ASSESSMENT OF *Listeria monocytogenes* AS PROBABLE CONTAMINANT OF GREEN LEAFY VEGETABLES AND IT'S ASSOCIATED ENVIRONMENT

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In partial fulfilment of the requirements for the Degree of

M A S T E R O F V E T E R I N A R Y S C I E N C E

IN

VETERINARY PUBLIC HEALTH

BY

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Enrolment No: V/16/345

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(INDIA)

2024

DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled **ASSESSMENT OF *Listeria monocytogenes* AS PROBABLE CONTAMINANT OF GREEN LEAFY VEGETABLES AND IT'S ASSOCIATED ENVIRONMENT** or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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Miss. KARPE SHIVANI SHIVAJI has satisfactorily prosecuted her course of research for a period of not less than two semester and that the thesis entitled **ASSESSMENT OF *Listeria monocytogenes* AS PROBABLE CONTAMINANT OF GREEN LEAFY VEGETABLES AND IT'S ASSOCIATED ENVIRONMENT** submitted by her is the result of research work is sufficient to warrant its presentation to the examination in the subject of **VETERINARY PUBLIC HEALTH,** for the award of **MASTER OF VETERINARY SCIENCE** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part thereof has not been previously submitted by her for a degree of any other University.

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CERTIFICATE

This is to certify that the thesis entitled, **ASSESSMENT OF *Listeria monocytogenes* AS PROBABLE CONTAMINANT OF GREEN LEAFY VEGETABLES AND IT'S ASSOCIATED ENVIRONMENT** to the Maharashtra Animal and Fishery Sciences University in partial fulfilment of the requirement for the degree of **MASTER OF VETERINARY SCIENCE** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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Date:

Place: Nagpur ***(Shivani Karpe)***

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LIST OF ABBREVIATIONS

α	:	Alpha
β	:	Beta
%	:	Per- cent
@	:	At the rate
µg	:	Microgram
µl	:	Microlitre
APS	:	Ammonium per sulphate
BHI	:	Brain Heart Infusion broth
Bp	:	Base pairs
CAMP	:	Christie, Atkins, Munch-Petersen
DW	:	Distilled water
DNA	:	Deoxyribonucleic acid
etc	:	et cetera (and other things
et al.	:	And others
Fig	:	Figure
gm	:	Gram
hr	:	Hour
HU	:	Haemolytic Unit
ISO	:	International Standards Organization
lit.	:	Litre
M	:	Molar
min	:	Minute
ml	:	Millilitre
mM	:	Millimolar
MR	:	Methyl Red
MTCC	:	Microbial Type Culture Collection
MW	:	Molecular weight

INTRODUCTION

In the modernized era of globalized food trade and rapid technological advancement, the issue of foodborne infections has taken on a significant and far-reaching economic and public health dimension. As our world becomes more interconnected and our diets evolve, driven by a growing demand for minimally processed, ready-to-eat, and refrigerated/frozen food products, the incidence of foodborne outbreaks has surged in recent years. These shifts in consumer preferences have, in many ways, created an environment where our food supply is more vulnerable to contamination from various sources. In the year 2016, the Produce Safety Rule (PSR) was finalized as part of the FDA Food Safety Modernization Act (FSMA), introducing science-based standards aimed at ensuring the safe cultivation of fresh produce intended for human consumption.

Foodborne listeriosis is an emerging bacterial disease and zoonotic public health hazard caused by *Listeria monocytogenes*. In 1980s with the publication of a recorded report of a Canadian listeriosis outbreak linked to tainted coleslaw, highlighted the significant significance of listeria as a food-borne human pathogen. (Schlech *et al*. 1983). Recently, the world's largest outbreak of listeriosis was reported in South Africa during 2017- 18 which was associated with ready-to-eat processed meat products in which 216 human mortality cases were reported among the 1060 laboratory-confirmed cases (Malla *et al.*, 2021).

Murray, Webb, and Swann made the discovery of *Listeria* in 1926 and named it as *Bacterium monocytogenes* considering its characteristic mononuclear leucocytosis (Murray *et al*. 1926). Pirie in 1927 discovered a unique microbe responsible for “Tiger River disease” in South Africa and named it as *Listerella hepatolytica*. Both these strains discovered by Murray and Pirie were found similar by National Type Collection at the Lister Institute in London and it named it as *Listeria monocytogenes* devoting it in honour of Lord Lister (Rocourt *et al.* 2007). Its significance as a food-borne pathogen in humans was recognised due to the ingestion

of tainted food in Canada, the United States, and Europe later in the 1980s (Mateus *et al*. 2013).

Listeria is a Gram-positive bacteria with a low G+C concentration which is related to *Bacillus, Clostridium, Enterococcus, Streptococcus,* and *Staphylococcus*. Together with the genus Brochothrix, the genus *Listeria* is a member of the phylum Firmicute, the order Bacillales, the class Bacilli, and the family *Listeriaceae* (Barbuddhe *et al*., 2021). *Listeria* consists of 27 species (Vishnuraj *et al*., 2023) of which *L. monocytogenes* is pathogenic to animals and human and *L. ivanovii* is pathogenic to animals (Arslan and Özdemir, 2020). *Listeria* species differentiation involves sub-typing molecular approaches, which demand thorough and precise optimization and validation. There are 14 recognized serotypes of which recently reported serovar 4h is a novel serotype of *L. monocytogenes* belonging to hybrid sub-lineage II which exhibit hypervirulent features (Feng *et al.*, 2020). The serotype 1/2a is most frequently isolated from food and serotype 4b causes the majority of epidemics in humans. Molecular characterization revealed 4b as predominant serotype in India (Barbuddhe, *et al*., 2016). The important virulence factors that are necessary for adhesion, intracellular multiplication and pathogenicity are the haemolysin, listeriolysin O (LLO), internalins, , fibronectin-binding protein, FbpA, ActA protein, two phospholipases, metalloprotease, Vip protein, a bile exclusion system (BilE) and a bile salt hydrolase (Dhama *et al.*, 2015).

It is a facultative anaerobic, ubiquitous, intracellular microorganism with flagellum-based tumbling motility at growth temperatures of 22–28°C (Mauder *et al*., 2008). *Listeria* can adapt and survive to a variety of food production environmental stress conditions, including temperatures, low and high pH, high salt concentration, ultraviolet lighting, presence of biocides, and heavy metals. *Listeria* spp. can be found in the water, soil, fresh vegetables, warm-blooded animals, and human intestinal tracts (Taormina, P.J. and Beuchat, L.R. 2002). Also the pathogen can survive for long time by forming biofilm on surfaces which increases the risk of food contamination (Osek *et al*., 2022). There is no significant change in the number of

live bacteria population under low temperature conditions. These has resulted in detection of bacteria in food products stored under refrigeration condition (Deka *et al.*, 2022). Recently CDC reported outbreaks of listeriosis connected to the consumption of frozen veggies in Washington (CDC, 2016).

In humans non-invasive and invasive are two types of listeriosis. Non-invasive listeriosis is mild form with short incubation period whose symptoms include diarrohea, muscle pain, fever, head ache. Invasive listeriosis is severe form of disease with incubation period from few days to upto 90 days. It affects mainly pregnant women, infants, people undergoing treatment for HIV, cancer, organ transplants and elderly people. The symptoms include fever, gastroenteritis meningitis, septicaemia, muscle pain (Posfay-Barbe and Wald, 2004). As compared to other healthy individuals pregnant women are 20 times more susceptible to contract listeriosis resulting in stillbirth, miscarriage and perinatal illness. Immunocompromised individuals like HIV patients are 300 times more susceptible (WHO, 2018).

Listeriosis affects sheep, goat, cattle, buffalo, horse, pig, camel, canine, rodent, wild animals, birds; sheep is mostly affected (OIE, 2014). Encephalitis is the most common form of disease which is also called as 'circling disease'. In ruminants listeriosis mostly occur in winter and early spring and its mainly caused by consumption of silage. Silage being one of the most potent cause of listeriosis it is also called as 'silage disease' (Fenlon, 1985). Listeriosis also causes abortion in last trimester of gestation, generally it is after 28 weeks in cattle and 12 weeks in sheep (Hird and Genigeorgis, 1990).

The vegetables due to its nutritional benefits forms an essential component of a healthy diet and its consumption is being actively promoted throughout the world. Majority of population prefer to consume vegetables in raw or in minimally processed form as part of new eating habit and lifestyle characterised by high convenience of use (Miceli and Miceli, 2014). The pathogen is commonly found in

vegetables. It is found with prevalence around 1 per cent in fresh cut (Tango *et al.* 2018) to upto 4 per cent in farmer market fresh produce (Miceli and Settanni, 2019). The detection of *Listeria* in vegetables poses a significant public health risk.CDC in 2015 reported listeriosis outbreak due to contaminated fruits and vegetables (apples, cantaloupe, celery). Therefore a proper microbiological analysis of *Listeria* in vegetables should be evaluated in order to safeguard consumer health.

The pathogen is present in an extensive range of animals, including humans, it can also be isolated from animal faeces, the land they inhabit, sewage, soils to which sewage is applied, and plants and vegetables, that are grown in these soils (Beuchat, 1996). It has been verified that *Listeria* can be detected in sewage and also sewage contaminated soil (Watkins and Sleath, 1981). *L. monocytogenes* could persist in soil for up to 45-100 days (Nicholson *et al.*, 2005). Soil is the origin of *L. monocytogenes* contamination of fruits and vegetables. The usage of contaminated manure as a fertilizer for soil and the quality of the water used to wash vegetables and fruits are the main sources of contamination. This bacterium showcases a remarkable life cycle adaptation. While it normally resides in the soil as a saprophyte, it can undergo a transformation into a pathogenic form when it infiltrates the cells of humans or animals (Freitag, *et al*., 2009).

Organic vegetables in relation to the method of cultivation and processing, which uses natural fertilizers like animal manure and no chemical treatments to lessen the microbiological loading of the raw product or to extend its shelf life, it have been considered to pose an increased risk to public health. There may be a higher chance that these vegetables will become and persist contaminated with potentially pathogenic species from enteric sources (McMahon and Wilson, 2001). *Listeria* spp. have been detected in both conventional and organic vegetable farms. However, understanding of the differences in *L. monocytogenes* contamination among conventionally grown, organically grown, and hydroponically grown vegetables is currently limited.

Being the foodborne disease with highest hospitalization and mortality rate (99.1 and 15.6%, respectively) listeriosis is characterized by a consistent social and public health impact (Scientific EFSA and ECDC 2015). It is the third main cause of death due to food-borne bacterial pathogens, with the fatality rates exceeding that of *Salmonella* and *Clostridium botulinum* (Ramaswamy *et al.*, 2007). However, precise epidemiological information is lacking due to underreporting and inadequate diagnostic capabilities. To determine the precise state of the disease globally, studies on the prevalence of *Listeria* infection in various emerging nations are required. To effectively prevent and control this infection across health, agricultural, and environmental systems, it is essential to implement strong quality control measures alongside appropriate preventive strategies.

