DETECTION AND ESTIMATION OF BACTERIAL LOAD IN SELECTED LEAFY GREENS COLLECTED FROM DIVERSE MARKETPLACES OF DHAKA CITY IN BANGLADESH

BY

RAIHAN FERDOUS

DEPARTMENT OF PLANT PATHOLOGY

FACULTY OF AGRICULTURE

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

DHAKA- 1207

JUNE, 2022

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BY

RAIHAN FERDOUS

REGISTRATION NO.: 20-11106

A Thesis
Submitted to the Faculty of Agriculture,
Sher-e-Bangla Agricultural University, Dhaka,
In partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE (M.S.)
IN
PLANT PATHOLOGY
SEMESTER: JANUARY-JUNE, 2022

Approved by:

Dr. Nazneen Sultana
Professor
Department of Plant Pathology
Sher-e-Bangla Agricultural University, Dhaka
Supervisor

Dr. Md. Belal Hossain
Professor
Department of Plant Pathology
Sher-e-Bangla Agricultural University, Dhaka
Co-Supervisor

Abu Noman Faruk Ahmmed
Professor and Chairman
Department of Plant Pathology
Sher-e-Bangla Agricultural University, Dhaka

Dr. Nazneen Sultana
Professor
Department of Plant Pathology
Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207
Mobile: +880-1733955171
E-mail: nazneen@sau.edu.bd

CERTIFICATE

This is to certify that the thesis entitled, ***"DETECTION AND ESTIMATION OF BACTERIAL LOAD IN SELECTED LEAFY GREENS COLLECTED FROM DIVERSE MARKETPLACES OF DHAKA CITY IN BANGLADESH*** submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of ***MASTER OF SCIENCE (M.S.) IN PLANT PATHOLOGY,*** embodies the result of a piece of bona fide research work carried out by ***Raihan Ferdous, Registration No. 20-11106,*** under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma elsewhere.

I further certify that such help or source of information as has been availed of during the course of this investigation has duly been acknowledged and the contents and style of the thesis have been approved and recommended for submission.

Dated:
Dhaka, Bangladesh

Professor Dr. Nazneen Sultana
Supervisor
Department of Plant Pathology
Sher-e-Bangla Agricultural University
Dhaka-1207

DEDICATED

TO

MY BELOVED

PARENTS AND SISTER

ACKNOWLEDGMENTS

The author would like to express his praise and gratefulness to the almighty Allah for owing mercy upon him and for imbibing confidence in him to complete the research work and to prepare this manuscript for the degree of Master of Science (M.S.) in Plant Pathology.

It is a great pleasure to articulate the author's gratitude to his respected parents, for their blessings and the constant support that they have had for him in prosecuting his studies, thereby receiving proper education.

It is recognized that "many assisting hands make the hard task easier". A good number of people assisted the author in various ways in the preparation of this thesis. Without their generous contributions and support the thesis could not see the light of day. The author is indeed grateful to those who have offered support, instructions, and suggestions for the accomplishment of this work.

The author would like to express his utmost gratitude, and indebtedness to his research supervisor ***Professor Dr. Nazneen Sultana****, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207, for her persistent encouragement, precious suggestions, constant inspiration, and timely advice along with providing an atmosphere of freedom in the research work and for taking care in preparing this manuscript.*

The author expresses his vast appreciation, heartfelt gratitude, and indebtedness to the respected Co-Supervisor ***Prof. Dr. Md. Belal Hossain****, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207 for his cooperation, criticisms on the manuscript, and helpful suggestions for the successful completion of the research work.*

Prof. Abu Noman Faruq Ahmmed *Chairman and all respected teachers, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, were kind enough to extend their cooperation whenever the author asked for their suggestions and opinions. The author is really grateful to all of them for their sincere cooperation.*

The author would like to convey his gratitude and thanks to ***Rifat Ara Sultana****, batchmate, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka who cordially helped him a lot during the lab work.*

The author remembers the enormous support and cordial help that Azwad Razeen provided him during the entire period of the study and the author is grateful for that.

There is also a group of special people; the author's friends- Jannatul Mauya Aishwarja, Riaz Rahman Patwary, Yobaida Afrin Ara Eti, Moin Uddin Kawser, Ariful Islam, Dewan Warina Wasek, Samia Tasnim, Robayet Islam, Ismam Aurin, Mahmud Solvey, Tithi Sarker, Sonia Hamid, Jannatul Mowya Nizhum, Sharowar Evan, Tohidur Rahman, Shamim Reza, and Atkiya Rahman who inspired him throughout his study period in Sher-e-Bangla Agricultural University, Dhaka. The author is also thankful to them.

The author expresses his thanks and sincere gratitude to the staff of the Department of Plant Pathology, SAU for their amiable support during the study period.

Furthermore, the author would like to express his heartfelt gratitude and appreciation to the Ministry of Science and Technology, Government of the People's Republic of Bangladesh, for bestowing upon the author the prestigious National Science and Technology (NST) Fellowship. This esteemed award has played a crucial role in supporting and enabling the successful completion of this study.

Finally, the author found no words to thank his beloved Father ***Late Md. Rejaul Karim,*** *Mother* ***Mst. Anju Ara****, Sister* ***Rezuana Ferdausi Tusy****, and Brother-in-law* ***Abu Sayed Sumon*** *for their love and continuous support, constant encouragement, their sacrifice, never-ending affection, immense strength, and their faith in him which always kept him focused on his objectives and helped to achieve his goals.*

June, 2022 The Author

SAU, Dhaka

DETECTION AND ESTIMATION OF BACTERIAL LOAD IN SELECTED LEAFY GREENS COLLECTED FROM DIVERSE MARKETPLACES OF DHAKA CITY IN BANGLADESH

ABSTRACT

A study was performed to detect the human pathogenic bacteria and to estimate the colony-forming units (CFU) and bacterial incidence from 3 selected leafy greens viz. coriander, lettuce, and mint leaves. The study was conducted in the MS Laboratory of the Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University during the period from July 2021 to June 2022. In total 27 samples were collected from 9 different marketplaces in Dhaka city. The bacteria were isolated by five-fold dilution from the stock solutions after being grown on NA media. Bacteria were identified by performing cultural, morphological, biochemical, and molecular studies. The biochemical tests were Gram-staining, KOH solubility, Oxidase, Catalase, Motility, Simmon's citrate utilization, Casein hydrolysis, Starch hydrolysis, Gelatin liquefaction, Levan production tests, and the culture on selective and semi-selective media were EMB agar, SS agar, Cetrimide agar, and Bacillus cereus agar. In molecular analysis, the DNA samples were put through to PCR using universal primer 27F: AGAGTTTGATCMTGGCTGAG and primer 1942R: CGGTTACCTTGTTACGAC TT that targeted the 16s rRNA gene at 1465 bp. Initially, Nine different bacterial genera viz. *Bacillus*, *Escherichia*, *Pseudomonas*, *Neisseria*, *Klebsiella*, *Enterobacter*, *Shigella*, *Vibrio*, and *Staphylococcus* were detected, and the incidence was 93%, 67%, 44%, 30%, 26%, 26%, 11%, 7%, and 7% respectively. A total of twelve bacteria have been identified from these nine genera viz, *B. subtilis, B. velezensis, B. altitudinis, B. cereus, E. coli, P. aeruginosa, Neisseria* spp., *K. pneumoniae, E. aerogenes, S. aureus, Vibrio* spp., and *Shigella* spp. Among them, *B. cereus, E. coli, P. aeruginosa, K. pneumoniae, E. aerogenes, S. aureus,* and *Shigella* sp. were detected as human pathogenic bacteria reported in several pieces of literature. The highest CFU was found in mint (4.27×10^9 /g) followed by lettuce (2.87×10^9 /g) and the lowest in coriander (2.43×10^9 /g). Considering marketplaces, the highest bacterial CFU was observed in the samples of roadside stand bazaars (5×10^9 /g) followed by local markets (2.7×10^9 /g), and the lowest was in supermarkets (1.9×10^9 /g). However, further advanced research is needed for the identification of undetected bacteria and to understand the specific conditions that lead to bacterial contamination in leafy greens.

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LIST OF SYMBOLS AND ABBREVIATIONS

et al.	=	And others
i.e.	=	That is
eg.	=	Example
etc.	=	Etcetera
viz.	=	Namely
Sl No.	=	Serial Number
%	=	Percentage
<	=	Less than
ml	=	Mili Liter
gm/g	=	Gram
cm	=	Centimeter
PSI	=	Pounds per square inch
Ibs	=	Pounds
MT	=	Metric Ton
Tk	=	Taka
°C	=	Degree Celsius
hr.	=	Hour
sp.	=	Species
spp.	=	Species
C	=	Coriander
M	=	Mint
L	=	Lettuce
Sp	=	Supermarket
Lc	=	Local market
Rs	=	Roadside stand bazar
H_2S	=	Hydrogen Sulfide
KOH	=	Potassium Hydroxide
H_2O_2	=	Hydrogen Peroxide
J.	=	Journal

LIST OF SYMBOLS AND ABBREVIATIONS (Cont'd)

STEC	=	Shigatoxin-producing *Escherichia coli*
Vtx	=	Verotoxin
EC	=	*Escherichia coli*
EMB	=	Eosin Methylene Blue
SS	=	*Salmonella Shigella*
NA	=	Nutrient Agar
SIM	=	Sulfide Indole Motility
CFU	=	Colony Forming Unit
TTP	=	Thrombotic Thrombocytopenic Purpura
HUS	=	Hemolytic Uremic Syndromes
UTIs	=	Urinary Tract Infections
LGV	=	Leafy Green Vegetable
RTE	=	Ready-to-eat
WHO	=	World Health Organization
CSPI	=	Center for Science in the Public Interest
BBS	=	Bangladesh Bureau of Statistics
UNB	=	United News of Bangladesh.
CDC	=	Centers for Disease Control and Prevention
FAO	=	Food and Agricultural Organization
CAC	=	Codex Alimentarius Commission.
ACMSF	=	Advisory Committee on the Microbiological Safety of Food
FSN	=	Food Safety News
PHE	=	Public Health England
FDA	=	US Food and Drug Administration
BLAST	=	Basic Local Alignment Search Tool
PCR	=	Polymerase Chain Reaction
NCBI	=	National Center for Biotechnology Information
MEGA	=	Molecular Evolutionary Genetics Analysis

CHAPTER I

INTRODUCTION

INTRODUCTION

Leafy greens are plant leaves eaten as a ready-to-eat vegetable, sometimes with fragile petioles and shoots attached. They are also known as Potherbs, Greens, Vegetable greens, Culinary herbs, or Salad greens. These leafy greens are crucial parts of a balanced diet for humans because they provide fiber, vitamins, and minerals that help ward off disease and malnutrition (Olson *et al.,* 2011; Nesamvuni *et al.,* 2001; Steyn *et al.,* 2001). A diet rich in vegetables and fruits has been shown to protect against cancers and chronic illnesses such as coronary heart disease (CAC, 2010). Similarly, leafy greens are an essential part of every population's diet around the world, but especially in Asia, Africa, and Oceania where they meet both medicinal and nutritional needs (Andres and Lebailly, 2011). In general, leafy greens have few calories, little fat, a lot of protein per calorie, and more dietary fiber, calcium, magnesium iron, and phytochemicals including vitamins C, K, Carotenoids, Lutein, and Folate. Leafy greens are grown in Bangladesh in roughly 107 different varieties such as Water spinach (Kolmi), Red amaranth (Lal shak), Coriander (Dhoniya), Mint (Pudina), Lettuce, etc. (Rafiul, 2015).

Leafy green vegetables (LGV) are divided into two categories based on processing. First, vegetables like water spinach, cabbage, and other leafy vegetables are only consumed after cooking. These types of vegetables are mainly used in the preparation of curry. Second, there are items that are ready-to-eat (RTE), such as coriander, mint, pudina, etc., which are consumed fresh or with little to no processing before being consumed without cooking. These RTE leafy greens are used in salads, fast foods, juices, and so on.

Lettuce (*Lactuca sativa* L) is one of the most consumed salad ingredients worldwide which is a member of the Asteraceae family. It is a milky-juicy herb with leaves. Early in the season, it grows a short stem with a cluster of leaves that vary greatly in shape, character, and color according to the variety (Ryder, 1979). Lettuce consumption is rising daily in metropolitan areas. Fresh lettuce is prized for its delicate, crunchy texture and somewhat bitter flavor. For a diet that is well-balanced, lettuce is a healthy food that has few calories and no fat. It is a beneficial source of folic acid and vitamin A (Herbst, 2001). Huge amounts of lettuce are consumed in Bangladesh at fast food restaurants and many five-star hotels as a fresh vegetable salad.

Coriander (*Coriandrum sativum* L.) is a notable annual spice crop in the Apiaceae family produced for both the seeds and the leaves throughout the nation. The crop's soft green leaves are used as a culinary herb, while the seeds are utilized as a spice. Coriander leaves are used in different types of traditional foods such as chicken curry, salads, pulses, hotchpotch, soup, etc. as well as in fast food such as chotpoti, pasta, and others. Since inflammation is linked to several brain disorders, such as Parkinson's, Alzheimer's, and multiple sclerosis, coriander's anti-inflammatory effects may protect against these conditions (Chen *et al.,* 2016). In addition, intestinal gas is alleviated, urine secretion and emission are increased, and fever is decreased by coriander. Regular coriander water consumption lowers blood cholesterol. Dodecenal, a coriander component, may resist *Salmonella* bacteria, which can cause catastrophic food poisoning (Kubo *et al.,* 2004). In the Rabi season, it has been discovered to be a lucrative crop. In Bangladesh, coriander production is growing both geographically and numerically (BBS, 2010).

Mint (*Mentha* sp.), also referred to as Pudina, is a perennial plant in the Lamiaceae family and has over 25 different species of mint (Harley and Brighton, 1997). The most common species of mint include *M. piperita* (Peppermint), *M. spicata* (Spearmint), *M. arvensis* (wild mint), and others. All mints include the volatile oil menthol, which is what gives mint its distinctively refreshing, cooling sensation (Curci, 2012). In addition to being high in vitamins A and C, mint also has modest amounts of vitamin B2 in it. It is generally known that the chemical compound menthol, which is made from mint oil, has therapeutic effects on the chest and respiratory system. One of the most widely used varieties of the mint plant is peppermint, which is used in products like toothpaste, chewing gum, mouthwash, soaps, candies, balms or creams, cough medicine, etc (Alvi *et al.,* 2001). Bangladesh is one of the countries where it is used in salads, juices, kabab, curry, fuchka as well as in cosmetics and medicines.

Leafy vegetables are easily contaminated by bacteria during and after harvest, from handling, storing, shipping, and purchasing (Inderbinen and Stephan, 2016; Martnez-Vaz *et al.,* 2014; *Barrera et al.,* 2012). At the field level, there are a variety of environmental contamination risks, including wildlife entry, animal dung, soil amendments, water, and cross-contamination from sanitized equipment and workers.

(Beuchat *et al.,* 1998). Some of these bacteria are pathogens, while others are potential pathogens or nonpathogens.

RTE leafy greens are a convenient approach to ensure the consumption of vegetables, but consumers must feel convinced that the food is safe to intake. To preserve the nutrients, green leaves and stalks should be consumed raw or minimally cooked. These leafy vegetables lose some of their nutritious content when heated during cooking. People are more likely to consume these leafy vegetables in raw or fresh condition because they don't want to lose their taste, flavor, or nutritional value. Leafy greens cannot be completely sanitized by washing. This is because bacteria can adhere to leaves' exteriors and even penetrate them. Due to LGV being consumed raw and there is no killing step of pathogens (e.g., heating) at any stage in the chain to prevent transmission so that the contaminated pathogenic bacteria are still alive after consumption. You might get sick if you will intake contaminated leafy greens raw, like in a salad or on a sandwich. Therefore, ensuring the microbiological safety of fresh LGV offers a special difficulty (Gil *et al.,* 2015). Additionally, while foodborne illnesses, sometimes known as food poisoning, can affect anyone, some groups of people are more susceptible to contracting them and developing serious illnesses. These groups of people include adults (aged 65 and older), children (younger than 5 years), people who have health problems or take medicines that lower the body’s ability to fight germs and sickness icons, and pregnant women.

LGV consumption may have health benefits, but recent reports of infectious disease outbreaks from the Centers for Disease Control and Prevention (CDC), US Food and Drug Administration (FDA), World Health Organization (WHO), and Center for Science in the Public Interest (CSPI) have raised questions about the safety and quality of vegetables. These alterations are mostly the result of human diseases' ability to adapt their ecology to survive in non-host situations. In general, over the past 20 years, more RTE vegetables have been consumed, and at the same time, more outbreaks of food-borne illness have been linked to the intake of leafy green vegetables (Castro-Ibanez *et al.,* 2017; Olaimat and Holley, 2012). In such outbreaks, poor sanitation practices were invariably mentioned. The use of chemical fertilizers, hormones, pesticides, and their residues in food are additional possible risks that could raise the risk of foodborne illnesses linked to fresh produce (Chen, 2007).

Fresh leafy greens are generally known to have significant bacterial populations, some of which may be plant endophytes, plant pathogens, or human pathogens (Leff and Fierer, 2013). The adaptation of pathogens to the host's defensive response, physiology, immunity, native microflora, physical obstacles, mobility, and temperature is one of the most crucial aspects of plant host colonization. Therefore, it is possible to identify several types of bacteria from leafy greens that may cause serious illnesses due to food poisoning in humans. *Escherichia coli* can be found in the intestines of animals and humans and can be transmitted to leafy vegetables through contaminated water or soil. Some strains of *E. coli* can cause serious illness in humans. *Salmonella* can also be transmitted to leafy vegetables through contaminated water or soil and can cause food poisoning in humans. *Listeria monocytogenes* can grow at refrigeration temperatures, it can be present in the soil, water, and on the surface of some animals. It can cause serious illness and even death, particularly in vulnerable populations such as pregnant women and immunocompromised individuals. *Staphylococcus aureus* is commonly found on the skin and in the noses of humans and animals, it can be found on the surface of leafy greens. It can cause food poisoning if consumed in large enough numbers. *Shigella* can be found in the feces of infected humans and animals and can cause diarrhea and other symptoms of food poisoning. *Yersinia* may also be present in animal feces, and can cause serious illness if consumed. *Neisseria*, the genus of these bacteria includes several species, such as *Neisseria meningitidis* and *Neisseria gonorrhoeae*. These species are known to cause serious infections in humans, such as meningitis and gonorrhea. *Klebsiella* can be found in various environments, including soil, water, and human guts, and can be transmitted to leafy vegetables through contaminated water or soil. Some species of Klebsiella, such as *Klebsiella pneumoniae*, can cause infections in humans, such as pneumonia, urinary tract infections, and sepsis. *Enterobacter* also found same places as *Klebsiella*. Some species of *Enterobacter*, such as *Enterobacter sakazakii*, can cause infections in humans, especially in immunocompromised individuals, infants and newborns.

