A
Carrot juice(s107)	Ramallah	1.57055	0.0898	N/A
Carrot juice(s108)	Ramallah	0.00000	0.00002	N/A
Carrot juice(s109)	Ramallah	1.36260	2558022471.6	N/A
Carrot juice(s110)	Ramallah	0.00151	31750.40420	N/A
Carrot juice(s116)	Hebron	N/A	N/A	N/A
Carrot juice(s117)	Hebron	N/A	N/A	N/A

Carrot juice(s118)	Hebron	N/A	N/A	N/A
Carrot juice(s119)	Hebron	N/A	N/A	N/A
Carrot juice(s120)	Hebron	N/A	N/A	N/A
Carrot juice(s126)	Bethlehem	7579864006.74	N/A	N/A
Carrot juice(s127)	Bethlehem	N/A	N/A	N/A
Carrot juice(s128)	Bethlehem	8936579892.97	N/A	N/A
Carrot juice(s129)	Bethlehem	3041976204.194	N/A	N/A
Carrot juice(s130)	Bethlehem	290.93340	N/A	N/A

Appendix B

Matching Results of Culture Confirmation Assay and Real-Time Polymerase Chain Reaction (RT-PCR) for (106 samples)

Type raw vegetables	Governorates	Culture selective media	Catalase test	Hemolysis test	Results RT-PCR by different primers		
					ILMHL1YF	HF9	HR9
Lettuce (s81).	Ramallah	+	+	+	-	-	-
Lettuce (s111).	Ramallah	+	+	+	+	+	-
Lettuce (s112).	Ramallah	+	+	+	+	+	-
Lettuce (s113).	Ramallah	+	+	+	+	+	-
Lettuce (s114).	Ramallah	+	+	+	+	-	-
Lettuce (s6).	Nablus	+	+	-	-	-	-
Lettuce (s7) .	Nablus	+	+	-	+	-	-
Lettuce (s8).	Nablus	+	+	-	+	-	-
Lettuce (s9).	Nablus	+	+	-	+	-	-
Lettuce (s10).	Nablus	+	+	-	+	-	-
Lettuce (s3).	Tulkarm	+	+	-	+	-	-
Lettuce (s4).	Tulkarm	+	+	-	+	-	-
Lettuce (s5).	Tulkarm	+	+	-	-	-	-
Lettuce (s66).	Hebron	+	+	-	+	-	-
Lettuce (s67).	Hebron	+	+	+	+	-	-
Lettuce (s68).	Hebron	+	+	+	-	-	-
Lettuce (s141).	Bethlehem	+	+	-	+	-	-
Lettuce (s142).	Bethlehem	+	+	-	+	-	-
Lettuce (s143).	Bethlehem	+	+	+	+	-	-
Parsley (s11).	Tulkarm	+	+	-	+	-	-
Parsley (s12).	Tulkarm	+	+	-	+	-	-
Parsley (s13).	Tulkarm	+	+	-	+	-	-
Parsley (s14).	Tulkarm	+	+	-	+	-	-
Parsley (s15).	Tulkarm	+	+	-	+	-	-
Parsley (s16).	Nablus	+	+	-	-	-	-
Parsley (s17).	Nablus	+	+	-	+	-	-
Parsley (s18).	Nablus	+	+	-	-	-	-
Parsley (s19).	Nablus	+	+	-	-	-	-
Parsley (s20).	Nablus	+	+	-	-	-	-
Parsley (s76).	Hebron	+	+	+	-	+	-
Parsley (s77).	Hebron	+	+	+	-	+	-
Parsley (s78).	Hebron	+	+	-	-	-	-
Parsley (s136).	Bethlehem	+	+	-	+	-	-
Parsley (s137).	Bethlehem	+	+	-	+	-	-
Parsley (s138).	Bethlehem	+	+	+	+	-	-