As of now, there is no definitive epidemiological information that can determine the extent of contamination is prevalent in the majority of food-related listeriosis outbreaks. However, it has been approximated that the infectious doses of *L. monocytogenes* range from 10^7 to 10^9 colony-forming units (CFUs) for healthy individuals, while for high-risk individuals, it is considerably lower at 10^5 to 10^7 CFUs (Quereda, *et al*., 2021). Several countries, such as the India, USA, Australia, China, and Turkey, mandate the non-detection of *L. monocytogenes* in 25 grams of food, adopting a 'zero tolerance' policy for *L. monocytogenes* in all ready-to-eat foods (Churchill *et al*., 2006; Tao *et al*., 2016).

International Organization of Standardization (ISO), the United States Department of Agriculture (USDA) and. U.S.A. Food and Drug Administration (FDA); Bacteriological Analytical Manual method are the conventional culture methods for isolating *L. monocytogenes* from environment and food (Lourenco *et al.*, 2022). Culture based methods are ‘gold standard’ against which the other methods are validated and also results in obtaining pure culture which can be used for further studies (Matle *et al*., 2020). ISO method involves two step enrichment with Fraser broth with selective supplements followed by selective plating on Polymixin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) agar.

Listeria can be significantly recovered by these selective enrichment and selective plating as they reduces the number of contaminating microbes and allowing multiplication of *Listeria* (Nayak *et al.*, 2010). Reissbrodt (2004) reviewed a number of beneficial selective chromogenic plating media replacing conventional media for isolation of pathogenic *Listeria* spp. mainly in food industries; one of those is use of Fraser broth for the selective enrichment (ISO 11290-1-2017). This is followed by gram staining, biochemical test, motility test, sugar fermentation test, pathogenicity test for identification. The CAMP test, the PI-PLC assay, the haemolysis on sheep blood agar, and *in vivo* tests involving intraperitoneal inoculation of 3-week-old mice and chorioallantoic membrane inoculation of 10-day-old chicken embryos are all those methods utilized to assess the pathogenic potential of *Listeria* isolates (Khan *et al*., 2013).

Molecular approaches offer a significant advantage in achieving precise and sensitive identification compared to microbiological methods, which are phenotypic-based and often influenced by sub-varieties and natural variations (Barbuddhe *et al*., 2021). As an illustration, PCR technologies have been utilized to create specific primers and probes tailored to target individual *Listeria* species, especially *L. monocytogenes* (Azinheiro *et al*., 2022; Köppel *et al*., 2021). Amplification often focuses on virulence-associated genes, including *inlA, inlC, plcA, prfA, actA, hlyA*, and *iap*. Recent advancements in *Listeria* identification include whole genome sequencing methods (Pietzka *et al*., 2019), isothermal amplification techniques (Wachiralurpan *et al*., 2021), and real-time PCR, providing both detection and quantitative insights into listeriosis (Amagliani *et al*., 2021). Remarkably, the combination of high-resolution melt (HRM) analysis and real-time PCR stands out as a noteworthy and recently introduced technique in the field of molecular biology (Grazina *et al*., 2021). In epidemiological research, *L. monocytogenes* can be classified using genetic methods like pulsed field gel electrophoresis (PFGE), whole genome sequencing, multivirulence-locus sequence typing (MVLST), and multilocus sequence typing (MLST).

Understanding the ecology of human pathogens in agroecosystems is crucial to foresee how their circulation and incidence may be influenced in an era of fast global change, when agriculture faces significant adaptation challenges. Reassessing health risks is in fact important. *L. monocytogenes* should be considered as a useful model organism for this purpose. Looking into ubiquitous nature of the pathogen and lack of systematic work especially on its occurrence in the vegetables, the current investigation is proposed with the following objectives:

OBJECTIVES:

1. To screen vegetables sold in local market as well as grown in organic farming system in and around Nagpur for isolation of *Listeria monocytogenes*.

2. To confirm the isolates by biochemical and molecular assays.

3. To study the virulence characteristics of the isolates by *in-vitro* assays including PCR.

REVIEW OF LITERATURE

In the ever-evolving landscape of food safety and public health, the assurance of safe, wholesome produce has assumed paramount importance. Among the threats that loom over the food supply chain, *Listeria monocytogenes* stands out as a tough adversary. This bacterium, notorious for its ability to survive and thrive in a spectrum of environments, has emerged as a significant concern when it comes to the safety of green leafy vegetables.

Green leafy vegetables which are celebrated for their abundance of essential nutrients, have witnessed an increased in popularity as consumers increasingly prioritize health-conscious choices. However, this rise in consumption has been accompanied by a growing concern over the potential contamination of these greens with *Listeria monocytogenes*. This foodborne pathogen's unique attributes make it particularly challenging to control, as it exhibits remarkable resistance to adverse conditions and can multiply even at refrigeration temperatures. The consequences of *Listeria* contamination are grave, especially for segments of the population with weakened immune systems, such as pregnant women, the elderly, and individuals with certain medical conditions. *Listeria monocytogenes* can cause listeriosis, a severe and potentially life-threatening illness that underscores the urgency of comprehending the dynamics of its contamination in green leafy vegetables.

This chapter refers to the review of available literature related to the occurrence, isolation, characterization, and public health significance of pathogenic *Listeria* species.

2.1 Occurrence of *Listeria* spp. in Vegetables

The vegetables are one of the essential components of human diet due to their nutritional and health benefits and is actively promoted throughout the world. The vegetables are usually consumed raw or in minimally processed forms. The contamination of these vegetables with *Listeria* spp. can lead to infections in humans with grave consequences in immunosuppressed individuals.

2.1.1 Global scenario

Heisick *et al*. (1989) analysed 1,000 vegetable samples *viz*. broccoli, cabbage, carrots, cauliflower, cucumbers, lettuce, mushrooms, potatoes, radishes, and tomatoes, for *Listeria* spp. from two Minneapolis area supermarkets in USA. The vegetables were tested by the Food and Drug Administration method for isolation of *Listeria* spp. *L. monocytogenes* was found in cabbage, cucumbers, potatoes and radishes. *L. innocua* was isolated from cucumbers, lettuce, mushrooms, potatoes, and radishes. *L. seeligeri* was isolated from cabbage and radish and *L. welshimeri* was isolated from cucumbers, potatoes, andradishes. Only potatoes (25.8 per cent) and radishes (30.3 per cent) showed significant *L. monocytogenes* contamination. No *Listeria* spp. was recovered frombroccoli, carrots, cauliflower or tomatoes.

Arumugaswamy, *et al*. (1994) reported 22% prevalence of *L. monocytogenes* in leafy vegetables from Kajang, Serdang in Selangor and Kuala Lumpur.

Cheong, *et al*. (1994) examined a total of 280 different vegetables bought from four different market outlets in Kuala Lumpur, Malaysia for the presence of *Listeria* spp. The isolation procedure was based on the Food & Drug Administration method (modified) and isolation media used were *Listeria* Selective medium and LiCl- phenylethanol-Moxal*act*am agars. The identification of isolates was by means of conventional biochemical tests and API *Listeria* identification system. *Listeria* spp. was found in five samples. *Listeria monocytogenes* was isolated in lettuce, sengkuang (Pachyrrhizus erosus) and selom Oenanthe javanica) and *Listeria* innocua was isolated from seng kuang (Pachyrrhizus erosus) and pegaga (Hydrocotyle asiatica). *L. monocytogenes* was recovered in two of the 70 samples (2.8%) taken from the one supermarket outlet and one isolate of *L. monocytogenes* was found in 210 samples (0.5%) from the three wet markets. The overall isolation rate of *L. monocytogenes* is 1.1%.

Gunasena (1995) examined 55 vegetable samples including green leaves (17), cabbage (18), lettuce (20) collected from retail shops in Sri Lanka

for incidence of *L. monocytogenes*. Highest prevalence of 65% (11/17) in green leaves followed by lettuce 50% (10/20) and cabbage (6/18) 33% was recorded.

Lin *et al*. (1996) examined the occurrence of *Listeria monocytogenes* in 63 vegetable salads served at 31 food service facilities (four supermarkets, 14 fast food chain restaurants, and 13 family restaurants). Homogenized salad samples were incubated in the University of Vermont broth (UVM; BBL) followed by plating on modified Oxford agar. Bacterial colonies with characteristic features were confirmed biochemically and immunologically. *L. monocytogenes* was detected in one sample tuning to 1.58 % prevalence.

A total of 181 samples of minimally processed leafy salads were collected from retailers in the city of Sao Paulo, Brazil. *L. monocytogenes* was assessed by Fröder *et al*. (2007) in 181 samples using the BAX System and by plating the enrichment broth onto Palcam and Oxford agars. Suspected Listeria colonies were submitted to classical biochemical tests. *L. monocytogenes* was detected in only 1 (0.6%) of the 181 samples examined. This positive sample was simultaneously detected by both methods. The other *Listeria* spp. identified by plating were *L. welshimeri* (one sample of curly lettuce) and *L. innocua* (2 samples of watercress).

Seventy one vegetable samples comprising of lettuce, cucumber, cabbage, sweet pepper and carrot from supermarkets across Trinidad were analysed for *L. monocytogenes* by immunomagnetic separation (IMS), ELISA (enzyme linked immune sorbent assay) and conventional method by Hosein *et al*. (2008). Only one sample of lettuce was found positive for the *L. monocytogenes* and its prevalence of was 1.7 %

Between 2000 and 2005, 717 samples of three types of salads were analysed by Cordano *et al*. (2009) for *Listeria monocytogenes* in Santiago, Chile in order to provide information to Chilean health authorities on the presence of the pathogen in vegetable salad samples and to ascertain the risk of these products for consumers. Bacteriological analysis of the samples was performed as recommended by the FDA Bacteriological Analytical Manual. *L.*

monocytogenes isolates were found in 88 out of 347 (25.4%) samples of frozen vegetable salads and in 22 out of 216 (10.2%) freshly supermarkets prepared, cooked or raw ready-to-eat vegetable salads; no *Listeria* was isolated from 154 samples of raw minimally processed salads industrially prepared.

Ponniah *et al*. (2010) examined a range of commercially available vegetables (n = 306) that are consumed in the minimally processed state in Malaysia for the presence of *Listeria* spp. Analysis was carried by procedure described in FDA-BAM Standard for detection of *Listeria* (USFDA CFSAN BAM, 2003) and the most probable number–polymerase chain re*act*ion (MPN–PCR) method wherein PCR targeted 16S rRNA for *Listeria* spp. and the *hly* gene which is specific for *L. monocytogenes*. It was found that *Listeria* spp. and *L. monocytogenes* could be detected in 33.3percent and 22.5percent of the vegetables respectively. *Listeria* spp. were more prevalent in *Cosmos caudatus* (wild parsley) at 50 percent, *Cucumis sativus* (cucumber) at 43.8 percent and *Oenanther stolonifera* (Japanese parsley) at 39.4 percent. *L. monocytogenes* was more frequently detected in *Vigna unguiculata* (Japanese parsley) at 31.3 percent and Oenanther stolonifera (yardlong bean) at 27.2 per cent.

Moreno, *et al*. (2012) analysed 191 samples of vegetables from Spain for *L. monocytogenes* and the isolation rate of 4.19 per cent was obtained by culture technique. Multiplex PCR showed a greater number of positive samples (10.47 per cent). The detection and enumeration of *L. monocytogenes* were carried out as described in the ISO 11290-1:1996 and ISO 11290- 2:1998 protocols respectively. For detection and enumeration of *L. monocytogenes* by DVC-FISH an oligonucleotide probe complementary to a 16S rRNA region of *L. monocytogenes* was used.