Apart from these, also some bacteria are found in fresh leafy greens such as *Vibrio cholerae, Bacillus, Streptococcus, Campylobacter, Clostridium*, etc. which are responsible for food poisoning and linked to many outbreaks. Above all, Shigatoxin-producing *Escherichia coli* (STEC), and *Salmonella*, are the two main culprits behind polluting food. The signs of food poisoning might manifest as nausea, vomiting,

diarrhea, abdominal pain, fatigue, fever, muscle pains, constipation, and others within a few hours to many days of consuming food (UNB, 2021; Banglapedia 2021). In Dhaka, medical clinics treat between 300 and 1,000 patients each day on average, most of whom have diarrhea or cholera, which are frequently caused by contaminated food or drink. In addition to being vehicles of human pathogens, RTE salads may also be vehicles for bacteria with genes coding for resistance to specific antibiotics (Campos e*t al.*, 2013).

This research spotted light on types of bacteria that can be isolated from leafy vegetables, which enable us to detect the presence of pathogenic bacteria on the surface of Coriander leaves, Mint leaves, and Lettuce.

Objectives

1. To isolate and identify the bacteria associated with selected leafy greens
2. To detect human pathogenic bacteria
3. To estimate the colony-forming units and bacterial incidence

CHAPTER II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1. Present status of leafy greens (coriander, mint, and lettuce) in Bangladesh

According to a report in the Daily Star newspaper, a commercial farmer annually produced around 5.0 metric tons (MT) to 6.0 MT of mint (pudina) per acre, where the herb sold for about Tk 150 to Tk 200 per kg (Roy, 2022).

In Bangladesh, the total area under spice cultivation is 4.29 lakh hectares with an annual production of 35.93 lakh metric tons. In recent years, the production rate of spices that are used as an herb or leafy greens like coriander leaf, and mint are 6309 and 3.93 metric tons respectively (BBS, 2021).

Afroj *et al.* (2016), conducted a survey that revealed 96% of vegetable vendors know about lettuce, and out of them, 97% of vendors sold lettuce in the market. It also showed that 86% of vendors sold lettuce at Taka 50-100, while 9% and 5% sold at Taka 101-150 and Taka 151-300, respectively. Besides, 0-250 g lettuce was sold per day in the market by 68% of vendors while 250-500 g lettuce was sold by 18% and the lowest amount was sold at 750-1000 g by only 5% of vendors. 65% of vendors considered that lettuce may be collected from the vegetable market while 24% and 11% of vendors collected from regional producers and import companies.

Afroj *et al.* (2013), conducted research on the "Profitability of lettuce cultivation around Dhaka city of Bangladesh" and said that the yield of lettuce is 10.887 metric tons per hectare and the gross margin is about Tk. 2 lakhs per hectare.

2.2. Bacterial contamination in fresh leafy greens

CDC reported that leafy greens, such as lettuce, spinach, cabbage, etc. are sometimes contaminated with harmful germs like *E. coli* O157 infections, norovirus, *Salmonella*, *Listeria*, and *Cyclospora.* Washing leafy greens do not remove all germs. That's because germs like bacteria can stick to the surface of leaves and even get inside them. You might get sick if you eat contaminated leafy greens without cooking them first, such as in a salad or on a sandwich (CDC, 2022).

Andrea *et al.* (2022), conducted research where a sample of 57 fruits and 76 vegetables including coriander and lettuce obtained from 5 regions of Jakarta, Indonesia and reported the prevalence rate of *Vibrio cholerae* was 100%.

Sowmya *et al.* (2021), conducted research on "Comparative studies of microbial and heavy metal safety assessment of the herbs cultivated in hydroponically and regular soil system" and reported that the sample collected from soil-grown contained *Enterobacter cloacae, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*.

Balali *et al.* (2020), reviewed the study of sources of contamination and type of pathogenic etiological agents isolated from fresh fruits and vegetables including *Bacillus cereus, Campylobacter jejuni, Clostridium botulinum, E. coli* O157: H7, *Listeria monocytogenes, Salmonella* spp., *Shigella, Staphylococcus,* and *Vibrio* cholera.

Yu *et al.* (2019), said that *Bacillus cereus* is a food-borne opportunistic pathogen that can induce diarrheal and emetic symptoms. It is widely distributed in different environments and can be found in various foods, including fresh vegetables.

Houngla *et al.* (2019), operated research on "Microbial Contamination of Leafy Vegetables in Porto-Novo, Republic of Benin" and found the load ranged of faecal

Streptococci from 1.01×10^3 to 3.18×10^3 CFU/g and was significantly ($p < 0.05$) higher in amaranths than in lettuces and nightshades.

Saifullah *et al.* (2018), reported that in Quetta city of Pakistan, *Staphylococcus aureus* was found in 54 salad vegetable samples out of 100 samples.

Callejon *et al.* (2015), reported that the consumption of fresh produce is associated with a growing number of foodborne outbreaks due to bacterial contamination of these products.

Jay-Russell *et al.* (2014), stated that Leafy greens, like lettuce, spinach, and fresh herbs, are some of the vegetables that are most frequently linked to bacterial infections.

Bacteria can reach leafy vegetables easily at the time of harvest and post-harvest, from handling, storing, transporting, and at the grocery store, some of them are pathogens others are potential pathogens or nonpathogens when these vegetables are eaten, and illnesses caused by these microorganisms may occur (Inderbinen and Stephan, 2016; Martínez-Vaz *et al.*, 2014; Barrera *et al.*, 2012).

Robin *et al.* (2010), lead research on "Prevalence and quantification of *Vibrio parahaemolyticus* in raw salad vegetables at retail level" and studied the prevalence of *Vibrio parahaemolyticus* in Lettuce (12.5%).

The Food Safety Project reported that in the United States, from 1990 to 2005, at least 713 produce-related outbreaks were associated with foodborne diseases of which 12% involved fresh fruits and vegetables (FDA, 2004).

Larry (2002), reviewed a bunch of research papers and found that fruits and vegetables can become contaminated with pathogenic microorganisms such as *E. coli*, *Salmonella*, *Shigella*, *Listeria*, *Staphylococcus*, *Vibrio cholerae*, and *Bacillus cereus* while growing in fields, orchards, vineyards, or greenhouses, or during harvesting, post-harvest handling, processing, distribution, and preparation in food service or home settings.

Drawn together by investigating environmental factors that could lead to contamination, the following pathogens of concern were identified for fruits and vegetables eaten raw: *Salmonella*, *Shigella*, *E. coli*, *Campylobacter*, *Yersinia*, *Listeria*, *Staphylococcus*, *Clostridium* spp., *Bacillus cereus*, *Vibrio* spp., Viruses, and Parasites. Most produce is grown in the natural environment and is therefore vulnerable to contamination from many sources: e.g., from the soil, irrigation water, wild animals, personnel, harvesting equipment, and post-harvesting treatment and distribution (WHO, 1998).

2.3. Human illness due to the consumption of pathogenic bacteria from fresh leafy greens

In 2020, the Centers for Disease Control and Prevention (CDC) and the U.S. Food and Drug Administration (FDA) investigated several multistate outbreaks strain of *E. coli* O157:H7 infections which are linked to leafy green vegetables, at the end of the outbreak, 40 cases of illness were reported in the USA where 20 people were hospitalized, and 4 developed hemolytic uremic syndromes (HUS), a type of kidney failure. No deaths were reported (CDC, 2020).

Journalist Joe Whitworth reported in 2020 that A Shigella outbreak in Denmark that sickened more than 44 people including 30 women and 14 men between 0 and 75 years old was likely caused by imported fresh mint. Thirteen people were hospitalized (FSN, 2020).

Zhang *et al.* (2018) reported that *Klebsiella pneumoniae* is not only a major hospital-acquired pathogen but also an important food-borne pathogen that can cause septicaemia, liver abscesses, and diarrhea in humans.

Enterococcus faecalis, Pseudomonas spp. Enterobacter spp., Mycobacterium spp., and *Listeria monocytogenes* are also detected in leafy vegetables worldwide and are responsible for many illnesses (Murray *et al.,* 2017; Rivera *et al.,* 2015).

Illnesses represented by gastrointestinal tract infections, nervous system infections, bacteremia, and many other illnesses can be caused by *E. coli*, *Pesudomonas fluorescens, Shigella* sp. *Salmonella* sp. etc. (Inderbinen and Stephan, 2016; Scales *et al.,* 2014; Martínez-Vaz *et al.,* 2014).

E. coli, Salmonella, and *Shigella* were responsible for many deaths all over the world (Inderbinen and Stephan, 2016; Callejas *et al.,* 2011).

Kirk *et al.* (2014), stated that Enterotoxin-producing *Staphylococcus aureus* causes toxin-mediated food poisoning with an estimated 1300 cases reported annually in Australia.

E. coli O157:H7 secretes a very powerful toxin called verotoxin (Vtx) that binds to the receptors on the human kidney, brain, and gut cells bringing them to death. In addition, this strain of bacteria causes acute hemorrhagic diarrhea with abdominal cramps that in its severe cases result in hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP), and bloody diarrhea (Zhu and Hill, 2013; Hedican *et al.,* 2009; Murry *et al.,* 2007).

Neisseria is aerobic, gram-negative, oxidase-positive coccoid bacteria, and most species are found in pairs (diplococci) on examination of the Gram stain. Two main pathogenic species exist, *N. meningitidis* (commonly referred to as meningococcus) and *N. gonorrhoeae* (commonly referred to as gonococcus) (Ketunuti and Kronman, 2012)

The Advisory Committee on the Microbiological Safety of Food reported that in the UK, there were 531 cases of reported illness, including one death, related to the consumption of fruits and vegetables between 2008 and 2010 (ACMSF, 2011).

Scallan *et al.* (2011), estimated that each year 31 major pathogens acquired in the United States caused 9.4 million episodes of foodborne illness, 55,961 hospitalizations, and 1,351 deaths. Among the 31 pathogens, 21 of them were bacteria such as *E. coli* STEC O157, *Salmonella* sp. *Shigella* sp., *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio* sp. *Brucella* sp., etc.

Mitov *et al.* (2010) reported that pathogenic *Pseudomonas aeruginosa* is an opportunistic pathogen and one of the main bacteria that cause nosocomial infections in hospitals that affects immunocompromised persons.

WHO categorized leafy vegetables as fresh produce which is currently presenting the greatest concern in terms of microbiological hazards safety from a global perspective (WHO, 2008).

Chiang *et al.* (2008), stated that *Staphylococcus aureus* is a pathogenic bacterium which causes food borne illness along with several other diseases.

Pradel and Pages (2002) investigated that *Enterobacter aerogenes* is a multi-drug resistant to antibiotics that can be involved ithe n urinary tract, gastrointestinal, and bloodstream infections and are implicated as a potential cause of adult meningitis.

B. cereus causes two different types of food poisoning, one emetic (intoxication) due to a preformed small cyclic peptide, and one diarrhoeal (infection) due to enterotoxins (Granum and Lund, 1997).

Streptococcus pyogenes are responsible for many diseases such as pharyngitis, scarlet fever (rash), impetigo, cellulitis, or erysipelas. Invasive infections can result in necrotizing fasciitis, myositis, and streptococcal toxic shock syndrome. Patients may also develop immune-mediated sequelae such as acute rheumatic fever and acute glomerulonephritis. *S. agalactiae* may cause meningitis, neonatal sepsis, and pneumonia in neonates whereas adults may experience vaginitis, puerperal fever, urinary tract infection, skin infection, and endocarditis (Patterson, 1996).

2.4. Detection of bacteria from fresh leafy greens

Turki *et al.* (2020), conducted research on the "Effect of washing and prevalence of bacteria in leafy vegetable foods collected from Iraqi markets" where authors used 25 bundles of leafy vegetable samples for isolation. 20 g of each sample had been soaked in 800 ml of sterile distilled water to make stock solutions and then serial dilutions were made up to 1:10,000. 0.1 ml of each dilution had been inoculated onto nutrient agar media plates after that incubated at 37°C for 18 -24 hr. They identified 11 genera of bacteria which were *Staphylococcus, Bacillus, Enterobacter, Shigella, Escherichia coli, Klebsiella, Salmonella, Acinetobacter, Pseudomonas, Leclerciaa,* and *Neisseria*.

Kausar *et al.* (2015), operated research on the "Microbial Studies of Pathogenic *E. coli* from Coriander, Mint Leaves, and Lettuce Collected from Different Localities of Lahore" where a total of 300 samples of coriander, mint leaves, and lettuce were collected. Different selective media such as MacConkey agar, Eosin Methylene Blue agar, Sorbitol MacConkey agar, and EC medium were used to isolate pathogenic *E. coli* O157 and found that the surfaces of salad vegetables (coriander, lettuce, and mint leaves) showed 100% contamination even after the first wash, this pathogenic bacterium remains present on the surface of salad vegetables.

2.5. Identification of bacteria

2.5.1. Cultural and morphological characteristics of different bacteria

Basavaraju and Gunashree (2023) investigated the characteristics of *E. coli*, a gram-negative, straight, rod-shaped bacterium. Their study revealed that *E. coli* colonies on NA plates are 1-3 mm in size, round, greyish-white, convex, smooth, translucent, and opaque. These features serve as crucial identifiers for this commonly encountered bacterium.

Lu *et al.* (2018) focused on *Bacillus*, a gram-positive, rod-shaped bacterium. Through their research, they identified that *Bacillus* colonies on NA plates are medium-sized, flat, round with jagged edges, white, rough, and opaque. These distinct attributes aid in the accurate identification of *Bacillus* isolates.

Verma and Verma (2018) explored the properties of *Pseudomonas aeruginosa*, a gram-negative, rod-shaped bacterium. Their research highlighted that *Pseudomonas aeruginosa* colonies on NA plates are cream, opaque, and irregular, with a distinctive small, rough, and strongly cohesive structure. These features are instrumental in distinguishing this bacteria.

Gnanamani *et al.* (2017) delved into the characteristics of *Staphylococcus aureus*, a gram-positive, spherical-shaped bacterium. They discovered that *Staphylococcus aureus* colonies on nutrient-rich agar media appear yellow due to the presence of carotenoids produced by the organism. The yellow color, resembling gold, is a significant identifying factor for this bacterium.

Cosmas *et al.* (2016) conducted a study on *Enterobacter*, a rod-shaped, gram-negative bacterium. Their findings indicated that *Enterobacter* colonies on NA plates exhibit a cream, irregular appearance, and are small and raised with entire edges. Such unique colony characteristics provide valuable information for differentiating *Enterobacter* from other bacteria.

Hill *et al.* (2016) focused on *Neisseria,* a gram-negative, obligate, fastidious, diplococcus bacterium. Their research emphasized the importance of recognizing the distinctive characteristics of Neisseria for accurate identification.

Sankar *et al.* (2012) studied *Vibrio* spp., a gram-negative, rod-shaped bacterium. They observed that *Vibrio* colonies on NA plates are small (2-3 μm), yellow-green, raised, and smooth.

The website (universe84a.com) provided information on *Klebsiella*, a gram-negative, rod-shaped bacterium. According to this source, *Klebsiella* colonies on NA plates appear large, mucoid, and white in color due to the presence of capsular material produced by the organism. This mucoid nature of colonies serves as an essential characteristic for identifying *Klebsiella*.

Similarly, the website (onlinebiologynotes.com) provided information on *Shigella,* a gram-negative, rod-shaped bacterium. *Shigella* colonies on NA plates were described as smooth, circular, convex, greyish, or colorless, with a translucent appearance and a diameter of 2-3 mm.

2.5.2. Characteristics of different bacteria in biochemical tests and selective media

Aryal (2022), described that Eosin Methylene Blue (EMB) agar is a differential microbiological medium, which slightly inhibits the growth of Gram-positive bacteria and provides a color indicator distinguishing between organisms that ferment lactose such as *E. coli, Klebsiella pneumoniae,* and *Enterobacter aerogenes* which produced blue-black with or without metallic green sheen, mucoid pink, and pink without sheen respectively and those that do not such as *Salmonella, Shigella,* etc. In the case of SS agar, he reported that *Shigella, Salmonella, Klebsiella*, and Enterobacter developed colorless, colorless with a black center, cream, and cream color respectively. *Neisseria* was a gram-negative, diplococci-shaped bacteria and showed positive in catalase and oxidase tests and negative in nitrate reduction, lactose, sucrose fermentation, and other

tests. *Vibrio cholerae* was a gram-negative, flagellated, non-sporing, motile, rod-shaped bacteria that were positive in gelatin hydrolysis, citrate, indole, oxidase tests, and negative in urease, methyl red, and so on. *Staphylococcus* aureus was a gram-positive, non-capsulated, non-sporing, cocci-shaped, non-motile, bacteria that were found to be positive in catalase, citrate, lactose, gelatin hydrolysis, and nitrate reduction whereas negative in oxidase, indole.

Enez (2020), described that *Bacillus* sp. was a gram-positive, rod-shaped, motile, catalase, hemolysis, glucose, and lactose positive whereas oxidase, H_2S, and indole were found to be negative.

Wang *et al.* (2020) conducted a comprehensive investigation of Bacillus velezensis, reporting several biochemical traits. *B. velezensis* tested positive for gram staining, catalase, gelatin liquefaction, starch, and casein hydrolysis. On the other hand, it was negative in nitrate reduction and xylose tests. Furthermore, Zhou *et al.* (2022) found that *B. velezensis* was also negative in the citrate utilization test.

Sivakumar *et al.* (2019), stated that *Pseudomonas* sp. was a gram-negative, rod-shaped, and motile bacteria showing positive in catalase and oxidase tests whereas negative in endospore straining, indole, methyl red, and VogesProskauer tests. On the other hand, he also reported that *Bacillus* sp. was gram-positive, rod-shaped, motile bacteria showing positive in endospore straining but negative in catalase, oxidase, indole, methyl red, and VogesProskauer tests.

Al-Baer and Hussein (2017), stated that *E. coli* showed positive results in catalase, indole, and lactose fermentation tests and negative results in oxidase and citrate utilization tests.

Escherichia, Shigella, Salmonella, Yersinia, Enterobacter, Klebsiella, etc. are some of the most important genera that belong to the family Enterobacteriaceae which is a large,

diverse family and the members of this family are gram-negative, facultative, anaerobic, bacilli (rod-shaped) bacteria (PHE, 2015).

De Melo *et al.* (2012) investigated *Bacillus subtilis* and identified it as a promising levan producer. They found that *B. subtilis* effectively ferments sucrose and possesses a high levan-formation capacity.

Dela Cruz and Torres (2012) conducted research on *Bacillus subtilis, Staphylococcus aureus,* and *Escherichia coli.* They found that *B. subtilis* and *S. aureus* tested positive in the gelatin liquefaction test, while *E. coli* showed negative results in the same test.

Awais *et al*. (2007) investigated the characteristics of *Bacillus subtilis*, describing it as a gram-positive, rod-shaped, and motile bacterium. The study showed that *B. subtilis* tested positive in catalase, oxidase, citrate utilization, starch, and casein hydrolysis. However, it was negative in indole production, methyl red, and gas production from the glucose test.

Shivaji *et al.* (2006) studied *Bacillus altitudinis* and observed several biochemical traits. The bacterium tested positive for gram staining, motility, oxidase, catalase, starch hydrolysis, and gelatin liquefaction. However, it was negative in casein hydrolysis and citrate utilization tests.

Takahashi *et al.* (2002) explored the biochemical properties of Neisseria and reported that it tested positive for oxidase, catalase, and fermentation of glucose and maltose. However, it exhibited negative results in nitrate reductase and sucrose fermentation tests.

Holbrook and Anderson (1980), showed that Bacillus Cereus Agar is a selective media for the identification of *Bacillus cereus* where a distinct colonial appearance of this bacteria by producing a peacock blue color on the media.