Parsley (s61).	Ramallah	+	+	-	+	-	-
Parsley (s62).	Ramallah	+	+	-	-	-	-
Parsley (s63).	Ramallah	+	+	-	-	+	-
Parsley (86).	Ramallah	+	+	+	+	-	-
Parsley (s89).	Ramallah	+	+	+	+	-	-
Parsley (s90).	Ramallah	+	+	-	+	-	-
Water cress (s21).	Nablus	+	+	-	-	-	-
Water cress (s22).	Nablus	+	+	-	-	-	-
Water cress (s23).	Nablus	+	+	-	-	-	-
Water cress (s24).	Nablus	+	+	-	-	-	-
Water cress (s25).	Nablus	+	+	-	+	-	-
Water cress (s26).	Tulkarm	+	+	-	-	-	-
Water cress (s27).	Tulkarm	+	+	-	-	-	-
Water cress (s28).	Tulkarm	+	+	-	-	-	-
Water cress (s29).	Tulkarm	+	+	-	+	-	-
Water cress (s30).	Tulkarm	+	+	-	+	-	-
Water cress (s56).	Ramallah	+	+	+	+	-	-
Water cress (s57).	Ramallah	+	+	-	+	-	-
Water cress (s58).	Ramallah	+	+	+	+	+	-
Water cress (s96).	Ramallah	+	+	-	+	+	-
Water cress (s97).	Ramallah	+	+	+	+	+	-
Water cress (s98).	Ramallah	+	+	+	+	+	-
Water cress (s73).	Hebron	+	+	-	+	-	-
Water cress (s74).	Hebron	+	+	-	-	-	-
Water cress (s75).	Hebron	+	+	-	-	-	-
Water cress (s133).	Bethlehem	+	+	-	+	-	-
Water cress (s134).	Bethlehem	+	+	+	+	-	-
Water cress (s135).	Bethlehem	+	+	-	+	-	-
Spring Onion (s31).	Nablus	+	+	-	+	-	-
Spring Onion (s32).	Nablus	+	+	-	+	-	-
Spring Onion (s33).	Nablus	+	+	-	-	-	-
Spring Onion (s34).	Nablus	+	+	-	+	+	-
Spring Onion (s35).	Nablus	+	+	-	-	-	-
Spring Onion (s36).	Tulkarm	+	+	-	-	-	-
Spring Onion (s37).	Tulkarm	+	+	-	-	-	-
Spring Onion (s38).	Tulkarm	+	+	-	-	-	-
Spring Onion (s39).	Tulkarm	+	+	-	+	-	-
Spring Onion (s40).	Tulkarm	+	+	-	-	-	-
Spring Onion (s51).	Ramallah	+	+	-	-	+	-
Spring Onion (s52).	Ramallah	+	+	+	+	+	-
Spring Onion (s53).	Ramallah	+	+	-	-	-	-
Spring Onion (s91).	Ramallah	+	+	-	+	-	-
Spring Onion (s92).	Ramallah	+	+	-	+	-	-
Spring Onion (s93).	Ramallah	+	+	+	+	-	-
Spring Onion (s121).	Hebron	+	+	+	+	-	-
Spring Onion (s122).	Hebron	+	+	+	+	-	-
Spring Onion (s146).	Bethlehem	+	+	-	+	-	-
Spring Onion (s147).	Bethlehem	+	+	+	+	-	-

Spring Onion (s148).	Bethlehem	+	+	-	-	-	-
Carrot juice (s41).	Nablus	+	+	-	-	-	-
Carrot juice (s42).	Nablus	+	+	-	+	-	-
Carrot juice (s43).	Nablus	+	+	-	+	-	-
Carrot juice (s44).	Nablus	+	+	-	-	-	-
Carrot juice (s45).	Nablus	+	+	-	+	-	-
Carrot juice (s46).	Tulkarm	+	+	-	+	-	-
Carrot juice (s47).	Tulkarm	+	+	-	+	-	-
Carrot juice (s48).	Tulkarm	+	+	-	-	-	-
Carrot juice (s49).	Tulkarm	+	+	-	-	-	-
Carrot juice (s101).	Ramallah	+	+	-	+	+	-
Carrot juice (s102).	Ramallah	+	+	+	+	+	-
Carrot juice (s103).	Ramallah	+	+	+	+	+	-
Carrot juice (s104).	Ramallah	+	+	-	+	+	-
Carrot juice (s107).	Ramallah	+	+	+	+	+	-
Carrot juice (s108).	Ramallah	+	+	-	+	+	-
Carrot juice (s109).	Ramallah	+	+	+	+	+	-
Carrot juice (s117).	Hebron	+	+	+	-	-	-
Carrot juice (s119).	Hebron	+	+	+	-	-	-
Carrot juice (s126).	Bethlehem	+	+	-	+	-	-
Carrot juice (s127).	Bethlehem	+	+	+	+	-	-
Carrot juice (s128).	Bethlehem	+	+	+	+	-	-
Carrot juice (s129).	Bethlehem	+	+	-	+	-	-

Key: (+) positive; (-) negative.