Jamali *et al*. (2012) conducted a study to determine the prevalence of *Listeria spp*. specifically *Listeria monocytogenes* in ready-to-eat (RTE) food (salads and vegetables) sampled from street hawkers and hypermarkets in Selangor, Malaysia and reported 14.7 per cent samples were positive for *L. monocytogenes*. The ISO 11290 method was used for isolation and identification of *L. monocytogenes* in this study.

Sant'Ana *et al*. (2012) evaluated the prevalence and counts of *L. monocytogenes* in 512 packages of ready-to-eat vegetables marketed in São Paulo. Isolation was done by ISO 11290-1 and ISO 11290-2 methods for prevalence and enumeration of *L. monocytogenes*, respectively. *L. monocytogenes* was detected in 3.1% of the samples.

De Silva *et al*. (2013) screened 50 vegetables from Kandy district, situated in the Central province of Sri Lanka for the presence of *L.monocytogenes.* Isolation was done by cold enrichment method. Two per cent of the vegetables (lettuce, cabbage, mukunuwenna, gotukola and tomato) were contaminated with the organism.

Morokova *et al*. (2017) studied the incidence of *Listeria* spp. in 10 states of European Union. The examination was done using standard culture methods (EN ISO 11290- 1) and qPCR. A total of 175 vegetables including ready to eat vegetables, frozen vegetable, sprouts, were analysed which detected 2.1per cent, 20.9per cent, 2.9per cent incidence respectively for *L. monocytogenes*.

Li, *et al.* (2017) evaluated the microbiological quality/safety of fresh produce from farmers' markets (FM). In study I, 212 produce samples were tested for the presence of *Salmonella* and *Listeria* spp. using modified FDA-BAM methods. In total 212 fresh produce samples of tomato (64 samples, 13 vendors, 4–6 each), green pepper (54 samples, 11 vendors, 4–6 each), cucumber (35 samples, 5 vendors, 6–8 each), cantaloupe (16 samples, 12 from one vendor, 4 from the other vendor) and spinach (43 samples, 8 vendors, 5–7 each). Samples were aseptically collected from a total of 39 vendors from two farmers' markets in Morgantown, West Virginia, and one farmers' market in Bowling Green, Kentucky. The results of this study showed that *Listeria* spp. was isolated from 8 of 212 (3.8%) fresh produce samples, including 1 tomato, 3 green peppers, 2 cucumbers and 2 cantaloupes indicating a prevalence of 3.78%. Among those *Listeria* spp. isolates, *L. monocytogenes* was most prevalent (50%), including 1 tomato, 2 cucumbers and 1 cantaloupe sample, and the possible serovars were 1/2b, 3b, 4b, 4d and 4e based on the multiplex PCR results.

Roth, *et al*. (2018) analyze the microbial quality of fresh produce from farmers' markets and compare it with the quality of produce from traditional retail markets. Between July 2016 and April 2017, 401 fresh produce samples, including 301 samples from farmers' markers and 100 from supermarkets, were collected from 9 farmers' markets and 12 supermarkets in North and Central Florida. sample was prepared and analyzed based on the FDA Bacteriological Analytical Manual (BAM) The presence of *L. monocytogenes* was detected utilizing the SureTect *Listeria monocytogenes* PCR Assay with the PikoReal Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA) *L. monocytogenes* was detected in 3.9% (2 of 52) and 2.6% (2 of 77) of farmers' market spinach and leafy greens, respectively. No supermarket samples were positive for *L. monocytogenes*.

Following the current listeriosis outbreak in the nation, Kayode *et al*. (2022) investigated on the incidence of *Listeria monocytogenes* found in fruits and vegetables purchased from three District Municipalities in South Africa's Eastern Cape Provtangoince. The procedure outlined by the International Organization for Standardization EN ISO 11290:2017 Parts 1 and 2 was adopted for the isolation. Molecular detection of the pathogen and the presence of 10 virulence-associated markers were assessed. Investigation from 140 vegetable samples detected 42.86 per cent prevalence. Highest prevalence was recorded in tomato (65.52 per cent) followed by spinach (56.67 per cent), cabbage (38.10 per cent), apple (36.84 per cent), mushroom (29.41 per cent) and carrot (10 per cent).

2.1.2 Indian scenario

Pingulkar *et al*. (2001) examined a total of 116 samples of 11 different fresh vegetables from a local market generally consumed in raw form as well as 12 samples of ready-to-eat green salads procured from three grade 1 and 2 restaurants in Mumbai, India for incidence of pathogens such as *Listeria* and Yersinia. The samples included 26 leafy vegetables, 12 roots, 62 tomatoes and four samples each of cabbage, capsicum and cucumber. A two-stage enrichment procedure followed by plating on *Listeria* selective agar (LSA) and PALCAM was adopted to isolate *Listeria* spp. and further identified to species level as

described in the B*act*eriological Analytical Manual (BAM). Presence of *L. monocytogenes* was observed in 7 out of 62 tomatoes, 5 out of 10 coriander leaves, 2 out of 4 spinach samples and one from 4 cabbage sample.

Dhanshree *et al*. (2003) screened the vegetable samples from Mangalore, South India, for the presence of *Listeria* spp. The procedure adopted for the isolation of *Listeria* spp. was a modification of USDA and FDA methods. *L. innocua* was isolated from 10 per cent of the palak leaves and 30 per cent of the coriander leaf samples. *L. monocytogenes* was not detected in any of the cabbage samples.

A total of 200 vegetable samples were collected by Soni, *et al*. (2014) from the agricultural farm of the Indian Institute of Vegetable Research (IIVR), Varanasi, India (25° 08' N latitude; 83° 03' E longitude and 90 m from sea level), through October 2011 to February 2012. All the samples were examined following the standard double enrichment method as prescribed by ISO 11290:1 with slight modifications. Among the vegetables, 20 each were from brinjal (*Solanum melongena*), cabbage (*Brassica oleracea* var. *capitata*), broccoli (*Brassica oleracea* var. *italica*), cauliflower (*Brassica oleracea* var. *botrytis*), dolichos-bean (*Dolichos lablab*), palak (*Beta vulgaris*), tomato (*Solanum lycopersicum*), chappan-kaddu (*Cucurbita pepo*), chilli (*Capsicum annum*) and cowpea (*Vigna unguiculata*). Out of the 10 vegetables selected, 6 (brinjal, cauliflower, dolichos-bean, tomato, chappan-kaddu and chilli), 20 isolates (10%) tested positive for *L. monocytogenes*. The overall prevalence of *L. monocytogenes* in 200 vegetable samples was 20 (10%). Conversely, cabbage, broccoli, palak and cowpea and their respective soils tested negative.

Satish (2015) investigated the occurrence of *Listeria* spp. in raw vegetables, milk and meat products from Thrissur and Mannuthy district, Kerala. A total of 360 samples containing six varieties of raw vegetables comprising of amaranth (63), coriander (64), cabbage (58), carrot (57), onion (59), and tomato (59) were analysed. All the samples were analysed for *Listeria* spp. using USDA conventional microbiological technique *L. innocua incidence* was reported 6.66 per cent and *L. monocytogenes* incidence was

reported 0.277 per cent.

Mritunjay and Kumar (2017) evaluated the microbiological quality of raw salad vegetables (RSV) consumed in Dhanbad city, India. A total of 480 samples of 8 different raw salad vegetables from local market were examined for *Listeria monocytogene.* The isolation was as per ISO 11290-1-1996). 25-g samples were weighed into sterile nylon bags and homogenized with 225 mL of Fraser broth (Himedia). After homogenizing and pre-culturing at 37 ± 1 °C for 48 ± 2 h, the positive broth was streaked onto Listeria *Palcam* agar (Himedia) and incubated at 37 ± 1 °C for 24 ± 2 h. *L. monocytogenes* was detected in 3.5%, of the total samples.

Verma and Singh (2022) examined 10 types of vegetables from in and around Madhya Pradesh state for the occurrence of *L. monocytogenes.* Findings demonstrated the occurrence as 27.2 per cent in Cabbage, 5 per cent in Tomato, 25 per cent in Potato, 3 per cent in Spinach, 31.3 per cent in Yardlong, 9 per cent in Carrot, 6 per cent in Green peas, 7 per cent Eggplant, 2 per cent in Okra and 7 per cent in Sweet potato.

2.2 Occurrence of *Listeria* spp. in Organic Farm Samples

Organic farming practices including the use of animal manures may increase the risk of microbiological contamination as manure can act as a vehicle for transmission of foodborne pathogens

2.2.1 Global Scenario

McMahon and Wilson (2001) screened a range of commercially available organic vegetables (*n*=86) for the presence of *Salmonella, Campylobacter*, *Escherichia coli*, *E. coli* O 157, *Listeria* and *Aeromonas* spp. to provide information on the occurrence of such organisms in organic vegetables in Northern Ireland. No *Salmonella, Campylobacter, E. coli, E. coli* O 157, *Listeria* were found in any of the samples examined. *Aeromonas* species were isolated from 34percent of the total number of organic vegetables examined.

Ueda and Kuwabara (2002) conducted microbiological safety evaluations on organically grown vegetables, the soil from their growing fields and organic fertilizers in Japan. The organic produce collected from some farms in winter and spring carried mostly aerobic bacteria at the level of 10E5 CFU/g. Enteric pathogens, like diarrheagenic *E. coli*, *Salmonella* and *Listeria monocytogenes*, were not detected from any of the vegetable and soil samples.

Loncarevic *et al*. (2005) investigated b*act*eriological quality in organically grown 179 leaf lettuce samples in Norway. *L. monocytogenes* serogroups 1 and 4 were isolated from 2 samples, *E. coli* from 16 samples and no samples were detected positive for *Salmonella.*

Oliveira *et al*. (2010) investigated prevalence of pathogens in fresh lettuce grown in organic and conventional farms in Spain. Out of 72 lettuce samples none of the sample was positive for *L. monocytogenes.*

Park *et al*. (2014) analyzed the presence of microbes in organic farm produce, green chillies, lettuce, tomatoes, apples, pears, and rice from 47 production sites in Korea, which revealed no incidence of *Listeria spp*.

Tango, *et al*. (2014) screened leafy vegetables (spinach, romaine lettuce, and green sesame leaves) grown in organic and conventional systems from various areas in Korea and examined using standard culture methods to compare the microbiological quality of the produce grown in the two agricultural systems. To isolate *L. monocytogenes*, the homogenate was spread onto *Listeria* selective agar (Difco, BD) plates and incubated at 30°C for 48 h. Presumptive *Listeria* colonies were identified to species using the API system. Out of 354 samples the prevalence of *L. monocytogenes* was highest on organic romaine lettuce and spinach; it was found in 4 (6.4percent) of 63 samples of each type of vegetable.

Xu, *et al*. (2015) study at Maryland failed to detect any *Listeria* spp. or *Lm* in fresh produce samples. *L. monocytogenes* was not detected in tomatoes, leafy greens, peppers, cucumbers, and other produce (n = 177) from seven organic farms in Maryland. Well water and surface water (n = 29) were also tested at these

organic farms and *L. monocytogenes* was not detected

Kuan, *et al*. (2017) compared the microbiological status between organic and conventional fresh produce at the retail level in Malaysia. A total of 152 organic and conventional vegetables were purchased at retail markets in Malaysia. *L. monocytogenes* was detected in both organic (9.1 per cent) and conventional vegetables (2.7 per cent).