2.5.3. Molecular analysis of bacteria

Wang et al. (2020) isolated the Genomic DNA of Bacillus spp. and then the 16S ribosomal RNA (rRNA) gene was amplified using the universal primers 27F (5′-AGAGTTGATC CTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTAC GACTT-3′).

Nurfitri *et al.* (2019) successfully amplified the 16S rRNA gene in *Bacillus* spp. isolate using 27F and 1492R primers, resulting in an observed band size of approximately 1465± bp on a 0.8% agarose gel.

Galkiewicz and Kellogg (2008) performed PCR to identify bacteria using 12.5 µl AmpliTaq Gold, 1 µl each of 10 pM concentrations of forward and reverse primers, 9.5 µl sterile deionized water, and 1 µl DNA template, for a total volume of 25 µl.

Abdulmajeed *et al.* (2008) performed PCR in an automated thermal cycler with an initial denaturation at 95°C for 5min. followed by 30 cycles of 95°C for 30sec (denaturation), 52°C for 45sec (annealing), 72°C for 1.5min (extension) and 72°C for 10min (final extension). They run PCR products on 1% agarose in TAE buffer to confirm that the right product (1500bp) was formed.

2.6. Bacterial load in leafy greens

In their investigation in Cape Coast, Ghana, Yafetto et al. (2019) reported a bacterial load of 1.23×10^8 CFU/ml for lettuce leaves on NA plates.

Hougbenou et al. (2019) reported the presence of aerobic mesophilic bacteria in 3 selected leafy greens (Lettuce, amaranth and nightshades) at Porto -Novo in Republic of Benin. The highest total coliform counts were found in lettuces (3.21×10^3 CFU/g), while nightshades had the lowest (1.78×10^2 CFU/g), demonstrating significant differences ($p<0.05$).

In their study conducted in Chittagong, Bangladesh, Nipa et al. (2011) observed viable bacterial colonies in coriander leaves ranging from 5.87×10^5 to 1.8×10^6 CFU/g, and in peppermint leaves ranging from 2.2×10^5 to 7.7×10^5 CFU/g.

Santos et al. (2005) provided valuable guidelines for bacterial load related to food contamination, specifically focusing on ready-to-eat leafy green salads. The study categorized bacterial contamination levels into three groups: Satisfactory, Acceptable, and Not Acceptable, based on the total aerobic mesophilic bacteria (TAMB). For ready-to-eat leafy green salads, the "Satisfactory" contamination level for TAMB was found to be less than or equal to 10^4 CFU/g, indicating an acceptable range of bacterial presence. The "Acceptable" range was greater than 10^4 CFU/g and less than or equal to 10^6 CFU/g, signifying a moderate level of contamination. On the other hand, contamination levels exceeding 10^6 CFU/g were categorized as "Not Acceptable," suggesting a potentially hazardous level of bacterial load.

2.7. Bacterial incidence in leafy greens

Srisamran *et al.* (2022), reported the prevalence of many vegetables including leafy vegetables such as coriander, lettuce, etc. where was the incidence of bacteria in coriander for *E. coli* (100% in open market samples, 83.3% in supermarket samples, 91.7% in certified organic samples), *Salmonella* (12.5% in open market samples, absent in supermarket samples, 4.2% in certified organic samples), Shigella (absent in all samples). In the case of lettuce, the incidence was for *E. coli* (100% in open market samples, 91.3% in supermarket samples, 75% in certified organic samples), *Salmonella* (16.7% in open market samples, 8.7% in supermarket samples, absent in certified organic samples), Shigella (absent in all samples).

Turki *et al.* (2020), investigated the prevalence of pathogenic bacteria in leafy vegetables were *Staphylococcus, Bacillus, Enterobacter, Shigella, Escherichia coli* (100%); *Klebsiella, Salmonella* (92%); *Acinetobacter* (80%); *Pseudomonas* (72%); *Leclerciaa* (60%); and *Neisseria* (12%).

CHAPTER III

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Study site

The experiment was carried out in the MS lab of the Department of Plant Pathology at Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207.

3.2. Study period

The experiment was conducted from July 2021 to June 2022.

3.3. Selection of leafy greens in this study

In this study, 3 different leafy greens were used which are eaten by people raw (without cooking) that are considered ready-to-eat (RTE) leafy greens. These selected leafy greens were collected from diverse marketplaces at several locations in Dhaka City, Bangladesh (Table 1.)

Table 1. Detailed information of the collected leafy greens in this study

Sl. No.	Types of marketplaces	Location/name of marketplaces	Moisture condition of samples at marketplaces
1	Supermarkets	Unimart	Dry
2		Swapno	Dry
3		Meena Bazaar	Dry
4	Local markets	Rayer Bazaar	Moist
5		Karwan Bazaar	Moist
6		Mohammadpur Krishi Market	Moist
7	Roadside Stand Bazaar	Dhanmondi road	Moist
8		Zigatola road	Moist
9		Mohammadpur road	Moist

3.4. Collection of samples

27 samples have been collected from 9 different marketplaces during July and August 2021. Each sample was collected three times during each visit, with 100 grams of each being taken at random from each market. Only fresh and disease-free samples were collected where no leaf spot, blight, or rot was observed. An insulated box with ice and a sterile zipper bag was used to collect all the samples, which were then thoroughly examined and analyzed within an hour of being collected. (Plate 1).

3.5. Preservation of samples

For further research, the collected samples were placed in sterile zipper bags and kept in the normal chamber of the refrigerator.

Plate 1. Collection of Samples; A. Sample Collection from Super-market, B. Sample Collection from the Local market, C. Sample Collection from Roadside stand Bazar, D. Collection of samples in a zipper bag

3.6. Isolation of bacteria

3.6.1. Preparation of stock solution

The previously collected samples were first cut into small pieces (1-2 cm) with a sterile scissor. Then 100 gm pieces of each of the samples were taken in conical flasks and submerged in 1000 ml of sterile distilled water. All the conical flasks were then shaken, and the samples were macerated and kept for 30 minutes for bacterial streaming to get stock solutions. Each sample's stock solution was stored in a 50 ml sterile centrifuge tube in a refrigerator at 4°-6° C for further study, (Plate 2).

3.6.2. Dilution of stock solutions

Five sterile test tubes, each with 9 ml of sterile distilled water were taken and labeled as 1:10, $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$ and were kept in a test tube holder sequentially. From the stock solution, 1 ml of properly mixed sample was drawn into the pipette and transferred to the first test tube ($1: 10^1$) to make the total volume of 10 ml. This provided an initial dilution of 1:10 dilution. The dilution was thoroughly mixed by using a vortex mixer. Then 1 ml of the mixer was taken from the 1:10 and transferred into the second tube to get a 1: 100 dilution. The same process was then repeated for the remaining test tubes to get 1: 100000 dilution (Plate 2).

3.6.3. Preparation of nutrient agar (NA) media

In a conical flask, 14g of dehydrated NA medium was added to 500 ml of sterile distilled water for the preparation of 500 ml NA medium. The medium was shaken vigorously for a few minutes to dissolve the components properly. Aluminum foil paper was used to cover the opening and cotton was used to seal it. After that, it was autoclaved for 15 minutes at 121 ºC with 15 PSI (Plate 2).

3.6.4. Isolation of bacteria on NA plates through a spread plating method

Autoclaved NA media were then poured on Petri dishes for each of the samples which were marked as C-Sp, C-Lc, C-Rs, M-Sp, M-Lc, M-Rs, L-Sp, L-Lc, and L-Rs at three replications and then 0.05 ml of dilution of 1: 10^4 and 1: 10^5 were spread over the NA plates with the help of sterile glass rod as described by (Goszczynska and Serfontein, 1998). This process was done in a laminar airflow cabinet. The inoculated NA plates were kept in an incubation chamber at 30±1° C. The plates were observed after every 24 hours, (Plate 2).

3.6.5. Isolation of single colony from NA plates through a streak plating method

After 24 hours, the single colony grown over the NA plate was selected to streak on a fresh NA plate to get pure colonies from every individual sample. The streaking was done using a sterile loop. The inoculation loop was first sterilized by passing it through a flame. When the loop was cooled, an isolated colony was picked from the NA plate culture, and it was spread over the first quarter (approximately 1/4 of the plate) using close parallel streaks. The inoculation loop was then immediately streaked very gently over the first quarter of the plate using a back-and-forth motion. The loop was then re-sterilized, and the plate was turned at 90 degrees. Starting in the previously streaked section, the loop was dragged and extended the streaks into the second quarter of the plate. The loop was again re-sterilized, and the procedure was then repeated in the third and into the center fourth quarter of the plate (Plate 2; G). A total of 30 isolates were isolated from NA plates of all the samples in which 5, 3, 5, 2, 3, 2, 3, 4, and 3 isolates were isolated from C-Sp, C-Lc, C-Rs, M-Sp, M-Lc, M-Rs, L-Sp, L-Lc, and L-Rs on NA plates respectively (Table 2).

Table 2. List of Isolation materials of bacteria from coriander, mint, and lettuce

Leafy vegetables	Plate symbol	Isolate symbol	Sl. No.
Coriander leaves	C-Sp	C-Sp_1	1
		C-Sp_2	2
		C-Sp_3	3
		C-Sp_4	4
		C-Sp_5	5
	C-Lc	C-Lc_1	6
		C-Lc_2	7
		C-Lc_3	8
	C-Rs	C-Rs_1	9
		C-Rs_2	10
		C-Rs_3	11
		C-Rs_4	12
		C-Rs_5	13
Mint leaves	M-Sp	M-Sp_1	14
		M-Sp_2	15
	M-Lc	M-Lc_1	16
		M-Lc_2	17
		M-Lc_3	18
	M-Rs	M-Rs_1	19
		M-Rs_2	20
Lettuce leaves	L-Sp	L-Sp_1	21
		L-Sp_2	22
		L-Sp_3	23
	L-Lc	L-Lc_1	24
		L-Lc_2	25
		L-Lc_3	26
		L-Lc_4	27
	L-Rs	L-Rs_1	28
		L-Rs_2	29
		L-Rs_3	30

*C= Coriander, M= Mint, L= Lettuce, Sp= Super-market, Lc= Local market, Rs= Roadside stand Bazar.

3.6.6. Preservation of pure culture through slant culture

Nutrient Agar slants were prepared by bringing Nutrient Agar to the boiling point in a conical flask and pouring 10 ml of it into test tubes. The opening of the test tube was sealed with cotton and covered with aluminum foil paper and autoclaved. To make it a slant tube, NA media were allowed to cool with the tube laying at an angle that resulted in a large surface area for spreading the isolated pure culture of bacteria. Once the slants were cooled, a single streaking was done on the surface of the slanted media with the help of a sterile loop, (Plate 2). The inoculated slant tubes were kept in an incubation chamber at 30±1 °C. The plates were observed after every 24 hours. After growing of colony in the slant tubes it was stored in the normal chamber of the refrigerator for further study.

3.6.7. Preparation of young culture from slant culture

Young cultures were prepared on the fresh new NA plates by taking the inoculum of the slant culture tube and streaking was done with the help of a sterile loop. Every NA plate contained 3 isolates of pure cultures, (Plate 2). After growing young colonies of 24 hours were used for the chemical analysis.

Plate 2. Isolation process of bacteria; A. Preparation of stock solutions B. Preservation of stock solution C. Dilution of bacteria from stock solution D. Mixing the solution by using Vortex mixer E. Preparation of NA media F. Diluted plate culture G. Pure culture of an isolate H. Slant cultures of isolate I. Young cultures of isolate

3.7. Biochemical test

3.7.1. Gram's staining test

At first in a gram's staining, on a clean microscopic slide, a small drop of distilled water was mounted. After that, a tiny section of a young colony from an isolate was taken with the help of a sterile loop from the nutrient agar medium and then the bacterial smear was made on the slide. Air-drying was performed on the thinly applied bacterial film. To attach the bacteria on the glass slide, the underside was heated by running it over a spirit lamp's flame two or three times. After that, the slide was overflowed in crystal violet solution for roughly one minute. The slide was rinsed with a gentle stream of squirt bottled water for a maximum of 5-6 seconds to remove unbound crystal violet. Following that it overflowed in Lugol's iodine solution for approximately one minute. Subsequently, it was decolored with 95% ethanol for 5 seconds and submerged in a soft stream of water. Then it was counterstained with 0.5% safranine for about 1 minute and submerged with a soft stream of water for 5 seconds and air-dried. Then the microscopic glass slide was examined under 100x magnification using immersion oil (Plate 3; A).

3.7.2. KOH solubility test

In the case of KOH solubility test for Gram differentiation of plant pathogenic bacteria without staining, a 3% KOH solution was used (Suslow *et al.,* 1982). Two drops of 3% KOH solution were placed at the center of a clean glass slide. A small portion of young colonies of bacteria from the NA medium was added to the KOH solution and homogenized with a nichrome loop using a quick, 10-second cyclic motion. When drawn with a loop, the sticky strand formation transformed into a fine, 0.4–2.5 cm long thread of slime (Plate 3; B).

3.7.3. Catalase test

Two drops of freshly prepared 3% H_2O_2 solution were added at the center of a clean glass slide with 48 hours old young culture of bacteria from the NA medium. It was observed whether it produced bubbles within a few seconds or not (Plate 3; C).

3.7.4. Oxidase test

A 1% aqueous solution of N,N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride was applied as the test reagent for the oxidase test. Three drops of a freshly made 1% aqueous solution of this reagent were added to a strip of Whatman filter paper (color indicator). A tiny portion of young bacteria culture of 24 hours from the NA medium of each isolate was rubbed individually on the surface of the filter paper using a sterile toothpick. Finally, it was observed whether it showed purple color within 10 seconds or not (Plate 3; D).

3.7.5. Levan production test

Nutrient agar medium with 5% sucrose was used in the Levan production test. For this test, inoculated with pure young colonies of bacteria on the plate by streaking. Then incubated the plate at 30±1°C. After that, examined Levan plate cultures for the presence or absence of white, convex, domed, and mucoid colonies (Plate 3; E).

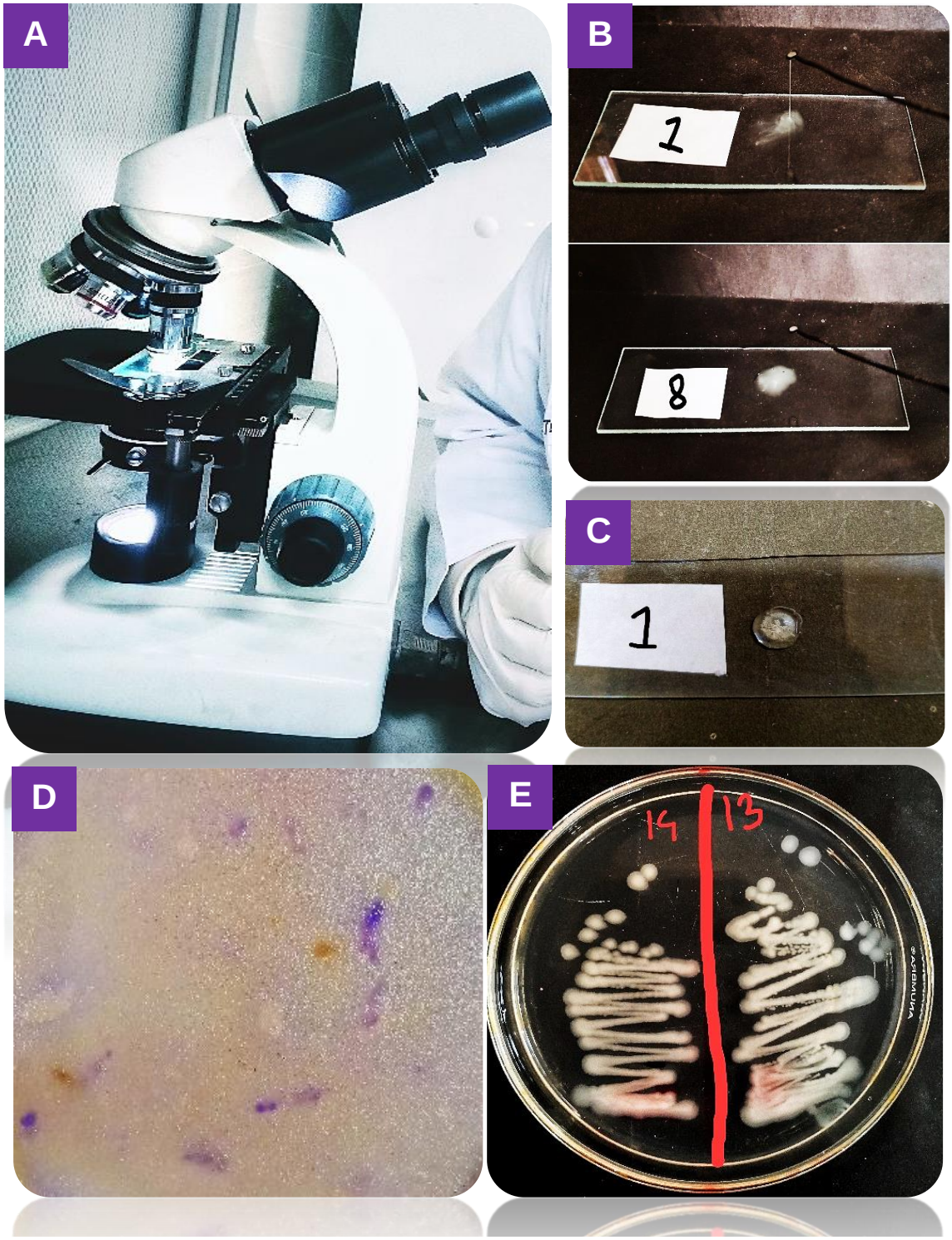


Plate 3. Biochemical test; A. Gram's staining test, B. KOH solubility test, C. Catalase test, D. Oxidase test, E. Levan production test

3.7.6. Starch hydrolysis test

A nutrient Agar plate containing 0.2% soluble starch was inoculated with pure young colonies of bacteria. Then it was incubated at 30°C for 48 hours in an incubation chamber. The plates were flooded with Lugol's iodine solution and observed whether a clear zone appeared around the colony or not (Plate 4; A).

3.7.7. Casein test

Skim milk agar was used in the casein test. For this, inoculated the bacteria on the plate are in a straight line. Incubated the plate at 30±1°C. Then examined the milk agar plate cultures for the presence or absence of a clear zone of proteolysis, surrounding the growth of each of the bacterial test organisms. Positive Test indicates clearing colony growth around and/or beneath colony growth. Whereas the negative test indicates no clear colony growth around and/or beneath the inoculum (Plate 4; B).