Appendix C

Figures

Figure 11

Gene expression analysis of *L. monocytogenes* in watercress in different governorate of Palestine. Folds (a-e) with difference superscripts difference significantly at ($P < 0.05$)

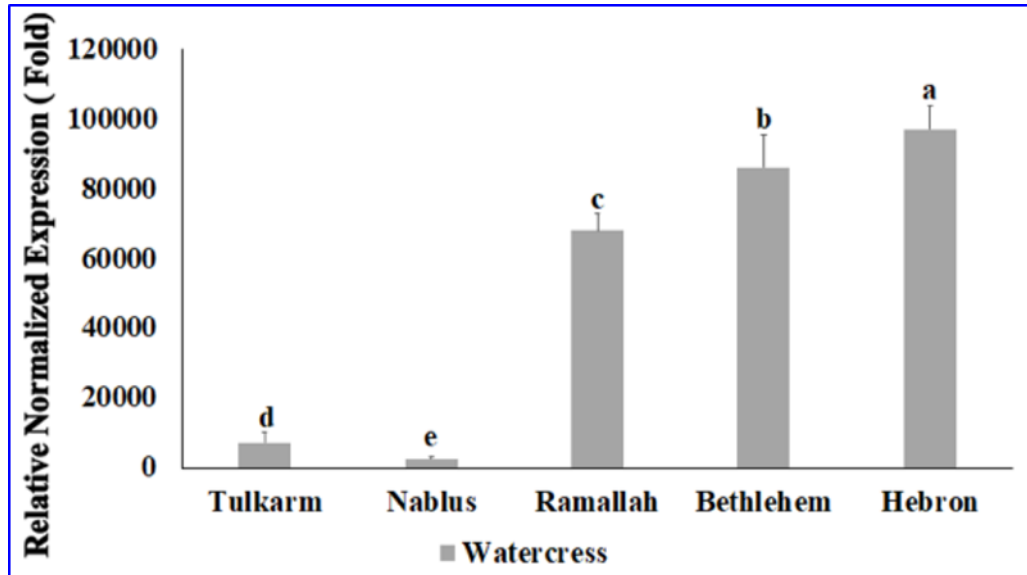


Figure 12

Gene expression analysis of *L. monocytogenes* in Spring onion in different governorate of Palestine. Folds (a-e) with difference superscripts difference significantly at ($P < 0.05$)