2.2.2 Indian Scenario

Richa Rautela (2018) examained five organic agricultural farms located at Kotabagh, Dhamola, Ramnagar and two locations of Pantnagar in Uttarakhand state, India. A total 500 samples, comprising 350 environmental samples viz; soil (n=227), manure (n= 66) and water (n=57) besides, 150 plant samples viz; rhizosphere (n=50), roots (n=50) and leaves/grains (n=50) were processed for isolation of *Salmonella* and *Listeria*. For isolation of *Listeria* samples USDA conventional microbiological technique was followed. A total of 11 *Salmonella* isolates were recovered with an overall prevalence of 2.2% while *Listeria* could not be isolated representing 0% prevalence.

2.3 Occurrence of *Listeria* spp. in Environment Samples

In a period of rapid global changes, when agriculture faces major adaptation challenges, understanding the ecology of human pathogens in agroecosystems is necessary to forecast how their circulation and incidence may be affected. The ubiquitious nature of *Listeria spp.* in conjunction with the use of surface waterways for discharge of sewage effluents, inevitably results in the presence of these organisms in a wide range of surface waters including lakes, rivers and streams. Soil is a complex ecosystem central to the function of the biosphere. Soil may play a pivotal role in the transfer of Human pathogens to cultivated plants and farm animals and the subsequent contamination of foodstuff. Considering *L. monocytogenes* ability to persist in soil, it is rather difficult to predict how anthropogenic-driven changes could modify their circulation and incidence in the biosphere.

2.3.1 Global scenario

Watkins and Sleath (1981) examined 52 sewage, river water, and industrial effluent samples from England for the presence of potentially harmful b*act*eria. There were effluents from slaughterhouses, cattle markets, and poultry packing operations. Samples were inoculated into double and single strength Nutrient Broth No. 2 (Oxoid) using a most probable number system and incubated at 4°C. The results indicated that *L. monocytogenes* is present in sewage and sewage sludge in considerable numbers and that this organism survives longer than *Salmonella* spp. on land sprayed with sewage sludge.

Van Renterghem *et al*. (1991) examined fresh pig feces, stored liquid manure, manured soil samples and water samples for *L. monocytogenes*. The prevalence was found to be 20 %, zero per cent, zero % and 50 % for fresh pig feces, stored liquid manure, manured soil samples and water samples respectively.

Ikeh *et al*. (2010) while studying the prevalence of *Listeria* spp. in food and environmental samples in Nsukka, Nigeria reported that the highest incidence (100%) was observed in the soil (5 samples) and surface swab samples (5 samples), in and around the slaughter houses. Pre-enrichment and enrichment were carried out according to the method of the United States Food and Drug Administration and Center for Food Safety and Applied Nutrition followed by plating on PALCAM agar.

Strawn, *et al*. (2013) performed a longitudinal field study on five produce farms in New York State. In total, 77 (68 surface and 9 engineered), 9 (all engineered), 45 (44 surface and 1 engineered), 18 (9 surface and 9 engineered), and 25 (all surface) water samples were collected from each of the five farms. Soil samples were pooled. Faecal samples represented only 10% of the 588 total samples. *L. monocytogenes* prevalence was estimated to be 15.0% (88/588) across all samples collected. The prevalence of *L. monocytogenes* was highest among water samples (48/174). All *L. monocytogenes*-positive water samples were from surface water (e.g., creek or pond water); none of the 28 samples from engineered water sources (e.g., municipal or well water) were

positive for *L. monocytogenes*

Linke, *et al*. (2014) studied, 467 soil and 68 water samples were collected from 12 areas in Austria between 2007 and 2009. Out of 467 soil samples, 30% (140) were determined to be positive for *Listeria* spp. of which 28 (16.77%) as *L. monocytogenes*, 8 (4.79%) as *L. welshimeri*, 73 (43%) as *L. seeligeri*,30 (17.9%) as *L. innocua*, 17 (10.18%) as *L. ivanovii* on the basis of PCR assay. Listeria was isolated from 26.5% of water samples.

Nassirabady *et al*. (2015) screened 150 water samples collected from different parts of Karun river (rural and urban environment), Iran. Isolation of *Listeria* was carried out by using Listeria enrichment broth (LEB) and Fraser *Listeria* selective enrichment broth as two step enrichment media and CHROM agar as selective agar. A total of 150 samples revealed 20 *L. monocytogenes* with detection of two pathogenicity genes: *hlyA* and *inlA* on amplification. Detection of *L. monocytogenes* with *hlyA* and *inlA* genes indicated that these strains may have the capacity to invade host cells.

Sarker and Ahmed (2015) reported *Listeria monocytogenes* prevalence of 15 per cent from surface water and 13.33 per cent of the total of 30 soil samples and 20 water samples from different areas of Dhaka city, Bangladesh. Samples were processed according to combined method of United States Department of Agriculture (USDA) and U.S. Food and Drug Administration (FDA).

Gholipour, *et al*. (2020) investigated the presence of *Listeria* spp. and *L. monocytogenes* via molecular methods in 126 wastewater effluent, sewage sludge and livestock manure in Iran as potential sources of *Listeria.* The results of survey showed no presence of *L. monocytogenes* in wastewater effluent and wastewater-irrigated soil and crop samples, whereas was detected in sewage sludge (50 percent) and manure (8 percent) samples.

2.3.2 Indian scenario

Moshtaghi *et al*. (2003) collected 136 soil samples from agricultural fields and animal-inhabited areas from Haryana and examined it for the presence of

Listeria. The microorganism was identified in 23 (17.7%) samples. *L. monocytogenes* was detected in 7 samples (5.4%), *L. ivanovii* in 2 (1.5%), *L. innocua* in 10 (7.7%) and *L. welshimeri* in 4 samples (3.1%). Prevalence of *Listeria* in soil from agricultural fields (17.5%) did not differ considerably from that in the soil and animal-inhabited area (18.0%), but *L. ivanovii* was isolated only from the latter source. The frequency of occurrence of different species of *Listeria* differed from place to place

Nightingale, *et al*. (2004) studied ecology and transmission of *L. monocytogenes* infecting the ruminants and in the farm environment. A total of 528 fecal, 516 feed and 1012 environmental soil and water samples were cultured for *L. monocytogenes*. Small ruminant farm showed a significantly ($P<0.01$) higher prevalence in case farms (32.9 %) than in control farms (5.95 %).

The occurrence of *Listeria* spp. in different animals and farms of Odisha were studied by Sarangi and Panda (2012). Screening of 70 environmental samples revealed isolation of four *Listeria* spp. with sewage having the highest prevalence followed by soil. Examination of 20 soil samples showed presence of one *L. monocytogenes*. The prevalence of *Listeria* in soil was five per cent.

Soni *et al*. (2013) conducted a study in which 100 water samples from the river Ganges were collected from Varanasi, Uttar Pradesh, during the period from June 2009-July 2010 and tested. The samples were enriched in half-Fraser broth, second enrichment was done by Fraser broth and subsequent spreading on PALCAM agar. Suspected colonies were confirmed by Gram staining, biochemical tests such as catalase test, methyl red, Voges-Proskauer (MR-VP) reaction, nitrate reduction and motility at 20–25 °C, acid production from rhamnose, xylose, mannitol, α-methyl-D-mannopyranoside and CAMP test. The positive isolates were confirmed by multiplex PCR assay, by which eight (8%) water samples were positive for *L. monocytogenes.*

Raorane *et al*. (2013) studied prevalence of *Listeria* spp. in animal and associated environment. A total 33 samples were collected from cattle and pig farms surroundings (soil and floor swabs) were screened to determine the

prevalence of *Listeria* spp. in Konkan region, India. Of these none were found to be positive. Isolation of *Listeria* spp. was attempted as per USDA–FSIS method (USDA, 2013).

Shantha and Shubha (2014) investigated the occurrence of *Listeria* species in environmental from Mysore city (moderately hot, semi–arid climate). Environmental samples included cow dung from cowshed, grazing pasture and soil samples from vegetable–cultivation land. Cold enrichment was used to recover *Listeria* species from cow dung (collected from individual animals) (n=130) and soil (n=100) samples. About 10 gm sample in case of cowdung and soil each were transferred to 90 ml BHI broth and incubated at 4°C for two weeks. Aliquots from the enriched broth were streaked on Oxford and PALCAM plates, at weekly intervals. *Listeria* isolates were subjected to phenotypic and genotypic characterization. Phenotypic characterization included standard biochemical tests such as catalase test, motility at 25 °C and 37 °C, acid production from the canonical panel of carbohydrates, nitrate reduction, aesculin hydrolysis, methyl red and Voges Proskauer reaction. Genus and species–specific primers were used for PCR differentiation of the isolates. *L. ivanovii* was isolated from 1% of the soil samples and *L. seeligeri* from 0.76% of the cowdung samples samples tested.

Sunitha *et al*. (2016) examined a total of 400 samples which included, 50 soil, 50 dung, 50 fodder, 50 water, 50 handwash and 50 udder wash samples collected from dairy farms of three different panchayaths namely Thariyode, Porhuthana and Kalpetta of Wayanad district, Kerala. Out of 400 samples, *L. monocytogenes* was isolated from three (6%) dung, three (6%) udder wash, two (4%) milker's hand wash, five (10%) fodder, seven (14%) soil and two (4%) water samples. A total of 22 (5.50%) samples were positive for *L. monocytogenes*.

Kulesh (2017) carried out study to access the prevalence of *Listeria monocytogenes* in ruminants, vectors and environment of organized farms. One soil sample was positive for *L. monocytogenes* indicating 5.88 per cent prevalence and none water sample was positive.

Tahir *et al*. (2022) studied the occurrence of *L. monocytogenes* in soil samples of Punjab province. The power soil DNA isolation Kit (MOBIO, West Carlsbad, CA, USA) was used for extraction of genomic DNA from the soil samples according to the manufacturer's protocol. Out of 970 soil samples the genome was detected in 17 samples by real time PCR indicating the prevalence of 1.7 per cent. The incidence was reported more at places near to water resources and having more than 1000 animals per village.

2.4 Isolation of *Listeria* spp.

Presumably, *Listeria* spp. is known on the basis of morphology (Gram-positive coccobacilli), biochemical tests i.e. catalase and oxidase tests, Haemolysis on 5% sheep blood agar, and char*act*eristic tumbling motility under a microscope. They can produce on a wide variety of non-selective plating media e.g. blood agar, chocolate agar, tryptic soy agar, and brain heart infusion agar.The isolation of *Listeria* organisms from food and clinical specimens that are not usually sterile requires a pre-enrichment step because injured organisms are likely to be present, pre-enrichment is followed by selective enrichment and then selective plating (Curtis and Lee, 1995).

In order to improve its isolation, Gray *et al*. (1948) observed the psychrophilic characteristic of *L. monocytogenes* and utilized cold enrichment (4°C) in a non-selective broth medium. This technique takes several weeks for separation and by that time other psychrophilic bacteria present overgrow, because of this disadvantage of this method, other methods of enrichment were developed.

Schuchat *et al*. (1991) utilized chemicals as selective enrichment media for *L. monocytogenes* which comprised acriflavine, glycine anhydride, lithium chloride, nalidixic acid, nitrofurazone, potassium tellurite, and potassium thiocyanate. The first selective plating agar for *L. monocytogenes* that produced recognizable light blue colonies was created by McBride and Girard in 1960.