3.7.8. Motility test

Motility by bacteria is mostly demonstrated in a semi-solid agar medium. In semi-solid agar media, motile bacterium 'swarms' and gives a diffuse spreading growth that can be easily recognized by the naked eye. The medium mainly used for this purpose is SIM medium (Sulfide Indole Motility medium). A straight needle was touched on a colony of a young (24 hours) culture growing on an agar medium for this test. Then stabbed once to a depth of only 1/3 inch in the middle of the tube and incubated at 35-37°C and examined daily for up to 7 days to observe for a diffuse zone of growth flaring out from the line of inoculation. Diffuse, hazy growths spread throughout the medium rendering it slightly opaque indicating a positive result. Whereas, growth that is confined to the stab line, with sharply defined margins and leaving the surrounding medium clearly transparent indicate a negative result (Plate 4; C)

3.7.9. Simmon’s citrate test

The Simmon's citrate agar medium was poured into test tubes and prepared slant. For this test agar was inoculated lightly on the slant by touching the tip of a needle to a colony that is 24 hours old. Then incubated at 35°C to 37°C for 24 hours. Observed the development of blue color, denoting alkalinization. In citrate utilization, positive growth will be visible on the slant surface and the medium will be an intense Prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raise the pH of the medium to around 7.6, causing the bromothymol blue to change from the original green color to blue. In the case of citrate negative trace or no growth will be visible. No color change will occur, the medium will remain the deep forest green color of the uninoculated agar. Only bacteria which can utilize citrate as the sole carbon and energy source will be able to grow on the Simmon's citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant (Plate 4; D).

3.7.10. Gelatin liquefaction test

A heavy inoculum of a 24-hour young bacteria was stab-inoculated into test tubes containing nutrients with 12% gelatin. The inoculated tubes and an uninoculated control tube were incubated at 25°C for up to 1 week and checked every day for gelatin liquefaction. Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes were kept in a refrigerator at 4°C for 30 minutes. Afterward, tubes were tilted to observe if the gelatin had been hydrolyzed. Hydrolyzed gelatin will result in a liquid medium even after exposure to cold temperatures while the uninoculated control medium will remain solid (Plate 4; E).

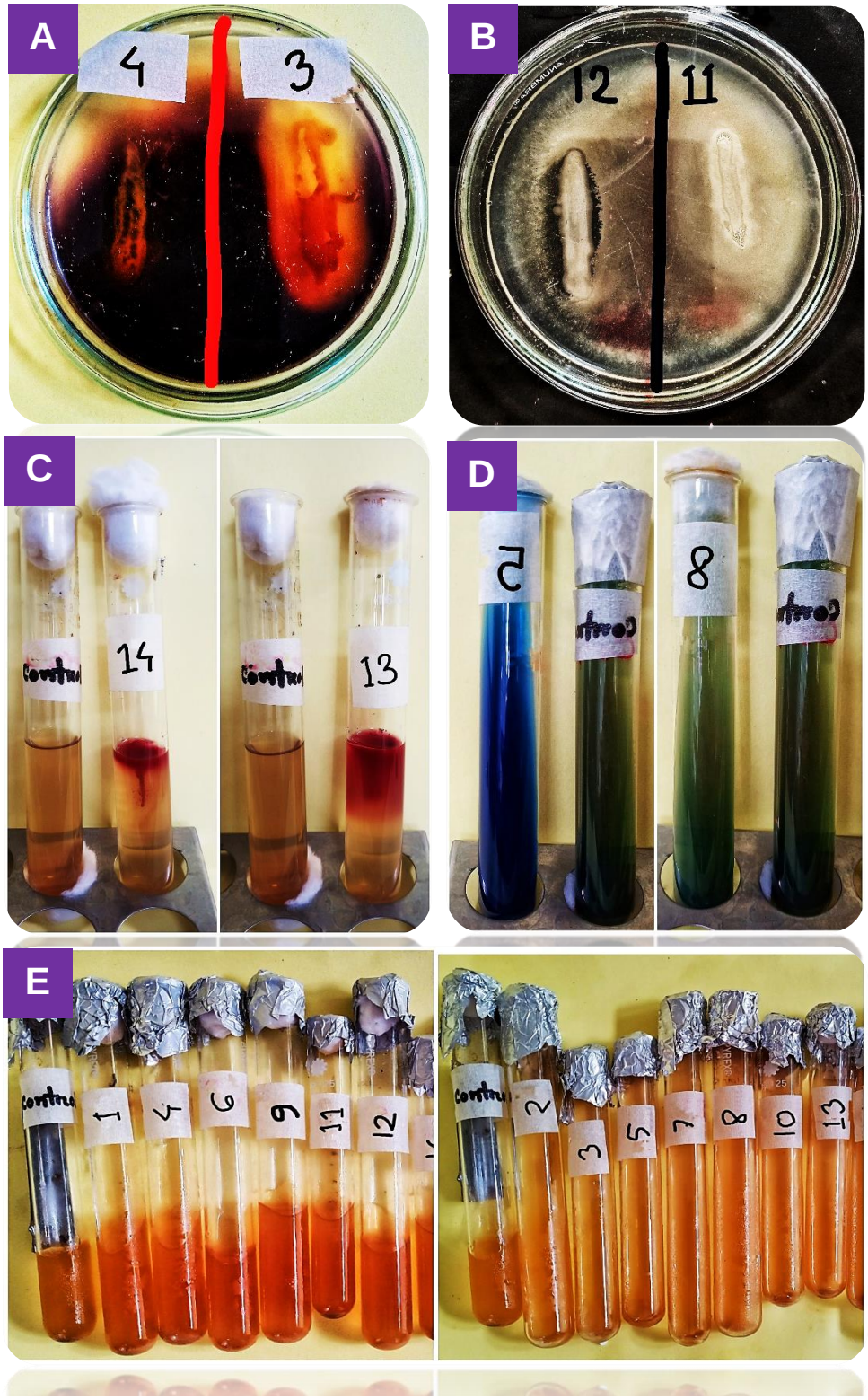


Plate 4. Biochemical test; A. Starch hydrolysis test, B. Casein test, C. Motility test, D. Simmon's citrate test, E. Gelatin liquefaction test

3.8. Isolation of bacteria on selective medium

3.8.1. Growth of bacteria on Bacillus Cereus Agar Base

Bacillus Cereus Agar is a highly specific and selective medium for isolating Bacillus cereus bacteria developed by Holbrook and Anderson (Holbrook and Anderson, 1980). For *B. cereus* and *B. thuringiensis* identification, suspend 20.5 grams of Bacillus Cereus Agar was mixed in 475 ml of purified distilled water (Plate 5; A). The mixture was heated to boiling to dissolve the medium completely. Then the media was sterilized by autoclaving at 15 lbs. pressure and 121ºC for 15 minutes. After that, it was cooled to 45-50ºC, and aseptically added 1 vial of Polymyxin B Selective Supplement and 25 ml of sterile Egg Yolk. Then the medium was poured into sterile Petri plates (Plate 5; B). Bacteria were streaked with a sterile loop (Plate 5; E) for isolation and incubated the plates aerobically at 30±1 ºC for 24-72 hours.

3.8.2. Growth of bacteria on Cetrimide Agar Base

Cetrimide Agar is a selective and differential medium for isolating and identifying *Pseudomonas aeruginosa*, a gram-negative bacterium, in various products (Brown and Lowbury, 1965). However, *Serratia*, *Klebsiella*, and *Acinetobacter* can be cultured. For the identification of *Pseudomonas aeruginosa*, 23.4 grams of Cetrimide Agar was mixed in 500 ml of purified distilled water, and aseptically added 5 ml of Glycerol was boiled to dissolve completely (Plate 5; A). Then the media was sterilized by autoclaving at 15 lbs. pressure and 121ºC for 15 minutes. After that, the medium was poured into sterile Petri plates (Plate 5; B). Bacteria were streaked with a sterile loop for isolation and incubated the plates aerobically at 30±1ºC for 24-72 hours. The plates were examined daily for colony morphology and growth characteristics.

3.8.3. Growth of Bacteria on Eosin Methylene Blue (EMB) Agar

Eosin Methylene Blue (EMB) agar is a differential medium, which slightly inhibits the growth of Gram-positive bacteria and provides a color indicator distinguishing between organisms that ferment the lactose (e.g., *E. coli*) and those that do not such as *Salmonella*, *Shigella*, (Levine, 1918). For the identification of *E. coli* and lactose fermenting bacteria like *Enterobacter,* and *Klebsiella*, suspend 18 grams of EMB Agar was mixed in 500 ml of purified distilled water. The mixture was shaken to boiling to dissolve the medium completely. Then the media was sterilized by autoclaving at 15 lbs. pressure and 121ºC for 15 minutes. After that, it was cooled to 45-50°C and shook the medium to oxidize the methylene blue (i.e., to restore its blue color) and suspend the flocculent precipitate. Then the medium was poured into sterile Petri plates (Plate 5; C). Bacteria were streaked with a sterile loop for isolation and incubated the plates aerobically at 35-37ºC for 18-24 hours and protected from light. The plates were examined the next day for colony morphology and growth characteristics.

3.8.4. Growth of Bacteria on Salmonella Shigella (SS) Agar

Salmonella Shigella (SS) Agar is a moderately selective and differential medium for the isolation, cultivation, and differentiation of Salmonella spp. and some strains of Shigella spp. (Leifson, 1935). For the identification of *Salmonella* spp. and *Shigella* spp., suspend 30 grams of SS Agar was mixed in 500 ml of purified distilled water. The mixture was heated to boiling to dissolve the medium completely. It was not autoclaved to avoid destroying the selectivity of the media. The media was mixed well and poured into sterile Petri plates (Plate 5; D). Bacteria were streaked with a sterile loop for isolation and incubated the plates aerobically at 35-37ºC for 18-24 hours. The plates were examined the next day for colony morphology and growth characteristics.

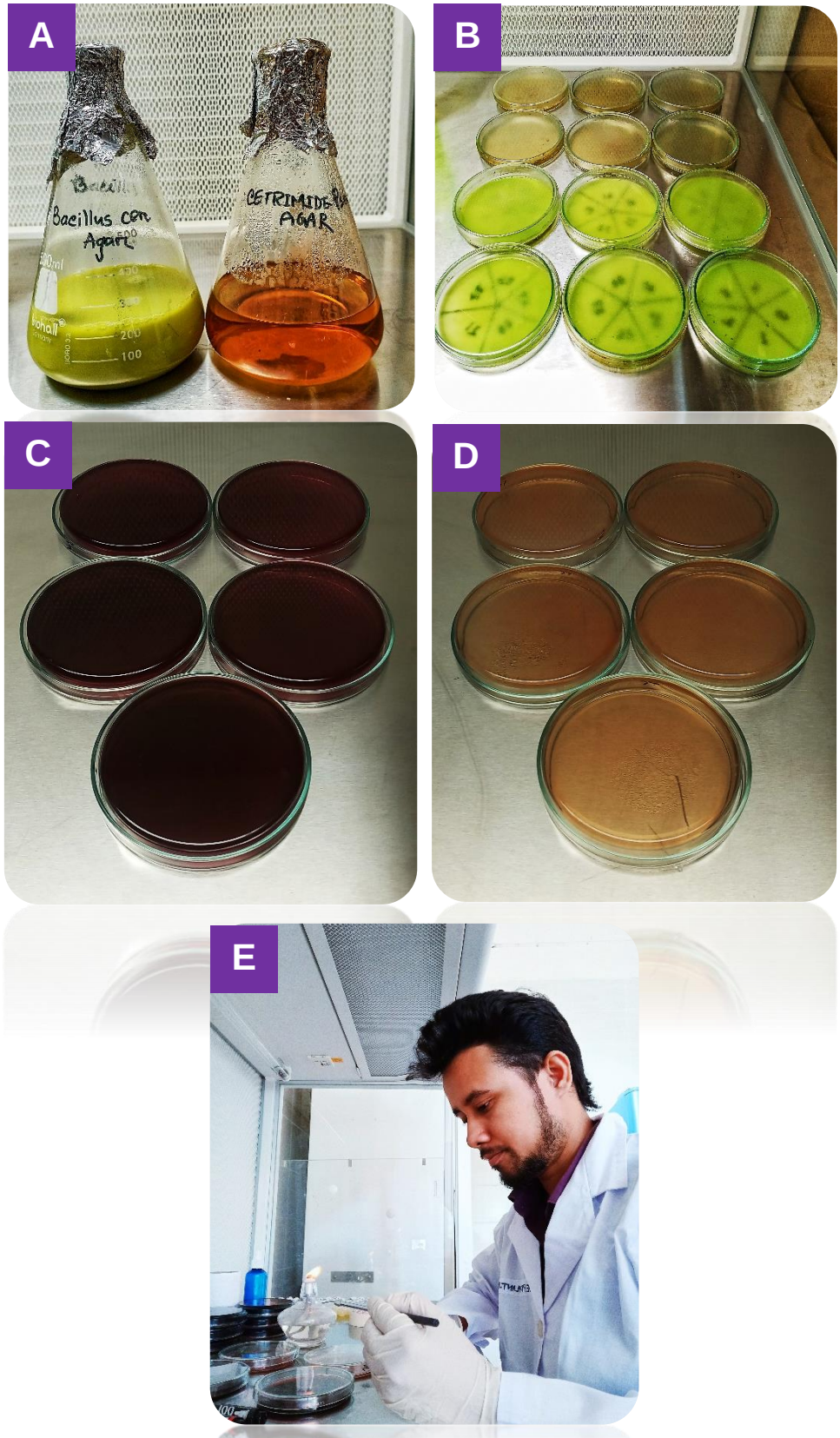


Plate 5. Bacteria isolation on selective media; A. Bacillus Cereus and Cetrimide Agar Mixture, B. Bacillus Cereus and Cetrimide Agar Petri plates, C. EMB Agar Petri plates, D. SS Agar Petri plates, E. Streaking on culture plate

3.9. Molecular analysis

3.9.1. DNA extraction

Materials and chemicals used

i. Overnight 3 different bacterial cultures (isolate no. 7, 13 and 17)
ii. 1.5ml microcentrifuge tubes
iii. Isopropanol
iv. Ethanol 70%
v. 50mM EDTA
vi. Lytic enzymes (e.g., lysozyme)
vii. Nuclei lysis solution
viii. RNase solution
ix. Protein precipitation solution
x. Rehydration solution

Procedure used

Initially, 1ml of overnight cultures was centrifuged at 15,000 × g for 2 minutes, and the supernatant was discarded, leaving behind the cell pellet. Next, the cells were resuspended in 480µl of 50mM EDTA, and 120µl of lytic enzymes (lysozyme) was added, followed by incubation at 37ºC for 60 minutes. The lysed cells were then centrifuged at 15,000 × g for 2 minutes, and the supernatant was discarded. For cell lysis, 600µl of nuclei lysis solution was added, and the mixture was incubated at 80ºC for 5 minutes, cooled to room temperature, and treated with 3µl RNase solution at 37ºC for 60 minutes. Protein precipitation was achieved by adding 200µl of protein precipitation solution, vortexing vigorously at high speed for 30 seconds to mix the protein precipitation solution with the cell lysate. The vortex mixture was incubated on ice for 5 minutes and centrifuged at 15,000 × g* for 3 minutes. The resulting supernatants were transferred to another clean tube containing 600µl of isopropanol, and DNA precipitation was facilitated by inverting the tube until visible DNA strands formed. After centrifugation at 15,000 × g* for 2 minutes and the pellet cells were removed. Then 600µl of room temperature 70% ethanol was added and gently inverted the tubes several times to wash the DNA pellet and centrifuged at 15,000 × g* for 2 minutes. The DNA pellet was air-dried for 15 minutes. Lastly, the DNA pellet was

rehydrated in 100µl of rehydration solution and incubated overnight at 4ºC. This method provides an effective means of extracting DNA from overnight bacterial cultures for downstream molecular biology applications.

3.9.2. DNA quantification

The quantification of extracted DNA was performed using a NanoDrop spectrophotometer. Prior to measurement, the DNA samples were prepared by transferring 1-2 µL onto the NanoDrop microvolume measurement pedestal. A blank measurement using the appropriate solvent was taken to calibrate the spectrophotometer. Subsequently, the absorbance of light at 260 nm and 280 nm was measured, allowing for the calculation of DNA concentration based on Beer's law. The purity of the DNA samples was assessed by determining the ratio of absorbance at 260 nm to 280 nm (A260/A280). A pure DNA sample typically has an A260/A280 ratio of around 1.8-2.0. The quantification and purity data were recorded for each DNA sample, and these values were used for subsequent molecular biology applications, such as PCR and DNA sequencing.

3.9.3. PCR amplification

After the quantification of extracted genomic DNA of 3 different bacteria isolates, the 16S ribosomal RNA (rRNA) gene was amplified using the universal primers 27F (5′-AGAGTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) which produced 1465-bp amplicons (Wang *et al.*, 2020). A 25 µL PCR mixture contained 12.5 µL of GoTaq™ G2 hot start master mix, 1 µL T DNA (25-65 ng/µl), 1 µL Primer 27F (10-20 pMol), 1 µL Primer 1492R (10-20 pMol), and 9.5 µL nuclease-free water (Galkiewicz and Kellogg, 2008). The PCR assay was performed at a volume of 25 µL in an automated thermal cycler (Gene Atlas, Model: G2). The PCR thermal protocol consisted of initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 52 °C for 45 s, and 72 °C for 90 s, and a final extension step at 72 °C for 10 min and finally hold at 4 °C for overnight (Abdulmajeed *et al.,* 2008). 5µl aliquot of each amplified PCR product was subjected to electrophoresis on 1% agarose gel. The PCR product was stored in a refrigerator at -20°C.

3.9.4. Electrophoresis and gel documentation

In the gel electrophoresis and gel documentation method, 1g of agarose powder (Cat: V3125) was mixed with 100 mL of Tris Borate EDTA (TBE) buffer in a 500 mL beaker using a magnetic stirrer (Abdulmajeed *et al.,* 2008). The mixture was then heated to 80°C for 5 minutes to ensure complete dissolution. After cooling for 2 minutes, 6 µl of ethidium bromide was added to the gel solution. The gel was poured onto a gel tray, and a comb with 20 teeth was placed to create sample wells. After 20 minutes, the DNA samples, along with a 1kb ladder as a size marker, were loaded into the wells. The electrophoresis chamber was filled with TBE running buffer, and the gel tray was carefully placed in the chamber. Electrophoresis was run at 90 volts for 30 minutes to separate the DNA fragments based on their sizes. After completion, the electrophoresis was stopped, and the gel was transferred to an gel documentation system (Alpha imager MINI) for visualization and imaging of the DNA bands.

3.9.5. DNA purification

DNA purification was performed following gel electrophoresis by excising the DNA band from the gel and placing the gel slice in a 1.5ml microcentrifuge tube. Next, 10µl of Membrane Binding Solution was added per 10mg of gel slice, and the tube was vortexed and incubated at 50–65°C until the gel slice completely dissolved. For processing PCR amplifications, an equal volume of Membrane Binding Solution was added to the PCR amplification products. Subsequently, the DNA samples were transferred to SV Minicolumns and incubated for 1 minute at room temperature. After centrifugation at 16,000 × g for 1 minute, the flowthrough was discarded, and the Minicolumns were washed twice using Membrane Wash Solution with ethanol. The Minicolumns were then centrifuged at 16,000 × g for 5 minutes. After emptying the Collection Tube, the column assembly was recentrifuged for 1 minute with the microcentrifuge lid open to allow ethanol evaporation. For elution, the Minicolumns were carefully transferred to clean microcentrifuge tubes, and 50µl of Nuclease-Free Water was added. After incubation at room temperature for 1 minute and centrifugation at 16,000 × g for 1 minute, the purified DNA was obtained and ready for downstream applications.

3.9.6. DNA sequencing

The purified DNA samples were sent to Bioneer (Seoul, Korea) for partial sequencing. The DNA sample was subjected to Sanger sequencing analysis. The raw sequencing data of 16s rRNA gene was saved in the AB1 file format. Using Chromas 2.6 software, the AB1 file was processed, and a FASTA file containing the partial 16s rRNA gene sequence was generated.