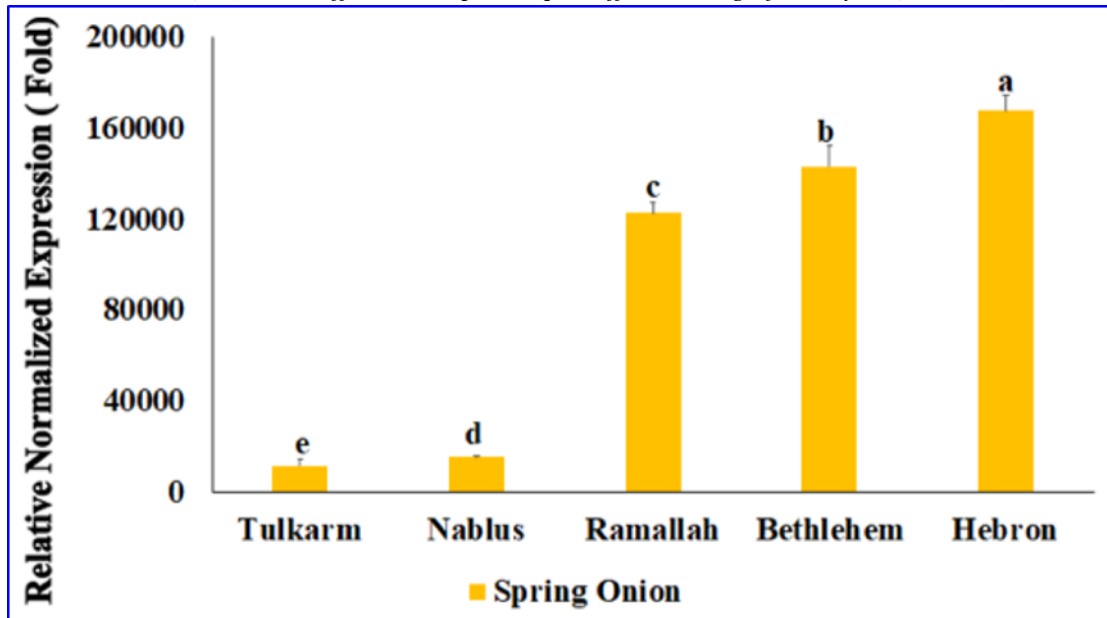


Figure 13

Gene expression analysis of *L. monocytogenes* in carrot juice in different governorate of Palestine. (a-e) folds with difference superscripts difference significantly at ($P < 0.05$).

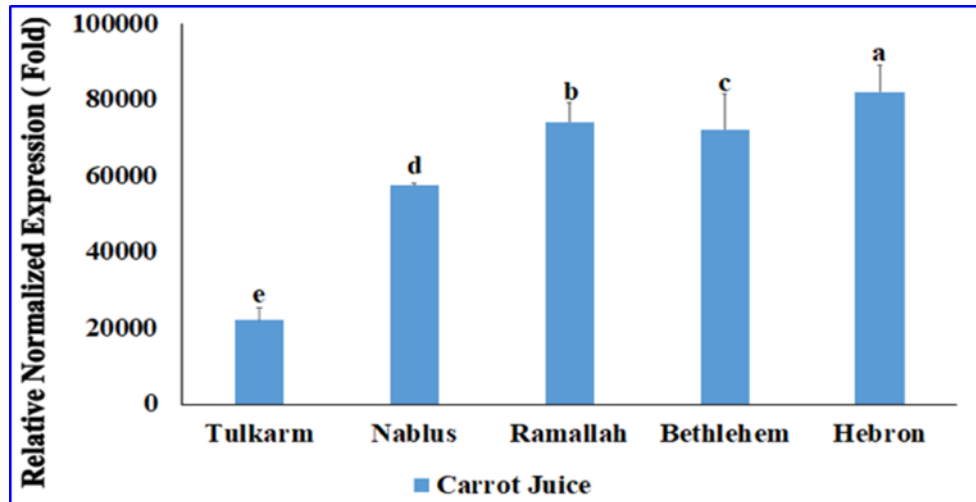
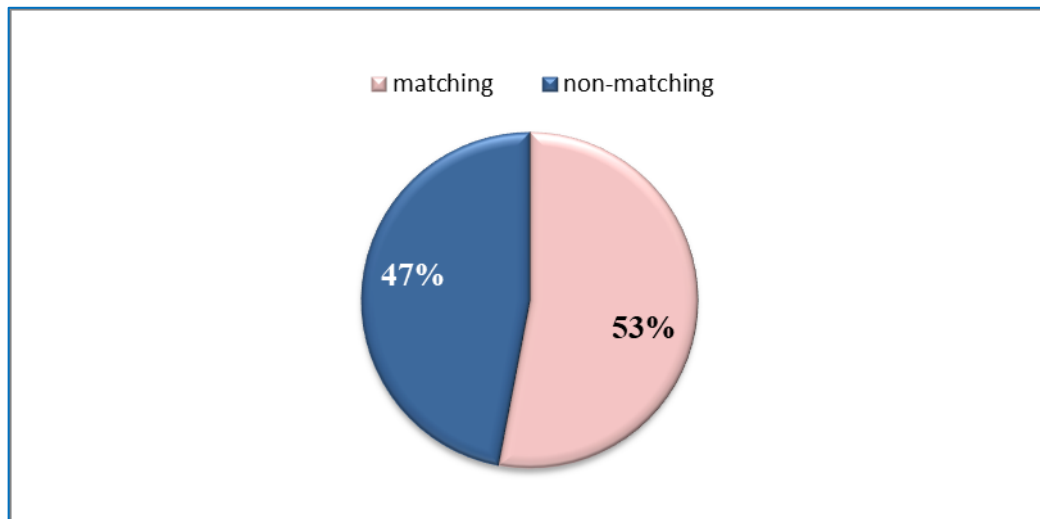


Figure 14

Matching and non-matching results between two methods culture and (RT-PCR).