Dominguez Rodriguez *et al.* (1984) defined a medium named DRIA (Dominguez–Rodriguez isolation agar) containing acriflavine, aesculin, and nalidixic acid for the selective as well as differential isolation of the *Listeria* spp. from severely contaminated samples. DRIA was chiefly used for the isolation of *L. monocytogenes* from meats.

Lee and McClain (1986) advanced an improved selective medium that contained moxalactam, lithium chloride, and phenyl ethanol, which enhanced the recovery of the pathogen from mixed cultures over the recovery on modified McBride Agar. Donnelly and Baigent (1986) modified one medium i.e. UVM (University of Vermont Medium) *Listeria* enrichment broth containing nalidixic acid (inhibits gram-negative b*act*eria) and Acriflavin hydrochloride (inhibits many gram-positive b*act*eria).

Van Netten *et al*. (1989) advanced a Polymixin Acriflavin lithium chloride ceftazidime aesculin mannitol (PALCAM) agar which contained Columbia agar as the base along with Acriflavin, lithium chloride, and antibiotics such as Polymixin B and ceftazidime. It was a double differentiating media with aesculin, ferrous ammonium citrate, mannitol, and phenol red. *Listeria* colonies appeared grey-green, approx. 2 mm in diameter, and have black sunken centers on PALCAM agar. They also had a black halo in contrast to a cherry red as the background. Aesculin and ferrous iron act as an indicator in both the agars that gives black colour to colonies.

Another selective medium, Oxford agar developed by Curtis *et al.* (1989) contains Acriflavin, lithium chloride, and antibiotics such as cefotetan, colistin, and fosfomycin, on which *L. monocytogenes* colonies seem black surrounded by a black halo. Further useful selective enrichment broths like University of Vermont-I (UVM I), University of Vermont-II (UVM II), Fraser broth (Fraser and Sperber 1988), Polymixin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol Egg Yolk (L- PALCAMY) and other selective media like modification of Vogel Johnson agar (MVJ) and a modified Oxford agar (McClain and Lee 1988).

The US Food and Drug Administration (FDA), the US Department of Agriculture (USDA), and the Food Sanitation Inspection Service (FSIS) are the two types of enrichment procedures that are most commonly employed in the USA (Lee and McClain 1988). They executed FDA and FSIS methods for dairy products and meat and poultry products, respectively wherein UVM broth was used for enrichment. Afterward, when food was contaminated with other types of microorganisms, the USDA modified a procedure for *Listeria* isolation. They used primary selective enrichment in UVM, followed by secondary enrichment in Fraser broth and selective plating on modified Oxford agar and Lithium Phenyl ethanol Moxalactam (LPM) agar. Cassiday and Brackett (1989) made the evaluation between three media viz. Lithium chloride phenyl ethanol moxal*act*am agar (LPMA), Agricultural Research Service-modified McBride agar (ARS-MMLA), modified Vogel Johnson agar with gum base-nalidixic acid tryptone soya medium for separation of *L. monocytogenes* from various foods like pasteurized whole milk, chocolate ice cream mix, brie cheese, and raw cabbage. They discovered, LPMA was the most suitable medium for examining brie cheese and raw cabbage, while gum base-nalidixic acid-tryptone-soya medium was appropriate for milk and chocolate ice cream mixture.

Al-Zorecky and Sandinew (1990) created the Al-Zoreky-Sandine *Listeria* Medium (ASLM), which contains aesculin as an indicator as well as ceftazidime, moxal*act*am, and Acriflavin as selective agents. The colonies seemed dark green with a black sunken center bounded by a black zone of aesculin hydrolysis. When they compared ASLM media with the *Listeria* Selective Agar (LSA), they found both were equally effective for the isolation of *Listeria* from food. ASLM media prevents the growth of gram-negative, micrococcus, and enterococcus, especially those strains that imitated *L. monocytogenes* on LSA (Farber and Peterkin, 1991).

For the purpose of isolating *Listeria* spp. Domjan and Ralovich (1990) compared six media, namely LPM agar, PALCAM agar, AC agar, oxford agar, trypaflavine nalidixic acid serum agar (TNSA), and forray's agar. They came to the conclusion that, when compared to other selective mediums, Oxford agar

produced the greatest results, while PALCAM was shown to be somewhat ineffective for the isolation of *Listeria* spp.

In order to isolate *L. monocytogenes* from 391 different meals, Art and Andre (1991) studied three selective isolation media: blood agar with nalidixic acid, PALCAM agar, and *Listeria* selective oxford medium (Oxford agar). The PALCAM and Oxford agar yielded the best recovery rates (8.43% each), whereas nalidixic acid agar yielded 8.18%.

Hayes *et al*. (1992) equated three selective enrichment methods for *L. monocytogenes* isolation from naturally contaminated foods in five different areas of the United States. A total 2, of 229 food samples were taken from the refrigerators of listeriosis patients. They compared U. S. Food and Drug Administration (FDA), U. S. Department of Agriculture (USDA), and Netherlands Government Food Inspection Service (NGFIS) methods and found both USDA and NGFIS methods were more sensitive with 74% sensitivity than FDA (65%). The isolation of *L. monocytogenes* was enhanced by means of a combination of two methods together and detected the highest isolation (91%) in the USDA-NGFIS combination subsequently 88% in USDA-FDA and 87% in NGFIS-FDA methods.

The effectiveness of PALCAM and Oxford agars for the isolation of *L. monocytogenes* from a variety of foods, including meat, were compared in a collaborative trial by Scotter *et al*. (2001), which involved 19 laboratories from 14 different countries. It was found that there was no appreciable difference in the effectiveness of these two media.

Ikeh *et al*. (2010) used UVM I and UVM II for enrichment for 24 and 48 hrs respectively for isolation of *Listeria* from food samples, after that selective plating on PALCAM agar plates. They observed grey-green colonies with a black background which were char*act*eristic of *Listeria* spp.

Park *et al*. (2014) described one new media i.e. Lecithin and levofloxacin (LL) medium for the isolation of *L. monocytogenes* from unspiked food samples.

The comparison was made between modified Oxford agar and two chromogenic media namely, Brilliance *Listeria* agar and CHROM agar *Listeria*. They found the specificity of LL medium was superior (96.0%) as compared to MOX (72.0%), whereas, specificities of Brilliance *Listeria* agar (96.5%) and Chromogenic *Listeria* Agar (CHROM agar *Listeria*) (94.5%) was similar. From 50 spiked food samples, LL medium and CHROM agar *Listeria* represented the highest detection sensitivities (96.0%), followed by Brilliance *Listeria* agar (92.0%) and MOX (54.0%). Additionally, the LL medium showed the highest confirmation rate (98.8%), followed by Brilliance *Listeria* agar (98.7%), CHROM agar *Listeria* (98.3%), and MOX (52.0%).

Soni *et al.* (2014) used Fraser both (Half and Full Fraser broth) followed by selective plating on PALCAM agar as selective agar, for isolation of *Listeria* spp from vegetable and soil samples.

2.5 Identification of *Listeria* spp. by Conventional methods

The conventional methods for identification of *Listeria* spp. are based on the result of biochemical tests and haemolytic reactions. Genus *Listeria*, aerobic to facultatively anaerobic, non-spore former, exhibits char*act*eristic tumbling motility at 20-25°C. All the *Listeria* spp. are catalase, MR (Methyl red), and VP (Voges- Proskauer's) test positive, while they are indole and oxidase negative. Moreover, they do not produce H_2S gas, hydrolyze urea, reduce nitrate, and do not liquefy gelatin.

Cruikshank *et al*. (1975) described various for *Listeria* species biochemical re*act*ions viz. catalase, oxidase, MR-VP, nitrate reduction and sugar fermentation tests.

Seeliger (1981) observed different expressions of hemolytic pathogenic species like *L. monocytogenes* and *L. ivanovii*, and non-hemolytic, non-pathogenic species (*L. innocua* and *L. welshimeri*). A positive CAMP re*act*ion, fermentation of rhamnose, and non-fermentation of xylose were the three in vitro

tests used to identify pathogenic *Listeria* spp. However, *L. seeligeri* was one species that was haemolytic but non-pathogenic.

All the *Listeria* spp. are differentiated on the basis of haemolytic properties on sheep blood agar, acid production from sugars (glucose, mannitol, xylose, and rhamnose) as well as CAMP (Christie, Atkins, and Munch-Peterson) test with *Rhodococcus equi* and *Staphylococcus aureus* (Farber and Peterkin 1991).

McLauchlin (1997) investigated a number of techniques for identifying *Listeria* species, such as conventional sugar fermentation, haemolytic re*act*ion, hydrolysis of the DL-alanine β-naphthylamide (DLABN), and the API *Listeria* identification test kit. He used conservative methods for the identification of *Listeria* spp. 99% of the isolates were correctly identified. The DLABN hydrolysis differentiated 98% of *L. monocytogenes* from another genus, while 97% of the culture identification was possible using the API *Listeria* test kit.

Variability in sugar fermentation was documented in studies conducted by Sakhare (2014), Khawase (2015), Dhote (2016), and Kulesh (2017).

2.6 *In vitro* pathogenicity tests

Hof and Rocourt (1992) stated that *Listeria* is heterogenous b*act*eria regarding its virulence, which is supposed to be a multif*act*orial phenomenon. Many in-vivo (mouse lethality, chick embryo lethality) and in-vitro [hemolysin on sheep blood agar, CAMP, Phosphotidylinositol-specific phospholipase C assay (PI-PLC)] have been performed by various researchers. The haemolytic activity of the former has made it possible to distinguish between haemolytic and pathogenic species (*L. monocytogenes* and *L. ivanovii*) and non-haemolytic and non-pathogenic species (*L. innocua* and *L. welshimeri*). Positive haemolysis demonstration of virulent *Listeria* can be done by Christie Atkins Munch Petersen (CAMP) test.

2.6.1 Haemolysis on sheep blood agar (SBA)

The test has been exploited for detecting the pathogenic strains of *L. monocytogenes* by many researchers (Cruischank *et al*, 1975; Barbuddhe *et al*., 2000 and Rawool *et al*., 2007).

Chaudhari *et al*. (2004) reported 2.4% *L. monocytogenes* from buffalo beef samples and all were reported pathogenic with characteristic haemolysis on SBA.

Kalorey *et al*. (2006) screened 50 faecal samples from different animals and confirmed eight (16%) as *L. monocytogenes* demonstrating hemolytic *a*ctivity on 5% sheep blood agar

Yadav *et al*. (2010) conducted a phenotypic char*act*erization of three *Listeria* isolates. These isolates were tested for the type (α, β) and degree (narrow or wider) of haemolysis on 7% sheep blood agar (SBA). A narrow zone of αhaemolysis was the char*act*eristic of *L. monocytogenes.*

The researchers viz; Bharate *et al*. (2012), Shelke *et al*. (2012), Suryawanshi (2012) and Dudhe *et al*. (2012) tested isolates of *Listeria* spp. obtained from different sources (foods of animal origin) in and around Nagpur city for production of haemolysin by streaking onto 7% sheep blood agar. And reported that all isolates showed haemolysis on sheep blood agar.