3.9.7. DNA analysis

To identify the sequence's origin and function of 3 selected bacteria isolates, the sequences were analyzed by using the BLAST (Basic Local Alignment Search Tool) on the NCBI website (https:// www.ncbi.nlm.nih.gov) for matching with existing nucleotide sequences in the NCBI GenBank database. The search yielded a significant match with a known sequence, and the accession numbers were obtained for the identified sequence. Phylogenetic analysis was performed by using MEGA 11 (Molecular Evolutionary Genetics Analysis) software where the maximum likelihood algorithm was selected to construct the phylogenetic tree with 1000 bootstraps and nucleotide Tamura-Nei substitution model.

3.10. Estimation of bacterial load

After 24 hours of inoculation, colony-forming units (CFU) were first counted and recorded on the same day. Data were recorded visually by observing the appearance of colonies behind the NA plates and counted manually by putting a dot of colored pencil on every single colony. During the estimation of the colony, the plate contained a low no. of colonies counted as a full plate whereas the plate contained a high no. of colonies counted as a half or even quadrant part of the plate and then multiply the colonies no. 2 and 4 respectively (Plate 6). At first, CFU was estimated for each ml of stock solution after that this value was multiplied by 10 (100 g samples submerged in 1000 ml stock solutions) to determine the CFU per gram of leafy green samples by following the equation:

$$\textbf{CFU/ml} = \frac{\text{No of colonies per plate} \times \text{Total dilution factor}}{\text{Volume of culture per plate in ml}}$$

Here, Total dilution factor = 10^5 (Five-fold serial dilution was performed)

volume of culture per plate = 0.05 ml

CFU/g= CFU/ml × 10

3.11. Estimation of bacterial incidence

Measurement of the incidence of different types of bacteria in leafy greens was determined by the following equation:

$$\textbf{Incidence of bacteria}\ (\%) = \frac{\text{No. of samples contaminated by bacteria}}{\text{Total no. of samples}} \times 100$$

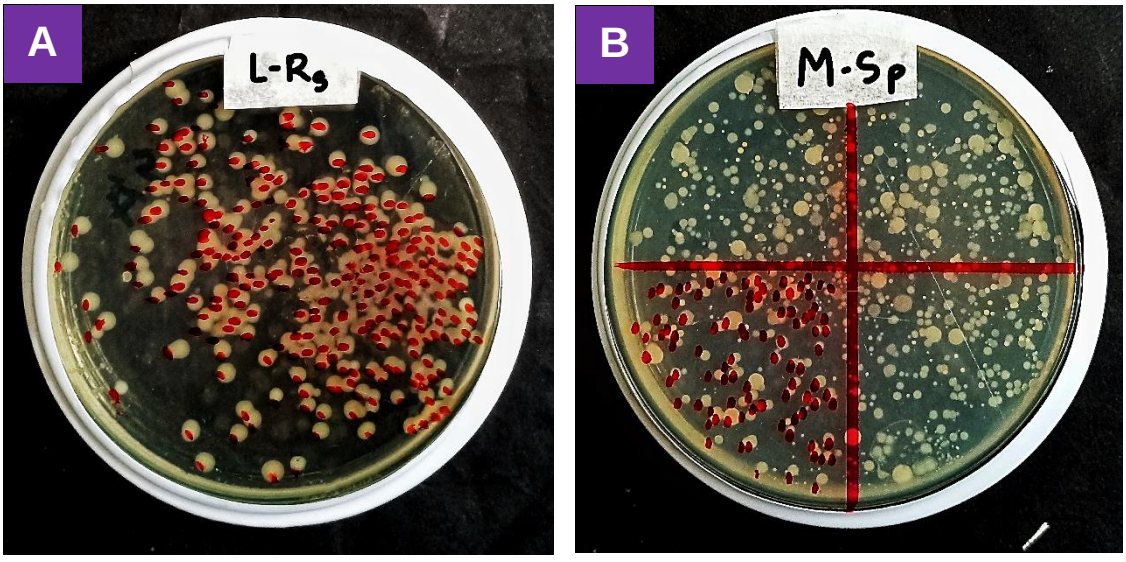
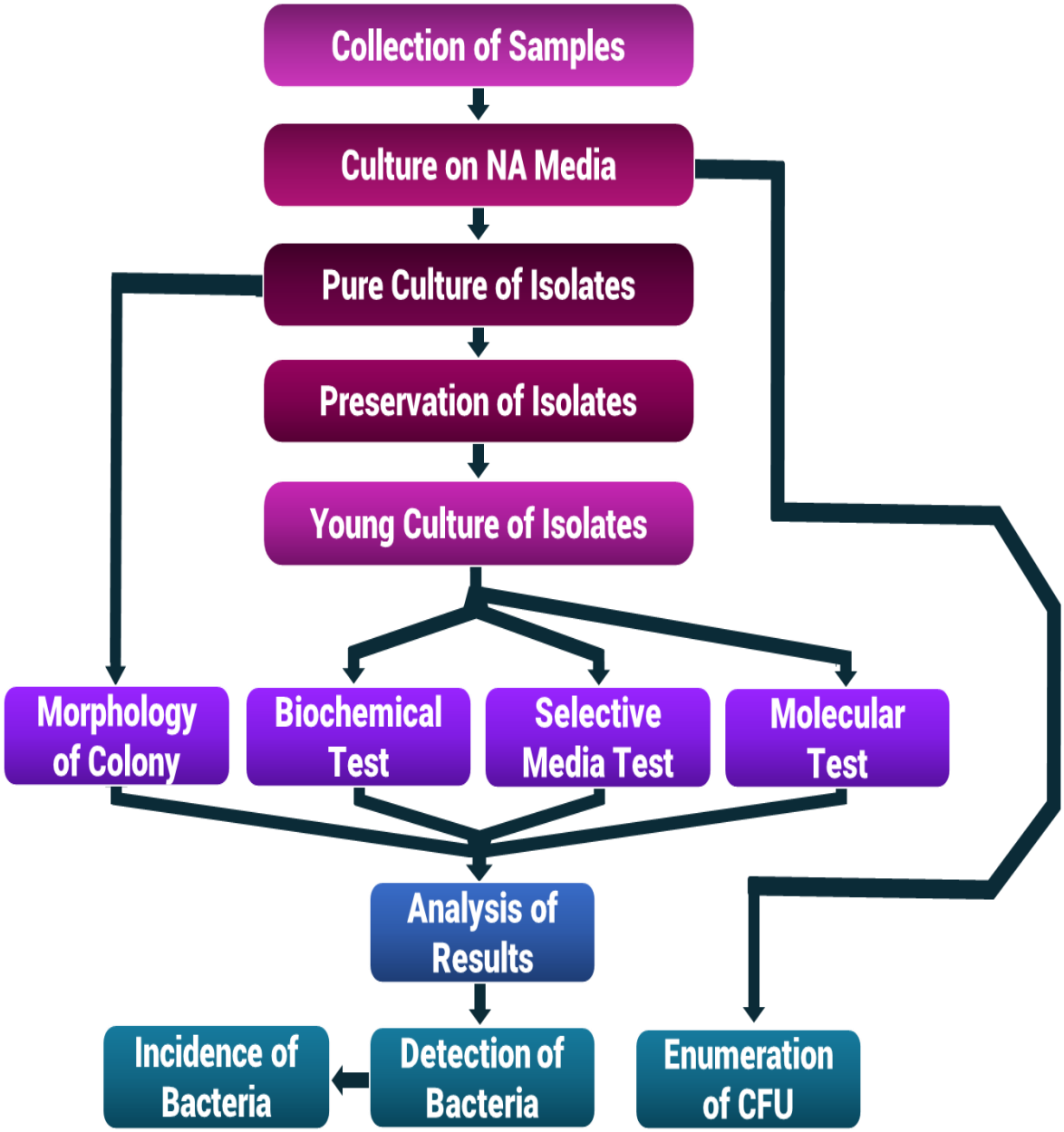


Plate 6. Count of CFU; A. Count the whole part of a plate, B. Count the quadrant part of a plate

3.12. A Flowchart of this study



CHAPTER IV

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1. Isolation and identification of different bacterial isolates from leafy greens

In this study, all the bacteria were isolated on the NA media through the plate and the streak method. After that, different cultural, morphological, and biochemical tests were analyzed and identified 9 genera of bacteria from 30 isolates of 3 selected leafy greens which were *Escherichia, Shigella, Klebsiella, Enterobacter, Bacillus, Pseudomonas, Staphylococcus, Vibrio,* and *Neisseria*. Observing cultural, morphological and biochemical tests, *Staphylococcus aureus was identified.* On the other hand, four selective and semi-selective media were applied and identified *E. coli*, *Klebsiella pneumoniae and Enterobacter aerogenes* in EMB Agar, *Pseudomonas aeruginosa* in Cetrimide agar, *Bacillus cereus in* Bacillus Cereus Agar and *Shigella* spp. in SS Agar. Besides, molecular analysis was done and identified *Bacillus subtilis, Bacillus velezensis* and *Bacillus altitudinis*.

4.1.1. Identified bacteria from coriander leaves

A total of 30 isolates, 13 isolates of bacteria were obtained from coriander leaves out of which 5, 3, and 5 isolates of bacteria were identified from the sample of Super-market, Local-market, and Roadside stand bazaar respectively (Table 3).

4.1.2. Identified bacteria from mint leaves

Regarding mint leaves, 7 isolates of bacteria were obtained out of which 2, 3, and 2 isolates of bacteria were identified from the sample of Super-market, Local-market, and Roadside stand bazaar respectively (Table 3).

4.1.3. Identified bacteria from lettuce leaves

Considering lettuce leaves, 10 isolates of bacteria were obtained out of which 3, 4, and 3 isolates of bacteria were identified from the sample of Super-market, Local-market, and Roadside stand bazaar respectively (Table 3).

Table 3. List of identified Bacteria from collected leafy green samples

Leafy vegetables	Types of marketplaces	Isolate Symbol	Identified Bacteria
Coriander leaves	Supermarket	C-Sp_1	*Escherichia coli*
		C-Sp_2	*Klebsiella pneumoniae*
		C-Sp_3	*Bacillus cereus*
		C-Sp_4	*Pseudomonas aeruginosa*
		C-Sp_5	*Vibrio* sp.
	Local market	C-Lc_1	*Enterobacter aerogenes*
		C-Lc_2	*Bacillus. subtilis*
		C-Lc_3	*Bacillus cereus*
	Roadside stand bazaar	C-Rs_1	*Pseudomonas aeruginosa*
		C-Rs_2	*Bacillus cereus*
		C-Rs_3	*Escherichia coli*
		C-Rs_4	*Neisseria* sp.
		C-Rs_5	*Bacillus altitudinis*
Mint leaves	Supermarkets	M-Sp_1	*Staphylococcus aureus*
		M-Sp_2	*Bacillus cereus*
	Local market	M-Lc_1	*Escherichia coli*
		M-Lc_2	*Bacillus velezensis*
		M-Lc_3	*Bacillus cereus*
	Roadside stand bazaar	M-Rs_1	*Bacillus cereus*
		M-Rs_2	*Pseudomonas aeruginosa*
Lettuce leaves	Supermarkets	L-Sp_1	*Bacillus cereus*
		L-Sp_2	*Neisseria* sp.
		L-Sp_3	*Klebsiella pneumoniae*
	Local market	L-Lc_1	*Enterobacter aerogenes*
		L-Lc_2	*Escherichia coli*
		L-Lc_3	*Neisseria* sp.
		L-Lc_4	*Bacillus cereus*
	Roadside stand bazaar	L-Rs_1	*Escherichia coli*
		L-Rs_2	*Pseudomonas aeruginosa*
		L-Rs_3	*Shigella* sp.

The present study focused on the identification of various human pathogenic bacterial species present in leafy greens. This study revealed the presence of several bacterial species (Table 3) and this identification adds to the growing body of knowledge concerning the microbial diversity associated with leafy greens and their potential implications for food safety and public health.

Remarkably, the results of this study align with previous research conducted by other researchers in this field. The Centers for Disease Control and Prevention (CDC) reported the presence of *E. coli* in leafy greens (CDC, 2022). Additionally, Andrea *et al.* (2022) identified *Vibrio cholerae* in their investigation, while Sowmya *et al.* (2021) detected *Klebsiella pneumoniae, Enterobacter cloacae,* and *Pseudomonas aeruginosa*. Moreover, Balali *et al.* (2020) and Turki et al. (2020) separately reported the occurrence of *E. coli, Bacillus* sp., *Pseudomonas* sp., *Shigella* sp., *Staphylococcus* sp. *Vibrio sp., Klebsiella* sp. *Neisseria* sp. *Enterobacter* sp. etc. in fresh fruits and leafy greens.

In this study, the absence of certain bacteria like *Salmonella* spp., *Listeria monocytogenes,* and *Campylobacter jejuni* was identified by Balali *et al.* (2020) may be due to geographic variability, seasonal variations, limited sample sizes, and the focus of the studies on other bacterial species. Comprehensive research across different regions with larger sample sizes and sensitive detection methods is needed to gain a more comprehensive understanding of bacterial diversity in leafy greens.

The presence of pathogenic bacteria among the identified species raises significant concerns about the potential risks they pose to public health. Yu *et al.* (2019) reported that *Bacillus cereus* is a food-borne opportunistic pathogen that can induce diarrheal and emetic symptoms. Additionally, *E. coli* and *Shigella* spp. have been responsible for numerous human deaths worldwide (Inderbinen and Stephan, 2016; Callejas *et al.*, 2011). Mitov *et al.* (2010) highlighted that *Pseudomonas aeruginosa* is an opportunistic pathogen and one of the main bacteria causing nosocomial infections in hospitals, particularly affecting immunocompromised individuals. Furthermore, Zhang *et al.* (2018) indicated that *Klebsiella pneumoniae* is not only a major hospital-acquired pathogen but also an important food-borne pathogen, capable of causing septicemia, liver abscesses, and diarrhea in humans. In addition to its pathogenicity, *Enterobacter aerogenes* is multi-drug resistant to antibiotics and can be involved in urinary tract, gastrointestinal, and bloodstream infections, posing a potential risk of adult meningitis

(Pradel and Pages, 2002). The study conducted by Kirk *et al.* (2014) sheds light on the significant impact of Enterotoxin-producing *Staphylococcus aureus* as a causative agent of toxin-mediated food poisoning.

4.2. Study on cultural characterization of identified bacteria

On the Nutrient agar plate (Plate 7), the shape, size, margin, pigment, elevation, and texture of the detected bacteria were examined to determine their cultural characteristics. The findings from the study of the bacterial colony characteristics are presented in (Table 4).

4.3. Study on morphological characterization of identified bacteria

In this study, a comprehensive morphological characterization of identified bacterial isolates was performed through Gram staining and subsequent observation under a compound microscope. Gram staining test was performed on all 30 bacterial isolates to determine their cell wall characteristics as well as the shape of bacterial cells. The Potassium hydroxide (KOH) test was also performed which is an alternative way to identify gram-positive or negative bacteria where a KOH-positive result refers to gram-negative bacteria and vice versa. The results showed that 12 isolates (40%) were gram-positive, and 18 isolates (60%) were gram-negative bacteria (Table 5). Among them, *Escherichia* and *Pseudomonas* were small rod-shaped; *Bacillus, Klebsiella, Shigella,* and *Enterobacter* rod-shaped; *Vibrio* coma-shaped; *Neisseria* bean/road-shaped and *Staphylococcus* spherical-shaped (Plate 8).

Table 4. Cultural characterization of identified bacteria on NA plates

Bacteria	Size	Pigment	Shape	Margin	Elevation	Texture
Escherichia	Small, Medium	Greyish white	Round	Even	Convex	Smooth, Translucent, Mucoid
Shigella	Small	Greyish white	Round	Even	Convex	Smooth, Translucent, Mucoid
Bacillus	Medium	White	Round	Jagged	Convex or Flat depressed	Smooth/ rough Opaque, Dry
Klebsiella	Medium-Large	White	Round	Even	Raised	Rough, Opaque, Mucoid
Pseudomonas	Small-Medium	Creamy	Round	Even	Flat	Rough. Opaque, Dry
Staphylococcus	Small	Golden-yellow	Round	Even	Raised	Smooth, Opaque, Dry
Enterobacter	Small	Creamy	Irregular	Even	Raised	Smooth, Translucent, Mucoid
Neisseria	Small	Grey	Round	Even	Convex	Smooth, Opaque, Dry
Vibrio	Small	Grey	Round	Even	Raised	Smooth, Translucent, Mucoid

Table 5. Analysis of Gram staining and KOH solubility test of the identified bacteria

Bacteria	Total isolates no.	KOH solubility test	Gram staining Test
Bacillus	25	-	+
Staphylococcus	2	-	+
Escherichia	18	+	-
Pseudomonas	12	+	-
Neisseria	8	+	-
Klebsiella	7	+	-
Enterobacter	7	+	-
Shigella	3	+	-
Vibrio	2	+	-

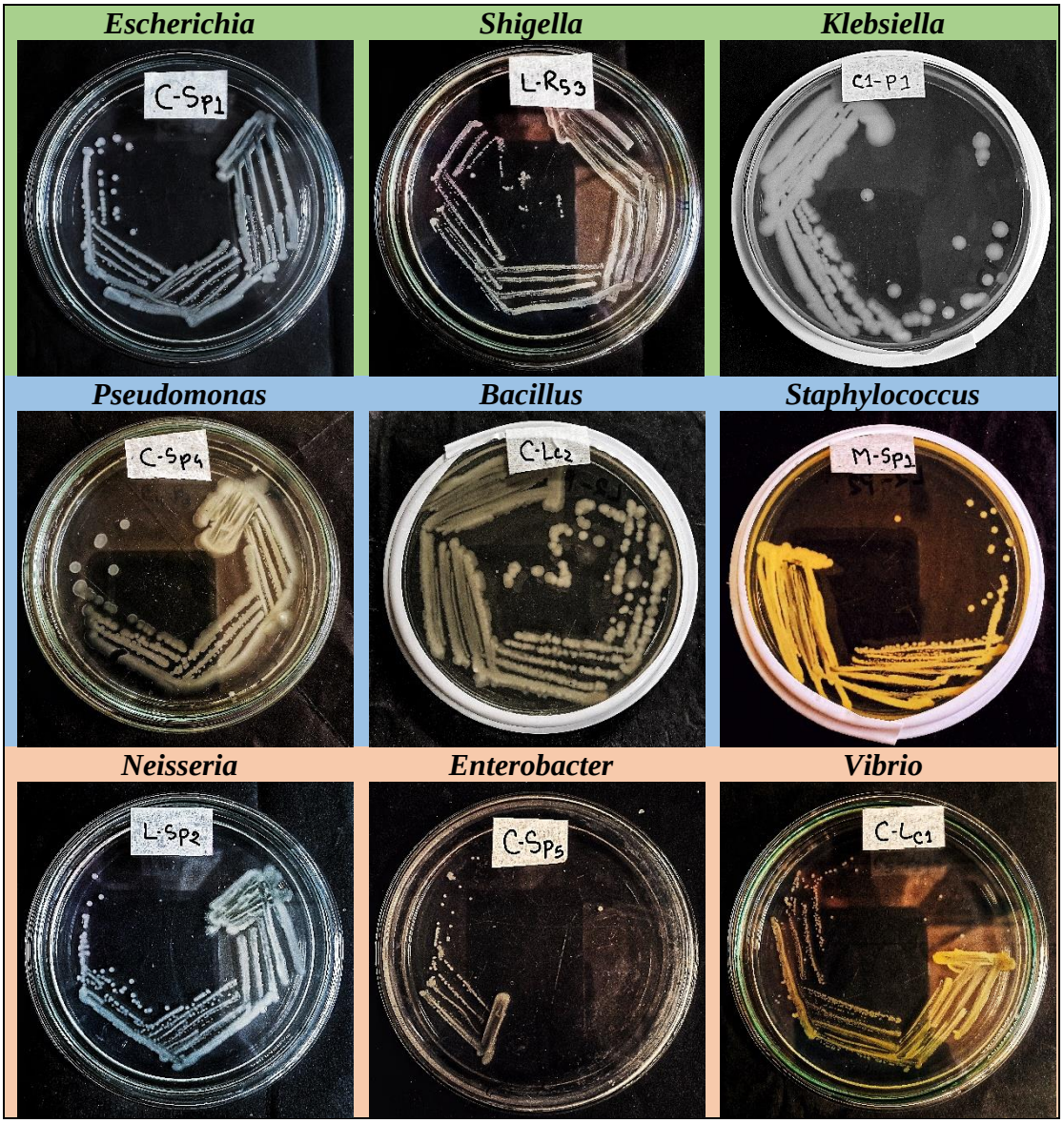


Plate 7. Pure culture plates of the identified bacteria on NA plates from the leafy green samples