جامعة النجاح الوطنية
كلية الدراسات العليا

الكشف الجزيئي عن بكتيريا الليستيريا مونوسايتوجينز في الخضروات الطازجة باستخدام تقنية RT-PCR في الضفة الغربية

إعداد

دانيا مفيد حسن العثمانة

إشراف

د. محمد التميمي

د. عبد الحفيظ دلعب

قدمت هذه الرسالة استكمالاً لمتطلبات الحصول على درجة الماجستير في التغذية وتكنولوجيا الغذاء، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس - فلسطين.

2024

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الملخص

الليستيريا المستوحدة هي أحد مسببات الأمراض الناشئة المنقولة بالغذاء والتي يمكن أن تنتقل عن طريق مجموعة واسعة من المواد الغذائية. هدفت هذه الدراسة إلى تقييم وجود الليستيريا المستوحدة في الخضروات الطازجة المختارة (الخس، البقدونس، الجرجير، البصل، عصير الجزر) في خمس محافظات في الضفة الغربية، فلسطين باستخدام الطرق التقليدية والجزيئية. تم شراء ما مجموعه 150 عينة من الخضروات الطازجة من محلات السوبر ماركت وأكشاك بيع الأطعمة المتجولة في الشوارع. تم التفريق بين العزلات المفترضة كيميائيًا وتم تأكيدها أيضًا بواسطة تفاعل البوليميراز المتسلسل في الوقت الحقيقي (RT-PCR) الذي يستهدف جين 16S rRNA لأنواع بكتيريا الليستيريا والجين *hly* الخاص بـ الليستيريا المستوحدة. أظهرت النتائج أن 71% من الخضروات الطازجة المفحوصة كانت إيجابية للبكتيريا الليستيريا المستوحدة بناء على انتقائية الوسط والخصائص الشكلية للمستعمرات. وكانت النسب المئوية للتوزيع النسبي للبكتيريا الليستيريا المستوحدة في الخضروات الطازجة 66%، 70، 70، 73 و 73% للخس، الجرجير، البصل، عصير الجزر، والبقدونس على التوالي. ومن ناحية أخرى، أشار التحليل الإحصائي لنتائج تفاعل البوليميراز المتسلسل العكسي إلى وجود فروق معنوية في مستويات بكتيريا الليستيريا المستوحدة بين عينات الخضروات ضمن نفس المحافظة. ففي طولكرم، كانت أعلى المستويات في عصير الجزر، بينما كانت أقلها في الخس.

وفي نابلس، كانت أعلى المستويات في عصير الجزر، بينما كانت أقلها في الخس والجرجير. كما كانت أعلى مستويات بكتيريا الليستيريا المستوحدة في كل من رام الله وبيت لحم والخليل في البصل الأخضر، بينما كانت أقلها في الخس. بالإضافة إلى ذلك، أشارت مقارنة مستويات بكتيريا الليستيريا المستوحدة عبر المدن لمجموعة مختارة من الخضروات إلى وجود فروق معنوية حيث كان أعلى انتشار للخس في بيت لحم، والبقدونس في بيت لحم، وكذلك الجرجير والبصل الأخضر وعصير الجزر في الخليل. في الختام، أظهرت هذه الدراسة ارتفاع معدل انتشار الليستيريا في الخضروات الطازجة باستخدام كلتا الطريقتين، إلا أن الطريقة الجزيئية كانت أكثر حساسية وموثوقية لتأكيد الليستيريا. يعد التلوث بالليستيريا في محلات السوبر ماركت وأغذية الشوارع أيضًا مصدر قلق عام كبير ويبرر المزيد من المراقبة واللوائح الصارمة للحد من التلوث المتكرر للخضروات الطازجة بالليستيريا المستوحدة.

الكلمات المفتاحية: الخضروات الطازجة، الليستيريا مونوسيتوجينز، أجار أكسفورد، PCR في الوقت الحقيقي،

الجين *hly*، داء الليستريات.