Further Sakhre (2014), Khawse (2015), Dhote (2016), Kulesh (2017) also reported haemolytic activity of *L. monocytogenes* isolates recovered from clinical samples of sheep goat cattle and meat from goat and pig respectively, from Nagpur region.

Vaidya *et al*. (2018) analyzed the haemolysin production of 17 isolates of *L. monocytogenes* on 7% sheep blood agar. All 17 isolates of *L. monocytogenes* were haemolytic and were considered pathogenic. They informed strong haemolytic activity for six isolates from the pig (4 pork and 2 faecal), moderate haemolytic activity by six other isolates of faecal origin (2 goats and 4 porcine),

and weak hemolytic zone by the remaining five goat isolates (3 chevon and 2 faeces).

2.6.2 Christie, Atkins, Munch-Petersen (CAMP) test

McKellar (1994) and Vazquez-Boland, *et al* (1989) discussed the factors related to the combined breakdown of red blood cells (RBC) and the use of the CAMP test to identify *L. monocytogenes* and *L. ivanovii* isolates. In case of *L. monocytogenes*, a 58-KDa protein called LLO, which is secreted by the bacteria, causes strong hemolysis when combined with *Staphylococcus aureus* culture but not with *Rhodococcus equi*. On the other hand, *L. ivanovii* secretes a 24-27 kDa protein called Sphingomylinase C, which causes strong hemolysis when combined with *R. equi* but not with *S. aureus*.

Vaz-VeIho *et al*. (2000), Chaudhari *et al*. (2004) and Rawool *et al*. (2007) exploited CAMP test for confirmation, identification and pathogenicity studies of *Listeria* spp. They reported CAMP positive *L. monocytogenes* isolates and accordingly designated as pathogenic.

Yadav *et al*. (2010) carried out pheno-genotypic characterization of *L. monocytogenes*. A total of three isolates of *L. monocytogenes* were recovered from 85 mastitic milk samples (47 buffalos and 38 cows) which on testing for CAMP gave positive results.

Suryawanshi (2012) studied five isolates of *Listeria* by subjecting to CAMP test. Two of them were designated as pathogenic *L. monocytogenes.*

Thomas *et al*. (2013) identified 18 field isolates obtained from cattle as *L. monocytogenes* by CAMP test. The isolates were designated as pathogenic as they showed char*act*eristic enhanced haemolytic zone with *S. aureus* on 5% sheep blood agar.

Raorane *et al*. (2014) subjected all biochemically identified *L. monocytogenes* and *L. ivanovii* spp. isolated from pigs, sheeps and goat samples

to CAMP test. *L. monocytogenes* showed increased zone of hemolysis toward *Staphylococcus aureus* while *L. ivanovii* towards *Rhodococcus equi.*

Further Sakhre (2014), Khawse (2015), Dhote (2016), Kulesh (2017) from department of Veterinary Public Health confirmed pathogenic *L. monocytogenes* isolates recovered from clinical samples of sheep goat cattle and meat from goat and pig respectively by employing CAMP test.

Vaidya *et al*. (2018) observed all 19 isolates showing enhanced haemolytic zone with *S. aureus*. These isolates showing strong haemolysis were interpreted as CAMP-positive pathogenic *L. monocytogenes*.

2.6.3 Phosphatidylinositol-specific phospholipase C (PI-PLC) Assay:

Notermans *et al*. (1991) studied phosphatidylinositol-specific phospholipase C (PI- PLC) based assay as reliable indicator to distinguish pathogenic and non-pathogenic *Listeria* species. They stated *L. monocytogenes* as positive for the *act*ivity.

Paziak-Domaneska *et al*. (1999) isolated 46 *Listerial* strains from meat and sausage and confirmed the pathogenicity on the basis of production of phosphatidylinositol-specific phospholipase C (PI-PLC). The workers reported all *L. monocytogenes* (41.30%) were PI-PLC positive.

Pimenta *et al*. (1999) studied a total of 30 strains of *L. monocytogenes* isolated from different food samples for production of phosphatidylinositol-specific phospholipase C (PI-*PLC*) enzyme. They reported PI-*PLC* production by 27 (90%) of the strains analyzed.

Kalorey *et al*. (2006) and Aurora *et al*. (2008) confirmed 105 and 18 *L. monocytogenes* isolates obtained from bovine raw milk samples based on PI*PLC* producing ability of the isolates.

Yadav et al. (2010) carried out PI-PLC for 3 *L. monocytogenes* isolated from milk samples and reported all positive.

Suryawanshi (2012), Bharate *et al*. (2012), Dudhe *et al*. (2012) and Shelke *et al*. (2012) streaked *Listeria* spp. recovered from goats, buffaloes and male cattle slaughtered in and around Nagpur city on L. mono differential agar and revealed typical blue coloured colonies after inoculation at 37℃ for 24 hours and designated them as PI-PLC producers.

Thomas *et al*. (2013) and Raorane *et al* (2013) conducted PI-PLC assay for pathogenicity detection of 18 and 11 isolates of *L. monocytogenes* obtained from cattle slaughtered in Nagpur region and from pigs, sheeps and goat samples respectively. The authors recorded all isolates positive for PI-*PLC* test and accordingly the isolates were designated as pathogenic.

Khawse (2015), Dhote (2016) and Kulesh (2017) confirmed PI-PLC producing pathogenic *L. monocytogenes* recovered from samples collected from clinical cases of sheep and goat and cattle respectively in and around Nagpur city.

Vaidya *et al*. (2018) screened 17 isolates of *L. monocytogenes* for pathogenicity by PI-PLC test employing L. momo differential agar. The study reported all isolates positive for PI-PLC test.

2.7 Molecular characterization of the *Listeria* isolates

To investigate the novel specific genes of *Listeria monocytogenes*, six genes related with virulence factor of *L. monocytogenes* were selected. These genes could be used as target genes for the detection of *L. monocytogenes*. *Listeria monocytogenes* causes severe infections in humans; therefore, interest in developing fast, economical and specific tests for the detection of *L. monocytogenes* has been increasing. However, one of the major problems involved in *L. monocytogenes* detection is the inability to distinguish *L. monocytogenes* from other non-pathogenic *Listeria* species, particularly *L. innocua* which may predominate in samples. Conventional methods of detection involve multiple selective enrichment steps which are time-consuming and generally require more than two days. The polymerase chain reaction (PCR) is specific, reliable and helps in rapid detection.

2.7.1 Characterization of genus and species specific genes by PCR

Dhanashree *et al*. (2003) performed PCR after confirming *Listeria* isolates by biochemical tests and used genus and species specific primers for *L. monocytogenes* avoiding confusion with another *Listeria* spp. Rawool *et al*. (2007) extracted DNA by snap chill method.

Rawool *et al*. (2016) confirmed as *Listeria monocytogenes* by using the PCR technique with *prs* (genus-specific) and *isp* (species-specific) with a product size of 844 bp and 713 bp, respectively.

Suryawanshi *et al*. (2023). In his study validated all three biochemically positive *Listeria* isolates using multiplex PCR, in which all three turned out positive for both the genes; *prs* 844 bp and *isp* 713 bp, endorsing their identification as *Listeria monocytogenes*.

2.7.2 Molecular detection of the virulence marker genes of *Listeria* species by PCR

Detection of multiple virulence associated genes in combination with in vitro pathogenicity tests may be required for confirming the pathogenic potential of *Listeria*. Use of molecular techniques employing specific primers have been widely used for differentiation and speciation as well as determination of pathogenic strains. The primers *iap*, *hly*, *prfA*, *actA* are being employed for study of virulence strains. Listeriolysin O is a major virulence f*act*or of *L. monocytogenes* which is encoded by *hlyA*.

Okwumabua *et al*. (2005) screened 21 isolates of *L. monocytogenes* from food and animal clinical cases. Serotyping results showed five of the isolates were of serotype 1/2a, six of 1/2b, nine of 4b and one was untypeable.

Kaur *et al*. (2007) standardized multiplex PCR for detection of virulence genes of *L. monocytogenes* targeting *hly*A, *prf*A, *plc*A, *act*A and *iap* gene. The study revealed presence of all five virulence-associated genes in two of the *L. monocytogenes* isolates recovered from placental tissue and urine whereas; the

three isolate was found to possess *hly*A, *act*A and *iap* genes; however, one isolate did not reveal any virulence-associated genes and hence confirmed as an atypical isolate.

Rawool *et al*. (2007) studied multiplex PCR to identify the virulence genes *plcA, hlyA, actA* and *iap* genes for confirmation of *L. monocytogenes* in milk. All the genes were detected in the isolates.

Yadav *et al*. (2010) screened three isolates of *L. monocytogenes* from 85 mastitic milk samples for genotypic char*act*erization by targeting five genes namely; *plc*A, *prf*A, *act*A, *hly*A and *iap*. It is reported that these five virulence associated genes detected in all three isolates.

Sunil *et al.* (2012) described that out of the 18 isolates of *Listeria* spp. obtained from fish, leafy vegetables and soil samples, all the isolates were positive for virulence genes *iap, hlyA, actA, prfA, plcA* and *inlA* and showed amplificationat 131, 456, 839, 1060, 1484 and 820 bp.

Negi *et al*. (2015) reported a diverse and a varied profile on screening of 36 *L. monocytogenes* isolates processed to determine presence of virulence associated genes viz; *prf*A, *plc*A, *hly, act*A by multiplex PCR. The study shows presence of all four genes in 22 *L. monocytogenes* isolates (human origin—6, animal origin—10, from food— 6), while the remaining 14 isolates failed to amplify the *prf*A and/or the *act*A or both the genes. The study evidenced that experimental data collected over the years had the strains of *L. monocytogenes* varied in virulence and pathogenicity.

Raorane *et al.* (2015) subjected 11 *L. monocytogenes* isolates for amplification of *hly*, *plc*A and *act*A genes to confirm pathogenic strains. The study confirms all isolates amplifying the virulence genes and was considered as pathogenic strains of *L. monocytogenes*.

Dhote (2016) confirmed five pathogenic *L. monocytogenes* isolates recovered from clinical cases of sheep and goats of Nagpur region after amplifying virulence associated genes viz; *hly*A, *plc*A, *act* A and *iap* gene.

Jagtap *et al*. (2017) detected virulence-associated genes of *L. monocytogenes* using multiplex PCR assay by targeting *plc*A, *hly*A, *act*A and *iap* genes. The study reports presence of all these genes in all four *L. monocytogenes* strains isolated from peridomestic birds and captive wild animals confirming their pathogenic nature.

Kulesh (2017) screened 15 isolates of *L. monocytogenes* for presence of *hly*A, *plc*A, *act*A and *iap* gene with product size of 456 bp, 1484 bp, 839 bp and 131 bp respectively. Of these, thirteen isolates of *L. monocytogenes* were positive for all four genes targeted in the study. Whereas, one each isolate of soil and tick sample showed positivity for *hly*A, *plc*A and *iap* except *act* A thus confirming all isolates as pathogenic in nature.

2.8 Serotyping of *Listeria monocytogenes* by PCR

The genus *Listeria* is composed of 17 species of which *Listeria monocytogenes* is considered the single pathogenic species that causes listeriosis in humans. Of the 13 serovars of *L. monocytogenes*, 1/2a, 1/2b and 4b are responsible for the majority of clinical cases.