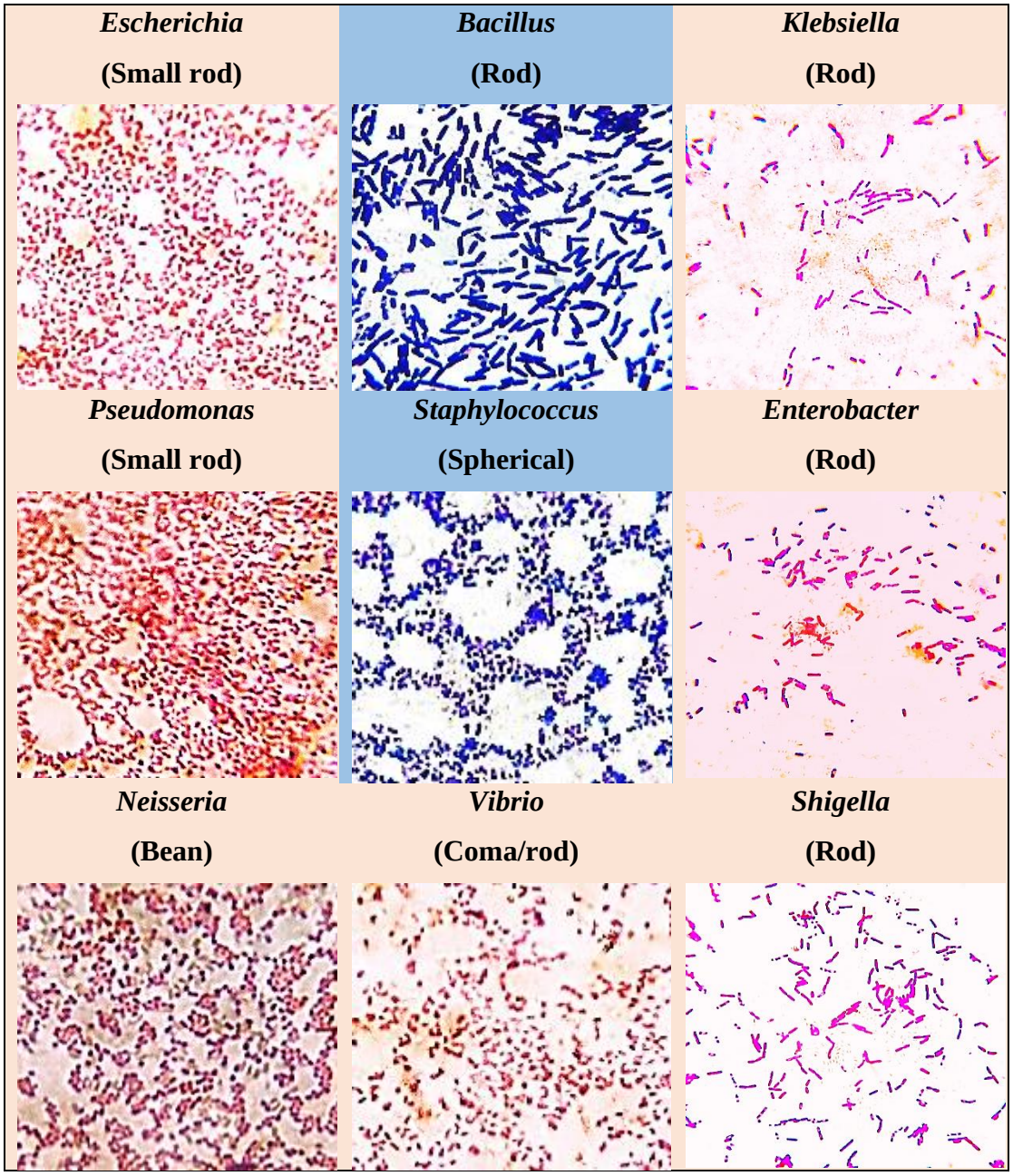


Plate 8. Cell morphology of identified bacteria in gram staining test under compound microscope (100 x)

The results of this study align with the findings of other researchers regarding the colony characteristics (Table 4) of different bacterial species on Nutrient Agar (NA) plates (Plate 7) and the morphological characters observed under the microscope (Plate 8) after the gram staining and KOH solubility test were performed (Table 5).

For *Escherichia coli*, both this study and the research by Basavaraju and Gunashree (2023) report gram-negative, rod cells and similar colony characteristics, including small to medium size, greyish-white color, round shape, convex morphology, and smooth, translucent, and mucoid texture.

In the case of *Bacillus*, these results align with the observations made by Lu *et al*. (2018), indicating gram-positive, rod cells, appeared medium-sized, white colonies with a round shape, jagged edges, and either convex or flat-depressed elevation. Additionally, both studies describe the colonies as having a rough and opaque texture.

Regarding *Klebsiella*, this study shows gram-negative, rod cells, medium to large-sized, white colonies with a round shape and even elevation, which corresponds to the findings of (https://universe84a.com), mentioning mucoid characteristics due to the production of capsular material.

Pseudomonas in this study exhibit gram-negative, rod cells, small to medium size, a creamy color, round shape, even elevation, and rough, opaque, and dry texture colonies, consistent with the results reported by Verma and Verma (2018).

The characteristics of *Staphylococcus* in this research, particularly gram-positive, spherical cells, and the small size, golden-yellow color, round shape, even elevation, and smooth, opaque, and dry texture colonies, align with the description provided by Gnanamani *et al.* (2017), who highlight the production of carotenoids giving the colonies a golden color.

For *Enterobacter*, these findings and the study by Cosmas *et al.* (2016) concur on the gram-positive, rod cells, small size, creamy color, irregular shape, even elevation, and raised morphology with smooth, translucent, and mucoid texture colonies.

Both these results and the information from (https://www.onlinebiologynotes.com) indicate that *Shigella* is a gram-negative, rod bacteria whose colonies on NA plates are small, greyish, round, convex, and smooth or translucent, agreeing on the general appearance of *Shigella* colonies.

The characteristics of *Vibrio* are a gram-negative, rod or coma-shaped bacteria whose colonies in this study, including small size, grey color, round shape, even elevation, smooth, translucent, and mucoid texture, are consistent with the observations made by Sankar *et al.* (2012).

Finally, Hill *et al.* (2016) focused on *Neisseria*, a gram-negative, obligate, fastidious, diplococcus bacterium that is also coherent with this study findings.

The results from this study align with previous research findings, providing further evidence of the consistent cultural and morphological characteristics exhibited by the identified bacterial isolates. These shared observations contribute to a better understanding of the distinctive features of each bacterial species, aiding in their accurate identification and potential applications in various fields such as clinical microbiology and food safety.

4.4. Study on biochemical analysis of the identified bacterial isolates

In this study, the biochemical tests conducted on the different isolates of bacteria obtained from leafy greens revealed significant variations in the biochemical characteristics. The motility test displayed a positive result in 67% of the isolates, indicating the presence of motile bacteria. Moreover, the starch hydrolysis and casein hydrolysis tests exhibited positive outcomes in 58% and 67% of the isolates, respectively, indicating the ability of certain bacteria to break down these substrates. The gelatin liquefaction test showed positive results in 50% of the isolates, suggesting the presence of gelatinase-producing bacteria. Interestingly, all isolates exhibited a positive reaction in the catalase test, indicating the presence of catalase-producing bacteria in every case. On the other hand, only 50% of the isolates displayed a positive result in the oxidase test, signifying the presence of oxidase-producing. The levan production test and citrate utilization test revealed positive results in 33% and 67% of the isolates, respectively, suggesting that certain bacteria could produce levan and utilize citrate as a carbon source. These diverse results highlight the heterogeneity of the bacterial populations present in the samples of leafy greens (Table 6).

Table 6. Analysis of different biochemical test results of selected isolates for the identification of different bacteria

Name of the Bacteria	Motility Test	Starch Hydrolysis Test	Casein Hydrolysis Test	Gelatin Liquefaction Test	Catalase Test	Oxidase Test	Levan Production Test	Citrate Utilization Test
Bacillus cereus	+	+	+	-	+	-	-	+
Bacillus subtilis	+	+	+	+	+	+	+	+
Bacillus velezensis	+	+	+	-	+	+	+	-
Bacillus altitudinis	+	+	-	+	+	+	+	-
Escherichia coli	+	-	-	-	+	-	-	-
Pseudomonas aeruginosa	+	-	+	+	+	+	+	+
Neisseria spp.	-	+	+	+	+	+	-	+
Klebsiella pneumoniae	-	-	+	-	+	-	-	+
Enterobacter aerogenes	+	+	+	-	+	-	-	+
Shigella spp.	-	-	-	-	+	-	-	-
Staphylococcus aureus	-	-	+	+	+	-	-	+
Vibrio spp.	+	+	-	+	+	+	-	+

*+ = Positive result, - = Negative result, +/-= Variable result

Table 6. represents the results of biochemical tests conducted on various bacterial species, including motility, starch hydrolysis, casein test, gelatin liquefaction, catalase, oxidase, levan production, and citrate utilization tests. Comparing these results with those from other researchers, the findings generally align with previous studies for each bacterial species.

Bacillus cereus was a motile bacterium that produced positive results for starch hydrolysis, casein hydrolysis, catalase, and citrate utilization tests and a negative result for oxidase, and gelatin liquefaction test, while a variable result was shown in levan production. Aryal (2022) confirmed its motility, catalase, and citrate utilization as positive results, while the gelatin liquefaction and oxidase were negative. However, the strain tested by Aryal (2022) did not show the results of levan production, casein, and starch hydrolysis test. *B. subtilis* was a motile bacterium that exhibits positive results for each test which aligns with the findings of De Melo *et al.* (2012), Dela Cruz and Torres (2012), and Awais *et al.* (2007), confirming its versatile metabolic capabilities. *B. velezensis* and *B. altitudinis* showed very similar results to *B. subtilis* except both were negative in the citrate utilization test while also negative in gelatin liquefaction and casein test respectively that is consistent with the findings of different studies (Zhou *et al.*, 2022; Wang *et al.*, 2020 and Shivaji *et al.*, 2006). Unlike *B. subtilis*, *Pseudomonas aeruginosa* was a gram-negative bacterium that also showed similar kind of results except for the starch hydrolysis test which was negative, and the findings were confirmed by Aryal (2022) and Sivakumar *et al.* (2019).

Escherichia coli was motile bacteria that showed negative results for each test except the catalase test and supported by two different findings of Al-Baer and Hussein (2017) and Dela Cruz and Torres (2012). Unlike *E. coli*, *Shigella* sp. was non-motile bacteria, but the other test results were the same as *E. coli* that consistent with the results of Aryal (2022).

In the case of *Enterobacter aerogenes* and *Klebsiella pneumoniae*, both were positive in casein, catalase, and citrate utilization tests while negative in gelatin liquefaction, oxidase, and levan production tests but *E. aerogenes* was motile bacteria that was capable of hydrolyzed starch, unlike *K. pneumoniae* that was a non-motile bacterium and negative in starch hydrolysis test (Aryal, 2022).

Neisseria spp. were non-motile bacteria that were positive for starch hydrolysis, casein hydrolysis, catalase, citrate utilization, gelatin liquefaction, and oxidase tests but showed variable results in levan production. Aryal (2022) and Takahashi *et al.* (2002) confirmed the positive results for catalase, oxidase, and citrate utilization and negative for motility tests which were consistent with the findings in Table 6. The variability in levan production was due to differences in strains or specific metabolic capabilities of *Neisseria spp*. *Vibrio* sp. was motile bacteria that exhibited positive results for starch hydrolysis, gelatin liquefaction, catalase, oxidase, and citrate utilization tests. They did not produce levan and could not hydrolyze casein. Aryal (2022) and Jayasree *et al.* (2006) confirmed the positive results for motility, gelatin liquefaction, catalase, oxidase, and citrate utilization tests, supporting the consistency of these tests for *Vibrio* sp. Aryal (2022), and Dela Cruz and Torres (2012) both found similar results for *Staphylococcus aureus* where it was positive for motility, gelatin liquefaction, catalase, oxidase, and citrate utilization tests. Their findings align with the results of Table 6.

The biochemical test results of the bacterial species examined are consistent with the results reported by other researchers in the literature. The agreement in the findings across different studies highlights the reliability and significance of these biochemical tests for bacterial identification and differentiation. However, it is essential to consider variations in strains and experimental conditions when interpreting the results of these tests for specific bacterial species.

4.5. Study on bacterial isolates on different selective and semi-selective media

By observing the appearance of bacterial colonies on four different selective and semi-selective (differentiated) media and identified *E. coli* (Blue colony without metallic green sheen), *Klebsiella pneumoniae* (Pink mucoid colony), *Enterobacter aerogenes* (Pink dull colony) on EMB Agar; *Pseudomonas aeruginosa* (Cream colony) on Cetrimide Agar; *Bacillus cereus* (Blue colony) on Bacillus cereus agar and *Shigella* sp. (Transparent colony) on SS Agar (Plate 9).

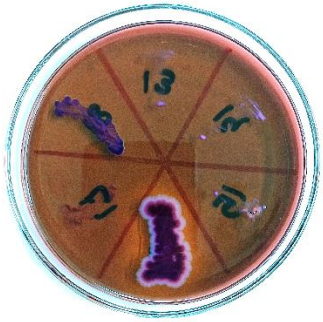
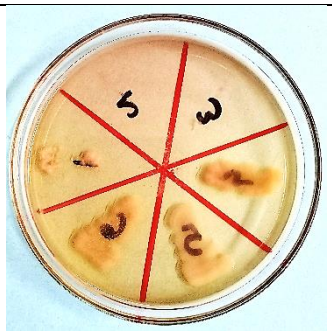
	EMB Agar plate	
E. coli	*K. pneumoniae*	*E. aerogenes*
		
Cetrimide Agar plate	**SS Agar plate**	**Bacillus cereus Agar plate**
P. aeruginosa	*Shigella* sp.	*B. cereus*
		

Plate 9. Cultural characteristics of different bacteria on four selective and semi-selective agar plates

The results (Plate 9) of this study revealed distinct colony appearances on these four different selective and semi-selective media. On Eosin Methylene Blue (EMB) agar, 3 different bacterial species were identified. *E. coli* produced a blue colony without a metallic green sheen, *Klebsiella pneumoniae* showed a pink, mucoid colony, and *Enterobacter aerogenes* exhibited a pink dull colony. These color variations are indicative of lactose fermentation, with each bacterium showing a unique color response due to the differential properties of the EMB agar. Aryal (2022) corroborated these findings, explaining that EMB agar is a differential medium that can inhibit the growth of Gram-positive bacteria while aiding in distinguishing lactose fermenters based on colony colors. The results from this study align with Aryal's description of the EMB agar's capabilities.

On Cetrimide Agar, *Pseudomonas aeruginosa* displayed a cream-colored colony. This selective medium is known for its ability to isolate and identify *Pseudomonas* species, particularly *Pseudomonas aeruginosa*, due to its characteristic growth and pigment production. The observed cream colony appearance is consistent with previous research on the distinctive colonial appearance of *Pseudomonas aeruginosa* on Cetrimide Agar (Brown and Lowbury, 1965).

Bacillus cereus agar was utilized to identify *Bacillus cereus*, and the results showed a blue colony for this bacterium. Holbrook and Anderson (1980) supported these findings, reporting that Bacillus cereus agar is a selective medium specifically designed for the identification of *Bacillus cereus*. The peacock blue color produced by *Bacillus cereus* on this agar medium is considered a distinguishing feature for the species.

Lastly, on SS Agar, *Shigella* sp. formed transparent colonies. SS Agar is a semi-selective medium that inhibits the growth of most Gram-positive bacteria while allowing the growth of Gram-negative enteric pathogens like *Shigella* and *Salmonella.* The transparent colony appearance aligns with the reported colorless characteristics of *Shigella* spp. on SS Agar as described by Aryal (2022).

4.6. Study on molecular analysis of bacteria

4.6.1. Gel electrophoresis of PCR products

The Gel-Doc system of PCR products using primers 27F and 1492R resulted in the amplification of a DNA band with a size of approximately 1465 base pairs (bp). The obtained DNA band was very close in size to the target band, indicating successful PCR amplification. A Bench Top 1kb DNA ladder was used as a size marker, confirming the size of the amplified DNA fragment. Lanes 1, 2, and 3, (Figure 1) represent the bacterial strains of *B. subtilis*, *B. velezensis*, and *B. altitudinis* respectively which exhibited the amplified DNA band. The results suggest that all 3 bacteria isolates contain the targeted DNA region, as indicated by the presence of the amplified band at the expected size. The Gel-Doc system confirms the specificity and successful amplification of the desired DNA fragment in the bacterial samples.