Doumith *et al.* (2004) first time developed a new multiplex PCR assay to separate the four major *Listeria monocytogenes* serovars (1/2a, 1/2b, 1/2c, and 4b) isolated from food and human patients into distinct groups. The PCR was successful in separation of 222 strains of *L. monocytogenes* into serovars 1/2a and 3a; 1/2cand 3c; 1/2b, 3b, and 4b, 4d, and 4e. The author also reported that amplifications of the four chosen serovar-specific fragments allowed separation of *L. monocytogenes* strains into four groups. Group 1 comprised strains of serovars 1/2a and 3a (amplification of only the *lmo0737* DNA fragment); group 2 comprised strains of serovars 1/2c and 3c (amplification of both *lmo0737* and *lmo1118* DNA fragments); group 3 comprised strains of serovars 1/2b, 3b, and 7 (amplification of only an *ORF2819* DNA fragment); and group 4 comprised strains of serovars 4b, 4d, and 4e (amplification of both *ORF2819* and *ORF2110* DNA fragments).

Okwumabua *et al.* (2005) screened a total of 21 isolates of *L. monocytogenes* originating from both food sources and clinical cases in animals and their serotyping analysis indicated that five of the isolates were classified as serotype 1/2a, six as 1/2b, nine as 4b, and one isolate could not be assigned a serotype.

The genotypic analysis of 17 *L. monocytogenes* isolates recovered from humans using multiplex serotyping PCR and allowing serovar predictions by conventional serology was carried by Kalekar *et al.* (2015). A multiplex-PCR based serotyping assay revealed 88.24% (15/17) of the strains belonging to the serovar group 4b, 4d, 4e and 11.76% (2/17) to the serovar group 1/2b, 3b. Conventional serology indicated that 13 (76.47%) *L. monocytogenes* isolates to be of serotype 4b, 2 (11.76%) serotype 4d and 2 (11.76%) serotype 1/2b. In addition, the author‘s concludes that predominance of *L. monocytogenes* serotype 4b is of concern, as this serotype has been most frequently associated with human listeriosis outbreaks.

Wang *et al*. (2015) conducted a study on 628 samples, comprising ready-to-eat (RTE) products sourced from both supermarkets and open-air markets in Nanjing, China, to investigate the prevalence of Listeria spp. The isolates obtained were subjected to analyses for serogroup, virulence genes, genotype, and antibiotic resistance. Their findings indicated that among the isolates, 45.5% belonged to serogroup 1/2a, 3a; 48.5% belonged to serogroup 1/2b, 3b, and 6.1% were classified into serogroup 1/2c, 3c

Terzi *et al*. (2015) confirmed four *Listeria monocytogenes* isolates from 100 ready to eat food products. The serotype distribution of *L. monocytogenes* isolates was determined by multiplex PCR. Among four *L. monocytogenes* isolates, two of them were identified as 4b (or4d, 4e) and the others as 1/2a (or 3a).

Barbuddhe *et al*. (2016) stated analysis report of identification of 60.35% 4b (239/396), 27.77% 1/2a (110/396), and 11.86% 1/2b (47/396) serotypes of *L. monocytogenes* collected during their investigation in a period of the year 2000-

2014 across India, wherein highest recovery of 4b serotype followed by 1/2a and 1/2b was observed.

Singh *et al*. (2016) who studied 29 *L. monocytogenes* isolates recovered from milk and meat products, vegetables and human clinical cases with bad obstetrics history at Nagpur, Maharashtra state and reported highest recovery of 1/2b and 4b serotypes.

Four *Listeria monocytogenes* isolates were serotyped using multiplex PCR by targeting *lmo0737*, *orf2819*, *orf2110*and *prs* gene by Jagtap *et al*. (2017). The study reveals four *L. monocytogenes* strains belonging to serogroup 4b.

Kulesh (2017) analysed 15 isolates of *L. monocytogenes* by serotype PCR. All isolates showed positivity towards *prs* gene which is specific for *Listeria* genus and 14 isolates of *L. monocytogenes* showed positivity towards *orf* 2819 and *orf* 2110, which indicate serovar specificity as 1/2b, 3b, 4b and 4b, 4d, 4e respectively, whereas; one isolate showed presence of *lmo* 0737 indicating 1/2a, 1/2c, 3a and 3c; 1/2b, 3b, and 4b, 4d, and 4e serotype.

MATERIAL AND METHODS

Listeriosis continues to be a notable economic threat in ruminants and other animals. Humans mainly contract the infection by consuming contaminated animal products, making infected ruminants a crucial source of the disease. Considering the ubiquitious nature and public health significance of the disease the present investigation was envisaged for the assessment of *Listeria monocytogenes* as probable contaminant of green leafy vegetables and it's associated environment. The prerequisites and techniques listed below were used.

3.1 Study area

The Nagpur district was selected as study area in current study. It is situated in Maharastra state in the geometric centre of the Indian subcontinent, exactly in the middle. It is referred to as the orange metropolis and is well known for its Nagpur Oranges. The Tiger capital of India is another name for it.

The Nag River is a river flowing through the city of Nagpur. Nagpur city derives its name from the Nag river. It forms a part of the Kanhan-Pench river system, originating from Ambazari Lake which is situated at upstream of Nagpur city. The length of Nag River around city boundary is 17 kms and this river basin is called as central zone. It is located at west part of city and it flows into Kanhan River and from there it is confluenced with Gosikhurd Project. Pili River, Pora River, Futala Nallah are its three tributaries. City of Nagpur has experienced very fast urbanization during the past 50 years. Exponential and rapid rate of growth has resulted Nag river in increased human interference and anthropogenic activites like discharge of untreated sewage and industrial effluents. However, there is a practice of cultivating the vegetables along the side of Nag river basin.

All samples in the current investigation were collected from local market, alongside Nag river basin and organic farms in and around Nagpur.

3.2 Place of work

The present investigation has been conducted in the Department of Veterinary Public Health & Epidemiology, Nagpur Veterinary College, Nagpur.

3.3 Glassware/ Plasticware/ Labware/ Media/ Chemicals/ Reagents/ Equipments/ Instruments/ Bacterial strains

3.3.1 Glassware/ Plastic ware/Labware

The glassware/ plasticware/ labware used in this study were obtained from Borosil Ltd (India), ABDOS (India) and Tarsons (India).

3.3.2 Media/Chemicals/ Reagents

Isolation, identification, and characterization of the *Listeria* spp. was carried out employing bacteriological media obtained from Hi-media Laboratories Pvt. Ltd. (Mumbai) and Sisco Research Laboratories Pvt Ltd. (SRL) (Mumbai). The oligonucleotides were obtained from IDT, Bengaluru. Mastermix, Nuclease free water and 100 bp ladder was procured from Promega (US). For gel electrophoresis reagents from Altrapure ™ (US) and Himedia (India) were used.

3.3.3 Equipments/ Instruments

Equipments and instruments used during the study were: Thermocyler (Applied Biosystems, United States), Deep freezers (-20°C) (Hoshizaki, Japan), Deep freezer (-80°C) (Eppendorf, Germany), Gel Documentation System (BIO-RAD, US), Gel Electrophoresis Apparatus (Cleaver Scientific Ltd. UK), Biosafety Cabinet Class 2 (Biobase, Germany), Vortex (Rivotek, India), Hot Water Bath, Cooling Centrifuge (Eppendorf, Germany), Spectrophotometer (Thermofisher Scientific, United States), Microscope (Carl Zeiss, Germany), Stomacher (Lab. Med. Ltd, England), Shaker incubator (Remi, Mumbai), Electronic weighing balance. Their usage is mentioned in the following text.

3.3.4 Bacterial strains

The standard strain of *Listeria monocytogenes* (MTCC 1143), *Staphylococcus aureus* (ATCC 12600) and *Rhodococcus equi* (ATCC 6939) were kindly spared by ICAR- National Meat Research Institute, Hyderabad. All the strains were stored in brain heart infusion (BHI) broth with 30% v/v glycerol at -20°C temperature. The cultures were periodically revived in BHI broth and agar for their usage in parameters.

3.4 Collection of samples

This study included environment and leafy vegetable samples including coriander (*Coriandrum sativum*)/ fenugreek (*Trigonella foenum-graecum)/* spinach (*Spinacia oleracea*)/ dill (*Anethum graveolens)/* amaranth (*Amaranthus*) collected from local market, cultivated alongside Nag river and at organic farm in and around Nagpur. Leafy vegetables and soil samples were also collected simultaneously in sterile zip lock bags. The water samples from these areas were collected in sterile bottles. The details of the samples are given in tabulated format in Table 3.1. All the samples were labelled properly including the type of sample, location, date, etc and immediately transported to the laboratory for cultural isolation and identification of *Listeria* spp. by using standard protocol (ISO 11290-1:2017).

Table 3.1 Details of samples collected

Source	Location	Sample	No of samples	Total
Local vegetable market	Kalamna market, Nagpur	Coriander	35	280
		Fenugreek	32	
		Spinach	28	
		Dill	21	
		Amaranth	26	
	Cotton market, Nagpur	Coriander	15	
		Fenugreek	14	
		Spinach	17	
		Dill	20	
		Amaranth	13	
	Gokulpeth market, Nagpur	Coriander	10	
		Fenugreek	2	
		Spinach	9	

		Dill	6	
		Amaranth	4	
	Hazripahad market, Nagpur	Coriander	4	
		Fenugreek	3	
		Spinach	4	
		Dill	2	
		Amaranth	3	
	Sakkardara Market, Nagpur.	Coriander	4	
		Fenugreek	1	
		Spinach	3	
		Dill	2	
		Amaranth	3	
Organic farm	Abhyuday rural mart, Vivekanand nagar, Nagpur	Coriander	1	58
		Fenugreek	3	
		Spinach	2	
		Dill	4	
		Amaranth	5	
	Organic Mandi, Gokulpeth, Nagpur	Coriander	1	
		Fenugreek	2	
		Spinach	3	
		Dill	4	
		Amaranth	3	
	Nagpur naturals, Gandhi nagar, Nagpur	Fenugreek	3	
		Spinach	1	
		Dill	2	
		Amaranth	3	
	Organic farm, Bhandara	Coriander	1	
		Fenugreek	1	
		Spinach	2	
		Amaranth	1	
	Arogya Kranti, Wardhaman Nagar	Coriander	1	
		Fenugreek	2	
		Dill	2	
	Green mart, Gondwana Square, Nagpur	Fenugreek	1	
		Spinach	1	
		Dill	2	
		Amaranth	1	
	Organic farm, Fetri,	Fenugreek	1	
		Spinach	1	

	Nagpur	Dill	1	
		Amaranth	3	
Nag river alongside	Shirpur, Kamptee	Coriander	1	37
		Fenugreek	2	
		Spinach	2	
		Dill	3	
		Amaranth	3	
	Sawangi, Kalmeshwar	Fenugreek	1	
		Spinach	2	
		Dill	2	
		Amaranth	2	
	Sonegaon, Kamptee	Coriander	1	
		Fenugreek	2	
		Spinach	1	
		Dill	3	
		Amaranth	2	
	Lihigaon, Kamptee	Coriander	1	
		Fenugreek	5	
		Spinach	0	
		Dill	1	
		Amaranth	3	
Environmental samples	Shirpur, Kamptee	Soil	4	50
		Water	4	
		Manure	1	
	Sawangi, Kalmeshwar	Soil	3	
		Water	3	
		Manure	1	
	Sonegaon, Kamptee	Soil	4	
		Water	4	
	Lihigaon, Kamptee	Soil	5	
		Water	5	
		Manure	1	
	Organic farm, Fetri, Nagpur	Soil	5	
		Water	4	
		Manure	2	
	Organic farm, Bhandara	Soil	4	
		Total	**425**	425

3.5 Isolation and Identification of *Listeria monocytogenes*

Samples collected aseptically were processed immediately after arriving at laboratory, for the isolation of *Listeria* species as per the protocol suggested by the ISO 11290-1:2017 guidelines with suitable modifications.