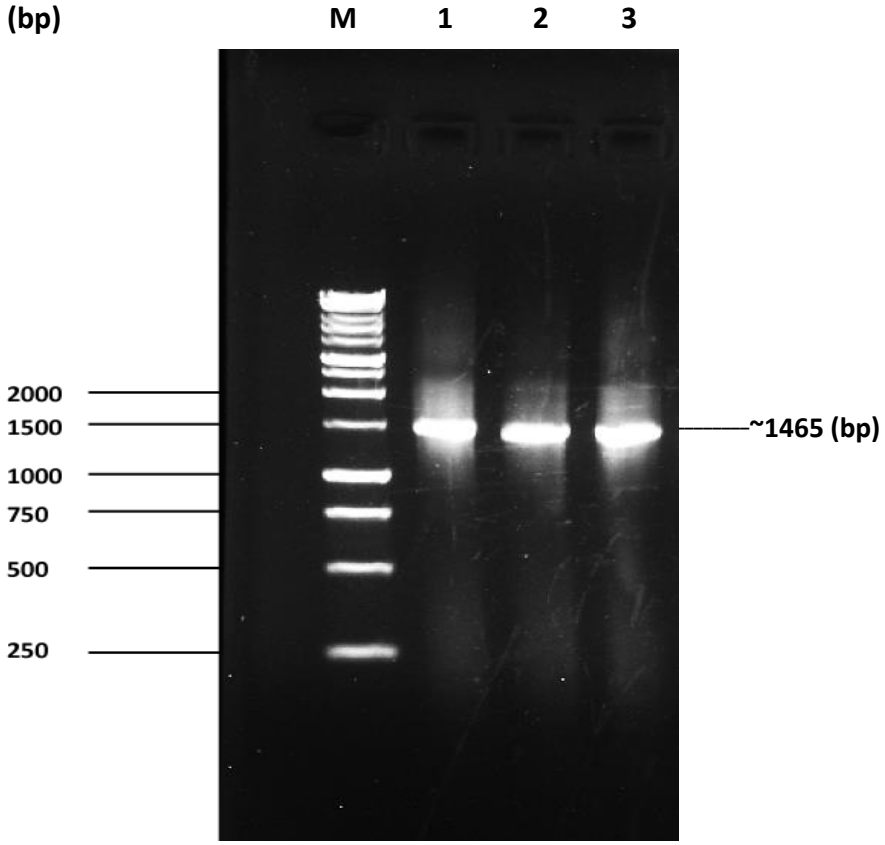


Figure 1. PCR amplified product from *B. subtilis* (1), *B. velezensis* (2) and *B. altitudinis* isolates of bacteria (3), M: denotes 1kb DNA ladder (Marker)

4.6.2. Analysis of DNA sequences

The sequences were analyzed by using the BLAST on the NCBI website (https:// www.ncbi.nlm.nih.gov) for matching with existing nucleotide sequences in the NCBI GenBank database. The partial sequences of 16S rRNA of isolated bacteria were submitted to NCBI for deposition in the GenBank and obtained accession number.

```
1     GCAGTCGAGC GAATGGATTA AGAGCTTGCT CTTATGAAGT TAGCGGCGGA CGGGTGAGTA
61    ACACGTGGGT AACCTGCCCA TAAGACTGGG ATAACTCCGG GAAACCGGGG CTAATACCGG
121   ATAACATTTT GAACCGCATG GTTCGAAATT GAAAGGCGGC TTCGGCTGTC ACTTATGGAT
181   GGACCCGCGT CGCATTAGCT AGTTGGTGAG GTAACGGCTC ACCAAGGCAA CGATGCGTAG
241   CCGACCTGAG AGGGTGATCG GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA
301   GGCAGCAGTA GGGAATCTTC CGCAATGGAC GAAAGTCTGA CGGAGCAACG CCGCGTGAGT
361   GATGAAGGCT TTCGGGTCGT AAAACTCTGT TGTTAGGGAA GAACAAGTGC TAGTTGAATA
421   AGCTGGCACC TTGACGGTAC CTAACCAGAA AGCCACGGCT AACTACGTGC CAGCAGCCGC
481   GGTAATACGT AGGTGGCAAG CGTTATCCGG AATTATTGGG CGTAAAGCGC GCGCAGGTGG
541   TTTCTTAAGT CTGATGTGAA AGCCCACGGC TCAACCGTGG AGGGTCATTG GAAACTGGGA
601   GACTTGAGTG CAGAAGAGGA AAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGAGATA
661   TGGAGGAACA CCAGTGGCGA AGGCGACTTT CTGGTCTGTA ACTGACACTG AGGCGCGAAA
721   GCGTGGGGAG CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA
781   AGTGTTAGAG GGTTTCCGCC CTTTAGTGCT GAAGTTAACG CATTAAGCAC TCCGCCTGGG
841   GAGTACGGCC GCAAGGCTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG
901   CATGTGGTTT AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCTCTGACAA
961   CCCTAGAGAT AGGGCTTCTC CTTCGGGAGC AGAGTGACAG GTGGTGCATG GTTGTCGTCA
1021  GCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCTTG ATCTTAGTTG
1081  CCATCATTTA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG AAGGTGGGGA
1141  TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTA
```

Figure 2. Nucleotide sequence of 16S rDNA from *Bacillus subtilis* strain SAUBD-B1 (Accession no. OQ740285.1)

```
1     GTCGAGCGGT ACAGATAGGG AGCTTCGCTC CCTGATGTTA GCGGCGGACG GGTGAGTAAC
61    ACGTGGGTAA CCTGCCTGTA AGACTGGGAT AACTCCGGGA AACCGGGGCT AATACCGGAT
121   GGTTGTTTGA ACCGCATGGT TCAAACATAA AAGGTGGCTT CGGCTACCAC TTACAGATGG
181   ACCCGCGGCG CATTAGCTAG TTGGTGAGGT AACGGCTCAC CAAGGCAACG ATGCGTAGCC
241   GACCTGAGAG GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG
301   CAGCAGTAGG GAATCTTCCG CAATGGACGA AAGTCTGACG GAGCAACGCC GCGTGAGTGA
361   TGAAGGTTTT CGGATCGTAA AGCTCTGTTG TTAGGGAAGA ACAAGTACCG TTCGAATAGG
421   GCGGTACCTT GACGGTACCT AACCAGAAAG CCACGGCTAA CTACGTGCCA GCAGCCGCGG
481   TAATACGTAG GTGGCAAGCG TTGTCCGGAA TTATTGGGCG TAAAGGGCTC GCAGGCGGTT
541   TCTTAAGTCT GATGTGAAAG CCCCCGGCTC AACCGGGGAG GGTCATTGGA AACTGGGGAA
601   CTTGAGTGCA GAAGAGGAGA GTGGAATTCC ACGTGTAGCG GTGAAATGCG TAGAGATGTG
661   GAGGAACACC AGTGGCGAAG GCGACTCTCT GGTCTGTAAC TGACGCTGAG GAGCGAAAGC
721   GTGGGGAGCG AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT GAGTGCTAAG
781   TGTTAGGGGG TTTCCGCCCC TTAGTGCTGC AGCTAACGCA TTAAGCACTC CGCCTGGGGA
841   GTACGGTCGC AAGACTGAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA
901   TGTGGTTTAA TTCGAAGCAA CGCGAAGAAC CTTACCAGGT CTTGACATCC TCTGACAATC
961   CTAGAGATAG GACGTCCCCT TCGGGGGCAG AGTGACAGGT GGTGCATGGT TGTCGTCAGC
1021  TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC AACCCTTGAT CTTAGTTGCC
1081  AGCATTCAGT TGGGCACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA GGTGGGGATG
1141  ACGTCAAATC ATCATGCCCC TTATGACCTG GGCTACC
```

Figure 3. Nucleotide sequence of 16S rDNA from *Bacillus velezensis* strain SAUBD-B2 (Accession no. OQ740286.1)

```
1     GTCGAGCGGA CAGAAGGGAG CTTGCTCCCG GATGTTAGCG GCGGACGGGT GAGTAACACG
61    TGGGTAACCT GCCTGTAAGA CTGGGATAAC TCCGGGAAAC CGGAGCTAAT ACCGGATAGT
121   TCCTTGAACC GCATGGTTCA AGGATGAAAG ACGGTTTCGG CTGTCACTTA CAGATGGACC
181   CGCGGCGCAT TAGCTAGTTG GTGAGGTAAC GGCTCACCAA GGCAACGATG CGTAGCCGAC
241   CTGAGAGGGT GATCGGCCAC ACTGGGACTG AGACACGGCC CAGACTCCTA CGGGAGGCAG
301   CAGTAGGGAA TCTTCCGCAA TGGACGAAAG TCTGACGGAG CAACGCCGCG TGAGTGATGA
361   AGGTTTTCGG ATCGTAAAGC TCTGTTGTTA GGGAAGAACA AGTGCAAGAG TAACTGCTTG
421   CACCTTGACG GTACCTAACC AGAAAGCCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
481   ACGTAGGTGG CAAGCGTTGT CCGGAATTAT TGGGCGTAAA GGGCTCGCAG GCGGTTTCTT
541   AAGTCTGATG TGAAAGCCCC CGGCTCAACC GGGGAGGGTC ATTGGAAACT GGGAAACTTG
601   AGTGCAGAAG AGGAGAGTGG AATTCCACGT GTAGCGGTGA AATGCGTAGA GATGTGGAGG
661   AACACCAGTG GCGAAGGCGA CTCTCTGGTC TGTAACTGAC GCTGAGGAGC GAAAGCGTGG
721   GGAGCGAACA GGATTAGATA CCCTGGTAGT CCACGCCGTA AACGATGAGT GCTAAGTGTT
781   AGGGGGTTTC CGCCCCTTAG TGCTGCAGCT AACGCATTAA GCACTCCGCC TGGGGAGTAC
841   GGTCGCAAGA CTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
901   GTTTAATTCG AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCTCTG ACAACCCTAG
961   AGATAGGGCT TTCCCTTCGG GGACAGAGTG ACAGGTGGTG CATGGTTGTC GTCAGCTCGT
1021  GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTTGATCTTA GTTGCCAGCA
1081  TTCAGTTGGG CACTCTAAGG TGACTGCCGG TGACAAACCG GAGGAAGGTG GGGATGACGT
1141  CAAATCATCA TGCCCCTTAT GACCTGGGCT ACCACGTGCT ACATGGGAGG AA
```

Figure 4. Nucleotide sequence of 16S rDNA from *Bacillus altitudinis* strain SAUBD-B3 (Accession no. OQ740287.1)

The results depicted in **Figure 1.** demonstrate a consistent detection of an approximately 1465± bp DNA band in the gel doc system, which aligns with Nurfitri *et al.* (2019) findings. This similarity is observed when using 27F and 1492R primers along with the 1 kb DNA ladder as a marker. The presence of this band indicates that the used primers and PCR conditions were amplified well in accordance with the primary target of the 16S rRNA gene. It has been observed in (Figure 2-4) that isolated *Bacillus subtilis* strain SAUBD-B1 (Accession no. OQ740285.1) showed 100% homology to the *Bacillus subtilis* strain Cu31 16s ribosomal RNA gene with accession no. KY085997.1, *Bacillus velezensis* strain SAUBD-B2 (Accession no. OQ740286.1) showed 99.75% homology to the *Bacillus velezensis* strain ND06 16s ribosomal RNA gene with accession no. ON386277.1 and *Bacillus altitudinis* strain SAUBD-B3 (Accession no. OQ740287.1) showed 99.83% homology to the *Bacillus altitudinis* strain LY43 16s ribosomal RNA gene with accession no. MZ067888.1.

4.6.3. Analysis of phylogenetic tree

The phylogenetic analysis was performed using genetic sequences of *B. subtilis, B. velezensis,* and *B. altitudinis* strains. The sequences were aligned, and a maximum likelihood phylogenetic tree was constructed using MEGA 11 software. The tree was rooted using an outgroup sequence and bootstraps values were calculated to assess the statistical support for the branching patterns (Figure 5).

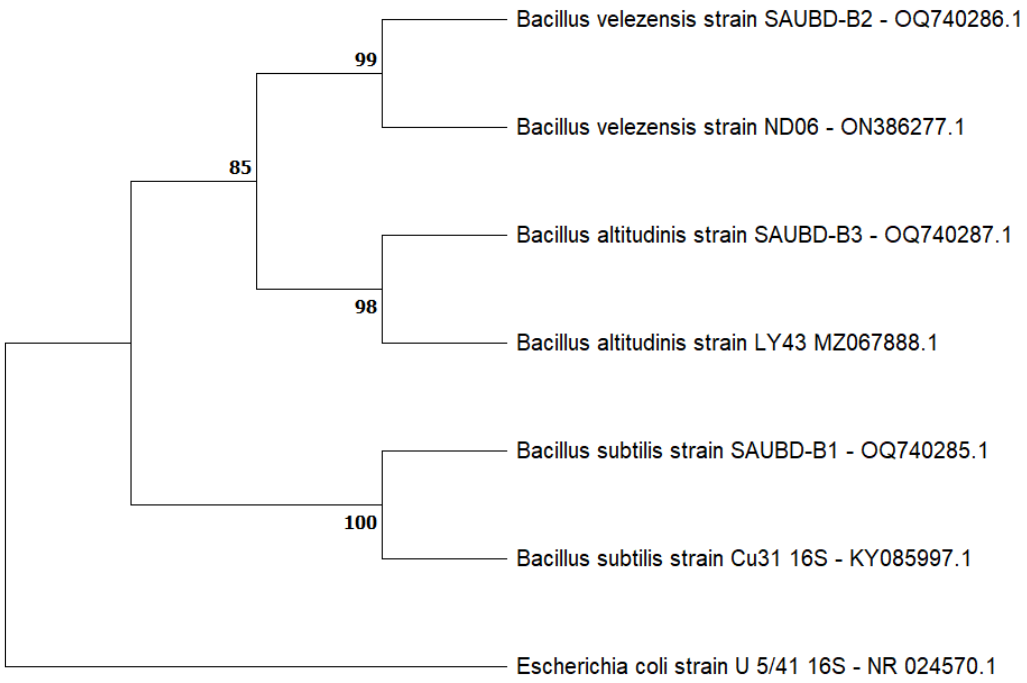


Figure 5. Analysis of phylogenetic tree of *B. subtilis, B. velezensis,* and *B. altitudinis* strains based on 16S rRNA sequences. The numbers at the branches are bootstrap confidence percentages from 1000 bootstrap trees. The *E. coli* strain U 5/41 NR_0245701 was used as the outgroup.

The resulting phylogenetic tree (Figure 5) revealed the relationships among the studied strains. Notably, *B. subtilis* strain SAUBD-B1 and *B. subtilis* strain Cu31 were found to form a single clade with strong bootstrap support. Similarly, *B. velezensis* strain SAUBD-B2 and *B.* velezensis strain ND06; *B. altitudinis* strain SAUBD-B3 and *B. altitudinis* strain LY43 were also grouped together in a well-supported clade. The identification of shared clades indicates a close evolutionary relationship between certain strains, supporting the notion that they belong to the same species.

4.7. Assessment of Bacterial load

The present study investigates the microbial contamination levels, specifically the colony-forming units per gram (CFU/g), in three selected leafy greens - coriander, lettuce, and mint. A total of 27 samples were collected, with each leafy green contributing 9 samples. These samples were obtained from 9 different locations, each representing a distinct market type - Supermarket, local markets, and roadside stand bazaars. All the collected samples of leafy greens were diluted up to $1:10^{-5}$ dilutions before plating on the NA plates and observed the total bacterial colony of each collected sample and the CFU was counted (Plate 10).

4.7.1. Bacterial load in coriander, mint, and lettuce samples

Among the collected samples, the highest mean of CFU was observed in the mint leaves followed by lettuce leaves whereas the lowest was in the coriander leaves (Table 7).

Table 7. The number of Colony Forming Units for different leafy greens

Name of the leafy greens	Market types	CFU/gm (Dilution 10^{-5})				Mean per leafy greens
		Market-1	Market-2	Market-3	Mean	
Coriander	Sp	1.3×10^9	1.3×10^9	1.6×10^9	1.4×10^9	**2.43×10^9**
	Lc	2.1×10^9	1.7×10^9	1.6×10^9	1.8×10^9	
	Rs	3.5×10^9	3.9×10^9	4.9×10^9	4.1×10^9	
Mint	Sp	2.2×10^9	1.7×10^9	1.8×10^9	1.9×10^9	**4.27×10^9**
	Lc	3.4×10^9	3.7×10^9	4.0×10^9	3.7×10^9	
	Rs	7.5×10^9	6.9×10^9	7.2×10^9	7.2×10^9	
Lettuce	Sp	2.7×10^9	2.1×10^9	2.1×10^9	2.3×10^9	**2.87×10^9**
	Lc	2.8×10^9	1.9×10^9	3.1×10^9	2.6×10^9	
	Rs	3.7×10^9	3.3×10^9	4.1×10^9	3.7×10^9	

*Sp= Supermarket, Lc= Local-market, Rs= Roadside stand bazaar

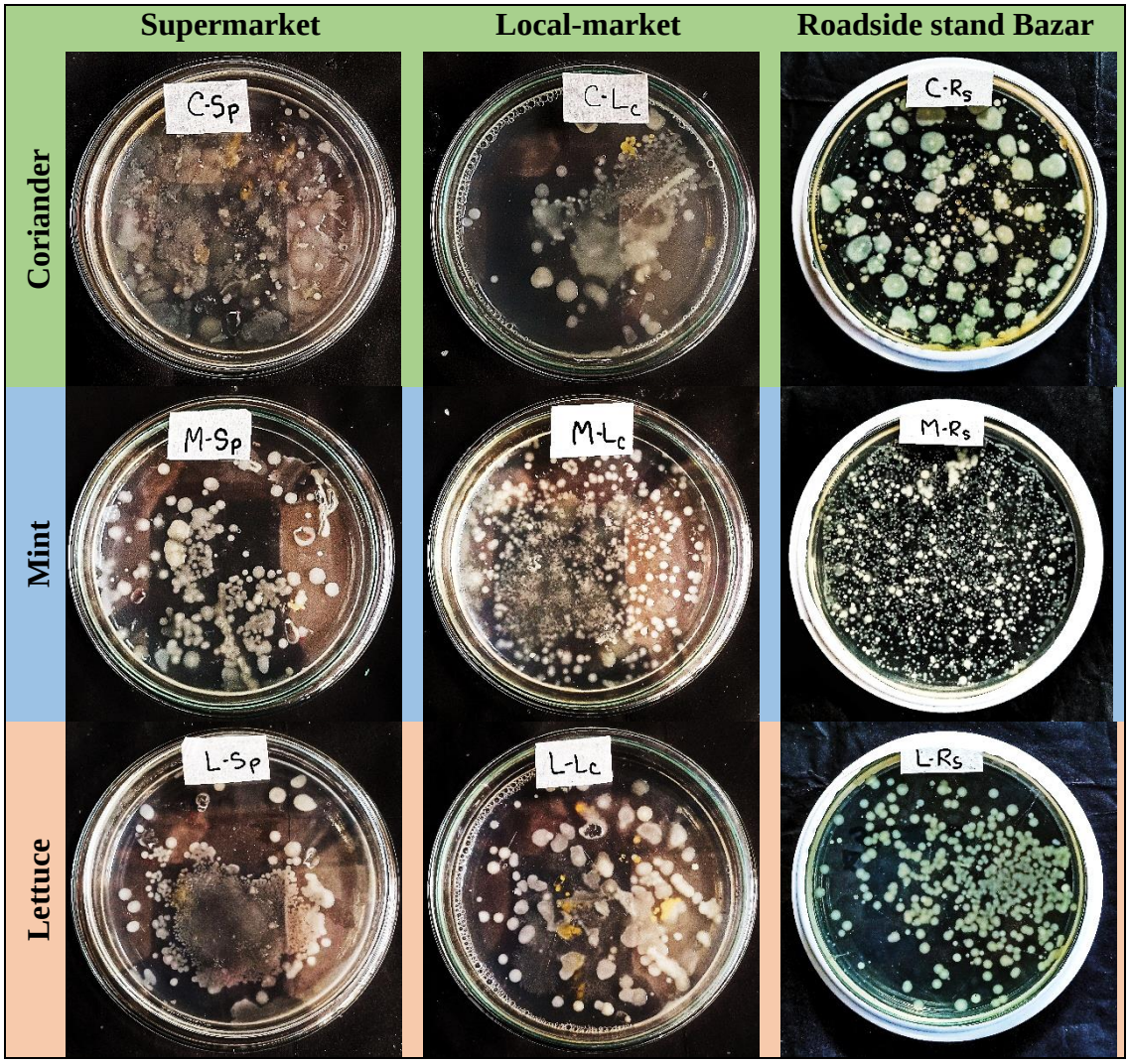


Plate 10. Growth of Bacterial colonies on NA plates of each sample at 1: 10^5 dilutions

4.7.2. Comparative analysis of Bacterial load for different marketplaces

Among the three different types of marketplaces, the maximum mean of CFU was obtained from the leafy greens in the roadside stand bazaar samples followed by the local market samples in contrast the lowest was found in the supermarket samples (Figure 6).

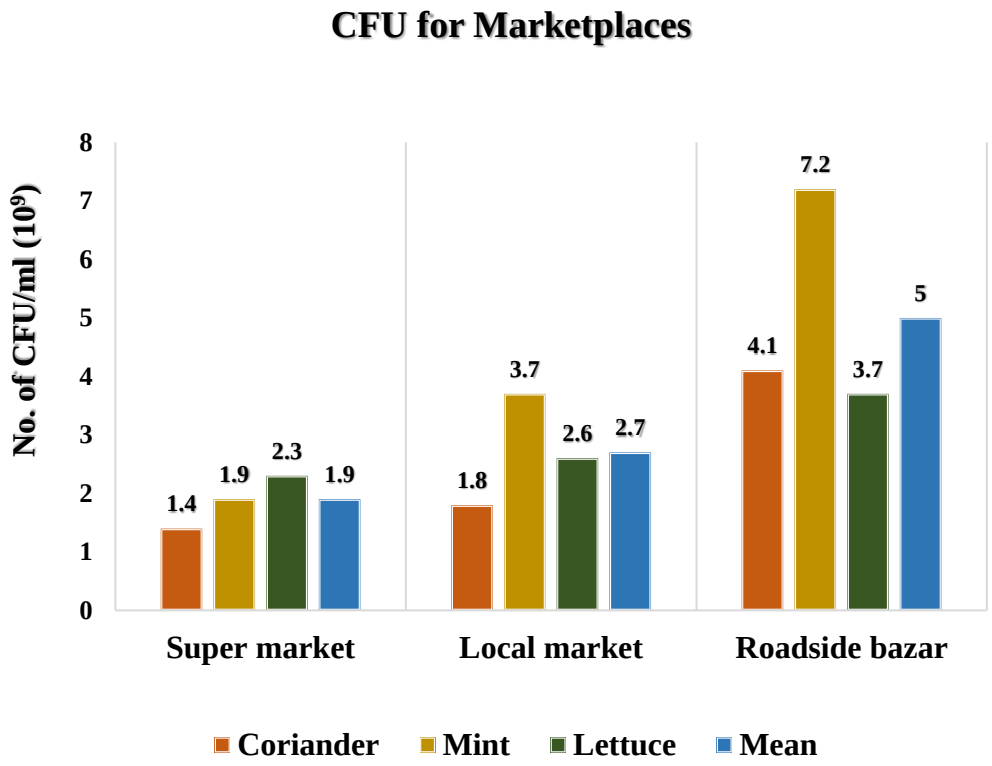


Figure 6. Bacterial load of each leafy green collected from different marketplaces

The findings of this study (Table 7; Figure 6) revealed varying bacterial prevalence in the selected leafy greens collected from diverse marketplaces in Dhaka city. Mint leaves had the highest bacterial prevalence (4.27×10^9 CFU/g), followed by lettuce leaves (2.87×10^9 CFU/g), and coriander leaves had the lowest (2.43×10^9 CFU/g). Moreover, considering different marketplaces, roadside stand bazaars exhibited the highest bacterial prevalence (5×10^9 CFU/g), followed by local markets (2.7×10^9 CFU/g), and supermarkets had the lowest (1.9×10^9 CFU/g).

These results indicate an overall very high prevalence of bacteria in all leafy greens from diverse marketplaces, highlighting the unhygienic conditions in Dhaka city's markets where it crosses the acceptable level of bacterial load according to the guidelines provided by Santos et al. (2005). In these guidelines, it was investigated that the bacterial load in ready-to-eat leafy green salads set a "Satisfactory" level of total bacteria contamination at less than or equal to 10^4 CFU/g, an "Acceptable" range from greater than 10^4 to less than or equal to 10^6 CFU/g, and a "Not Acceptable" level exceeding 10^6 CFU/g. These guidelines can serve as valuable references for ensuring food safety in the production and distribution of leafy green salads.

Comparing with other research findings, Yafetto *et al.* (2019) reported a bacterial load of 1.23×10^8 CFU/ml for lettuce leaves in Cape Coast, Ghana. Hougbenou *et al.* (2019) studied selected leafy greens in Porto-Novo, Republic of Benin, and found the highest total coliform counts in lettuces (3.21×10^3 CFU/g) and the lowest in nightshades (1.78×10^2 CFU/g). Nipa *et al.* (2011) observed viable bacterial colonies in coriander leaves ranging from 5.87×10^5 to 1.8×10^6 CFU/g and in peppermint leaves ranging from 2.2×10^5 to 7.7×10^5 CFU/g in Chittagong, Bangladesh.

The prevalence of bacteria in leafy greens from diverse marketplaces in Dhaka city, as well as findings from other studies, highlight the need for continuous monitoring and implementation of stringent food safety measures. By adhering to established guidelines and best practices, it is possible to mitigate bacterial load in leafy greens, ensuring the provision of safe and hygienic food products to consumers.

4.8. Assessment of bacterial incidence

Among the 27 tested samples, 9 genera of bacteria were identified where *Bacillus*, *Escherichia*, *Pseudomonas*, *Neisseria*, *Klebsiella*, *Enterobacter*, *Shigella*, *Staphylococcus*, and *Vibrio* were present in 25, 18, 12, 8, 7, 7, 3, 2, and 2 no. of tested samples respectively. The incidence of these genera of bacteria was shown in Figure 7.

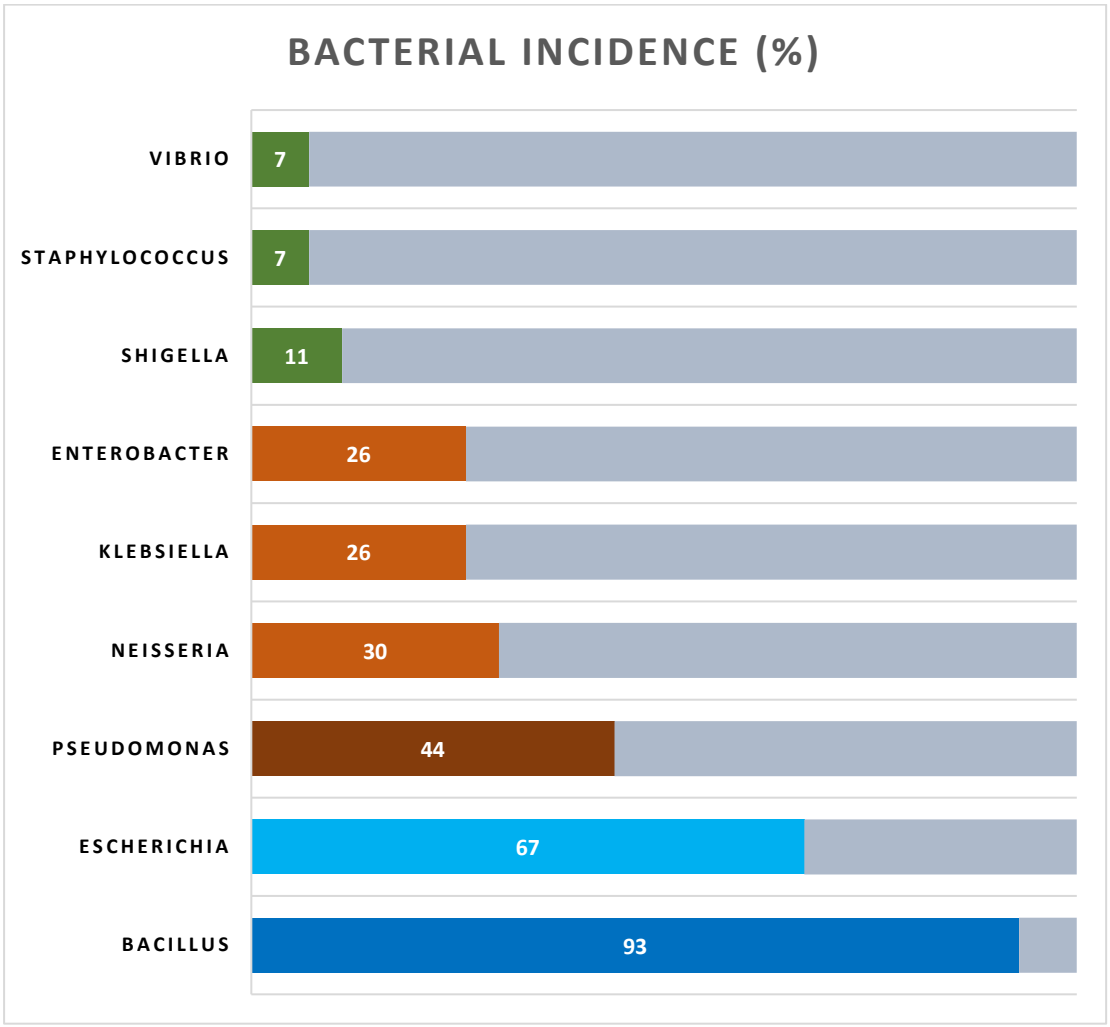


Figure 7. Bar chart showing the different types of bacterial incidence in leafy vegetables

Comparing these results (Figure 7) with the findings of Srisamran *et al.* (2022), their study reported a higher prevalence of *E. coli* in coriander and lettuce reaching 100% in open-market samples besides 83.3% and 91.75% in supermarket samples in coriander and lettuce respectively. Additionally, *Salmonella* was present (12.5% in open market samples, absent in supermarket samples) in different market samples, and *Shigella* was absent in all samples. These variations in bacterial incidence highlight the importance of considering the source and handling practices of leafy greens in different marketplaces.

Turki *et al.* (2020) also investigated the prevalence of pathogenic bacteria in leafy greens. Their study reported a 100% incidence of *Staphylococcus, Bacillus, Enterobacter, Shigella,* and *Escherichia coli*. *Klebsiella* and *Salmonella* also showed a high incidence at 92%, and *Pseudomonas* were found in 80% and 72% of the samples, respectively. *Neisseria* had a lower incidence of 12%.

These collective findings emphasize the variability in bacterial incidences in leafy greens across different studies and regions. Further research is essential to investigate the contributing factors behind these variations and to identify effective strategies to mitigate bacterial contamination in leafy greens. Implementing strict food safety measures and raising awareness among producers, vendors, and consumers about proper hygiene practices can play a crucial role in reducing the risk of bacterial contamination in leafy greens and ensuring food safety.

CHAPTER V

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

The present study was performed to isolate and detect the bacteria and after that to identify the human pathogenic bacteria from all the detected bacteria associated with fresh leafy greens viz. coriander, lettuce, and mint leaves. In addition, the estimation of bacterial load and bacterial incidence was also performed in this study. The study was conducted in the MS laboratory in the Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University during the period from July 2021 to June 2022. A total of 27 fresh leafy green samples were collected from 9 different markets or locations (3 supermarkets, 3 local markets, and 3 roadside stand bazaars) in Dhaka city. From these 27 samples, a total of 30 isolates of bacteria were obtained.

Initially, the cultural characteristics of bacterial colonies on NA media, the morphological characteristics of the bacterial cell under a compound microscope after the gram-staining test, and other biochemical tests were studied and detected a total of nine different bacterial genera from these 30 isolates which were *Bacillus, Escherichia, Pseudomonas, Neisseria, Klebsiella, Enterobacter, Shigella, Staphylococcus* and *Vibrio*. By performing the culture of bacteria on selective and semi-selective media and molecular analysis besides considering biochemical test results, a total of twelve bacteria have been identified from these nine genera namely, *B. subtilis, B. velezensis, B. altitudinis, B. cereus, E. coli, P. aeruginosa, Neisseria* spp. *K. pneumoniae, E. aerogenes, S. aureus, Vibrio* spp., and *Shigella* spp.

Bacterial species from the genera of *Shigella, Vibrio,* and *Neisseria* remained unidentified due to the lack of selective media for these 3 genera as well as molecular analysis. After reviewing many pieces of literature, this study found that seven of these twelve identified bacteria were responsible for many diseases of human beings, so these bacteria are well considered human pathogenic bacteria and these were *B. cereus, E. coli, P. aeruginosa, K. pneumoniae, E. aerogenes, S. aureus,* and *Shigella* spp.

This study also estimated the bacterial load on coriander, mint, and lettuce leaves and on different marketplace samples as well. The highest CFU was found in mint (4.27×10^9 /g) followed by lettuce (2.87×10^9 /g) and the lowest in coriander (2.43×10^9 /g). Considering marketplaces, the highest bacterial CFU was observed in the leafy green samples of roadside stand bazaars (5×10^9 /g) followed by local markets (2.7×10^9 /g), and the lowest in supermarkets (1.9×10^9 /g).

The highest prevalence of bacterial incidence was shown by *Bacillus* (93%) followed by *E. coli*, (67%), *Pseudomonas* (44%), *Neisseria* (30%), *Klebsiella* (26%), *Enterobacter* (26%), and the lowest by *Shigella* (11%), *Vibrio* (7%), and *Staphylococcus* (7%).

The study demonstrated the alarming presence of seven pathogenic bacteria in these three leafy green samples where these bacteria were highly prevalent and alive. While people consumed these leafy greens in a raw condition (without cooking) or without following proper sanitation processes they get infected by these identified bacteria which may cause various severe food-borne illnesses in the human body such as hemolytic uremic syndromes, kidney failure, respiratory infections, urinary tract infections, meningitis, pneumonia, gonorrhea vomiting, diarrhea, etc., and even cause death.

The results also showed the present hygiene condition of fresh leafy greens selling and purchasing areas in Dhaka city. This study gave a broad overview of the microbiological quality of fresh leafy greens in Dhaka city, which would make it easier to take the necessary precautions to ensure food safety when selling and purchasing leafy greens.

Due to some limitations and the small number of samples, more pathogenic bacteria may not be identified. Additionally, further research is needed to understand the specific conditions that lead to bacterial contamination of leafy greens and to develop effective interventions to prevent it.

CHAPTER VI

REFERENCES

REFERENCES

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APPENDICES

Preparation of Culture Media

The compositions of the media which were used in this thesis work are given below: Unless otherwise mentioned all media were autoclaved at 121 ºC for 20 minutes at 15 lb. pressure.

NA Medium for Bacteria Culture

Dehydrated Medium	14 g
Distilled Water	500 ml

Specific Medium for *Bacillus* sp. (Bacillus Cereus Agar Base)

Dehydrated Medium	20.5 g
Distilled Water	475 ml
Polymyxin B Selective Supplement	1 vial
Sterile Egg Yolk	25 ml

Specific Medium for *Pseudomonas* sp. (Cetrimide Agar Base)

Dehydrated Medium	23.4 g
Distilled Water	500 ml
Glycerol	5 ml

Specific Medium for *E. coli* (Eosin Methylene Blue Agar Base)

Dehydrated Medium	18 g
Distilled Water	500 ml

Specific Medium for *Salmonella* sp. and *Shigella* sp. (SS Agar Base)

Dehydrated Medium	30 g
Distilled Water	500 ml

Gram Staining Reagents

Gram's Crystal Violet	**100 ml**
➢ Crystal Violet (88% dye content)	0.5 g
➢ Ethanol	20 ml
➢ Distilled Water	80 ml
Gram's Iodine (Lugol's Solutions)	**100 ml**
➢ Iodine	1 g
➢ Potassium Iodide (KI)	2 gm
➢ Distilled water	97 ml
Gram's Decolorizer	**100 ml**
➢ Ethanol	95 ml
➢ Distilled Water	5 ml
Gram's Safranin	**100 ml**
➢ Safranin (2.5% Solution in 95% Ethanol)	10 ml
➢ Distilled Water	90 ml

KOH Solubility Test Reagent

KOH 3% Aqueous Solution	**25 ml**
➢ KOH Granules	0.75 g
➢ Distilled Water	24 ml

Catalase Test Reagent

H_2O_2 3% Aqueous Solution	**25 ml**
➢ H_2O_2 Absolute Solution	0.75 ml
➢ Distilled Water	24 ml

Oxidase Test Reagent

NNN'N-tetramethyl-p-phenylene-diamine dihydrochloride 1% Aqueous Solution	**10 ml**
➢ NNN'N-tetramethyl-p-phenylene-diamine dihydrochloride Granules	0.1 g
➢ Distilled Water	10 ml

Motility Test Reagent (SIM Medium)

Dehydrated Medium	15 g
Distilled Water	500 ml

Casein Hydrolysis Test Reagent (Skim Milk Agar Medium)

Skim Milk Powder	14 g
Trypton	2.5g
Yeast Extract	1.25 g
Dextrose	0.5 g
Agar	7.5 g
Distilled Water	500 ml

Starch Hydrolysis Test Reagent

Beef Extract	1.5 g
Soluble Starch	5 g
Agar	6 g
Distilled Water	500 ml
Gram's Iodine Solution	100 ml

Citrate Test Reagent (Simmons Citrate Agar Medium)

Dehydrated Medium	12.25 g
Distilled Water	500 ml

Gelatin Liquefaction Test Reagent

Beef Extract	1.5 g
Peptone	2.5 g
Gelatin	60 g
Distilled Water	500 ml

Levan Production Test Reagent

Sucrose	10 g
Peptone	2.5 g
Yeast Extract	2.5 g
Sodium Chloride (NaCl)	2.5
Agar	10 g
Distilled Water	500 ml

Reagents used for DNA sequencing

DNA isolation

Homogenizer	Pro Scientific, USA
Centrifuge Machine	Model: Z-216 M, HERMLE Labortechnik GmbH; Origin: Germany
Heat Block	Model: My Block, Benchmark, Origin: USA
Promega Wizard™ Genomic DNA Purification Kits	Catalog No: PR-A1120 Assecories, Mfr. No: A1120 , Origin: Promega, USA.

DNA quantification

NanoDrop Spectrophotometer	Model: ND2000, Origin: Thermo Scientific, USA

PCR

Gene Atlas	Model: G2, Origin: Astec, Japan
Primer 27F	AGA GTT TGA TCM TGG CTC AG
Primer 1492 R	CGG TTA CCT TGT TAC GAC TT
GoTaq® Green Master Mix (dNTPs, Buffer, MgCl2, Taq Pol),	Cat: M782A, Origin: Promega, USA.

Gel electrophoresis system

Horizontal	Model: Mini, Origin: CBS Scientific, USA
Agarose	Cat: V3125, Origin: Promega, USA
Bench Top1kb DNA Ladder	Cat: G754B, Origin: Promega, USA
Cat: G754B, Origin: Promega, USA	Cat: H5041, Origin: Promega, USA
TAE Buffer	Cat: V4251, Origin: Promega, USA.

Gel documentation

Alpha Imager	Model: mini, Origin: Protein Simple, USA


The End