3.5.1 Processing of samples

Isolation of *Listeria* spp. from vegetables, water and soil was attempted as per the ISO 11290-1:2017 guidelines after making suitable modifications. Processing of samples was done in 3 steps as pre-enrichment, enrichment, and selective plating.

3.5.2 Isolation of *Listeria* spp. by ISO 11290-1:2017 method

A. Pre enrichment of sample

The solid sample of vegetables, soil, manure was homogenized or macerated by using stomacher (Lab. Med. Ltd, England). Approximately 5 gm of sample was taken and added with the 45 ml of Half Fraser Broth Base supplemented with Fraser selective supplement, For liquid sample (water), 5 ml of sample was added to the Half Fraser Broth Base and incubated at 30°C for 24 hrs.

B. Enrichment

After incubation 0.1 ml of pre-enriched sample was transferred to the Full Fraser Broth Base supplemented with Fraser selective supplement and further incubated at 37°C for 24hrs for enrichment.

C. Selective plating

A loopful of innoculum from Full Fraser Broth was streaked onto sterile Polymixin Acriflavin Lithium chloride Ceftazidime Aesculin (PALCAM) agar plates and incubated at 37°C for 24 to 36 hrs. The typical greyish green, glistening, iridescent and pointed colonies of about 0.5 mm diameter surrounded by a diffuse black zone of aesculin hydrolysis presumed as of *Listeria*. Selected

single colony was again streaked on another PALCAM plate to get purified colonies. Then typical colony was picked up and subjected to Gram staining, motility, biochemical characterization, *in-vitro* pathogenicity and molecular characterization as stated below.

3.6 Staining and motility characterization of isolates

The selected presumptive *Listeria* colonies were subjected to Gram‘s staining. The Gram positive isolates having coccobacillary appearance were further used for motility (Low and Donachie, 1997). The motility of bacteria was examined by hanging drop method. The identified single colony was inoculated in Brain Heart Infusion broth (BHI) and incubated at 25°C for 8-12 hrs. The typical tumbling motility of isolate was examined under 40x magnification.

3.7 Biochemical characterization and sugar fermentation tests

The isolates showing typical tumbling motility were further streaked on to BHI agar plates for further biochemical characterization viz. catalase, oxidase, MR-VP, nitrate reduction and sugar fermentation tests, as per Cruikshank *et al*. (1975), Javaid and Rashid (2018) and Agarwal *et al*. (2003). The chemicals and reagents used for biochemical tests are stated in Appendix-I.

3.7.1 Catalase test

The selected presumptive *Listeria* colony from Brain Heart Infusion Agar (BHA) was taken with a sterile tooth pick onto a clean glass slide and a drop of 3% hydrogen peroxide (H_2O_2) was added on it. Development of prompt effervescence was considered as catalase positive.

3.7.2 Oxidase test

A strip of filter paper was soaked in freshly prepared solution of tetramethyl-p-phenylene-diamine (Hi-Media, Mumbai). The isolate to be tested was rubbed on the paper with sterile tooth pick. Development of deep purple colour within 5-10 seconds was recorded as oxidase positive reaction, while no coloration even after 60 seconds was considered as oxidase negative.

3.7.3 Methyl Red and Voges Proskauer (MR-VP) tests

The presumptive test culture was grown in BHI broth at 37°C for eight-10 hrs. A loopful of fresh growth of this test culture was added into five ml sterile solution of glucose phosphate peptone water (Hi Media, Mumbai) (Appendix-I) and incubated at 37°C for 36-48 hrs.

For MR test, development of bright red colour after addition of drop of methyl red indicator was considered as positive.

For VP test, 0.2 ml of potassium hydroxide (40 per cent) and 0.6 ml of α naphthol solution (5 per cent) was added and tube was shaken vigorously. The tubes were shaken intermittently for proper aeration and kept for an hour at room temperature. Development of pink red colour was recorded as positive test.

3.7.4 Nitrate Reduction test

The presumptive test culture was grown in BHI broth at 37°C for 8-10 hrs and a loopful of fresh test culture was inoculated in five ml of sterile nitrate broth which was incubated further at 37°C for 48 hrs. After completion of incubation, 0.1ml each of 0.8% sulphanilic acid and 0.6% α-napthylamine (ratio 1:1) was added to the culture. Nitrate will reduce to nitrite and leads to development of pink red colour which was considered as positive.

Table 3.2 Standard Biochemical characterization of *Listeria* spp.

Sr. No.	Test	*Listeria monocytogenes*	*Listeria ivanovii*	*Listeria innocua*	*Listeria seeligeri*
1.	Catalase	+	+	+	+
2.	Oxidase	-	-	-	-
3.	Methyl Red	+	+	+	+
4.	Voges-Proskuer	+	+	+	+
5.	Nitrate Reduction	-	-	-	-

(OIE terrestrial manual, 2021)

3.7.5 Sugar fermentation pattern

Sterile tubes containing five ml peptone solution added with required sugars and 0.2% bromocresol purple indicator were prepared. The test isolates were inoculated into these sugars and incubated at 37°C for 36-48 hrs. Tubes revealing change in colour of broth from purple to yellow was considered as positive as a result of carbohydrate fermentation. However, variation in sugar fermentation if any was also recorded.

Table 3.3 Standard Differentiation of *Listeria* species based on sugar fermentation tests

Test	X	MM	R
L.monocytogenes	-	+	+
L. grayi	-	+	V
L. innocua	-	+	+
L. seeligeri	+	-	-
L. ivannovii Sub sp. Ivannovii	+	-	-
L. welshimeri	+	-	+

(OIE terrestrial manual, 2021)

(X; Xylose, MM; Alpha Methyl-D-Mannoside, R; Rhamnose, V; Variable)

3.8 *In-vitro* pathogenicity tests

The biochemically characterized, *Listeria* isolates were subjected to haemolysis test on sheep blood agar (SBA), Christie, Atkins, Munch-Peterson (CAMP) test, and Phosphatidylinositol-specific phospholipase C (PI-PLC) activity. The isolates were further processed for detection of *hlyA, actA, plc* and *iap* genes.

3.8.1 Haemolysis on sheep blood agar (SBA)

Freshly grown *Listeria* isolates were streaked onto seven per cent sheep blood agar plates (SBA) prepared by using defibrinated sheep blood as described by Cruickshank *et al*. (1975). The inoculated plates were then incubated at 37°C for 24 hrs and observed for the zone of haemolysis around colonies (OIE, 2014).

3.8.2 Christie, Atkins, Munch-Peterson (CAMP) test

Listeria isolates were exposed to the CAMP test according to the method defined by Yadav *et al.* (2010). Freshly grown isolates of *S. aureus* (ATCC 12600) and *R. equi* (ATCC 6939) were streaked on freshly prepared 7% SBA plates in parallel straight lines with a distance of 3-4cm from each other.

In between the parallel streaks of *S. aureus* and *R. equi* the *Listeria* isolates were streaked at 90°C angle and 3 mm apart. The plates were incubated at 37°C for 24 hrs. Later incubation, the plate with an enhanced fractional or complete zone of haemolysis was taken as a positive for the CAMP test.

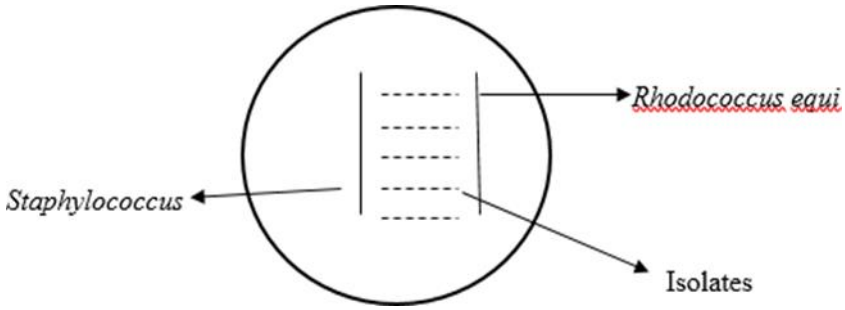

Fig. 3.1 Streaking for CAMP test on 7% sheep blood agar plate. (All dotted lines are horizontal streaking lines of suspected pathogenic strains of *Listeria* spp.*)*

Listeria isolates showing CAMP positive reaction against *S. aureus* were characterized as *L. monocytogenes*, while, those against *R. equi* as *L. ivanovii*. Typical haemolysis characters of *Listeria* spp. detected on the CAMP test are cited in Table 3.4

Table 3.4 Standard Haemolysis character of *Listeria* spp. on SBA and CAMP test

***Listeria* species**	**Haemolysison SBA**	**CAMP Test**	
		S. aureus	***R. equi***
L. monocytogenes	+	+	+/-
L. ivannovii	+	-	+
Remaining all non pathogenic *Listeria* spp.	-	-	-

(OIE terrestrial manual 2021)

3.8.3 Phosphatidylinositol–specific Phospholipase C (PI - PLC) assay

For PI-PLC reaction, all biochemically characterized *Listeria* isolates were screened as per the method standardized by Yadav *et al*. (2010). On seven per cent SBA plates *Listeria* isolates were grown overnight at 37°C. Further all haemolytic *Listeria* isolates were streaked on *L. mono.* Differential Agar (Hi Media Ltd, Mumbai, India) in order to measure PI-PLC activity. The inoculated plates were incubated at 37°C in a humidified chamber for 24 hrs. The isolates with light blue colonies were considered as positive for PI-PLC production

3.9 Polymerase Chain Reaction (PCR) based molecular detection of *Listeria* spp.

3.9.1 Extraction of genomic DNA by the snap chill method

About, 1.5 ml of freshly grown bacterial culture in BHI broth was taken in a 2 ml microcentrifuge tube and centrifuged at 15, 000 x g for 15 min. The supernatant was discarded and the pellet was washed twice in nuclease-free water by centrifuging at 15, 000 x g for 5 min. After washing, 200 µl of nuclease-free water was added to re-suspend the pallet and vertexed for 15 seconds. DNA was then extracted by the snap chill method. The re-suspended pellet was boiled in a water bath for 20 min and then cooled suddenly on ice for 15 min. The tube was centrifuged at 15,000x g for 5 min, the supernatant containing DNA was collected and the pellet was discarded. DNA aliquot was used instantly or stored at -20°C for future use (Rawool *et al*. 2007).

The quality and quantity were checked via quantifying DNA with help of a Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA) by checking of concentration by absorption of nucleic acids at 260 nm and 280 nm wavelength and the ratio of quantified DNA should not be less than 1.9 to use in PCR.

3.9.2 PCR based molecular detection of genus *Listeria* and species *L. monocytogenes*

Following the use of standard biochemical tests for the identification of *Listeria* spp. and sugar fermentation, additional confirmation using molecular

techniques, particularly PCR, was carried out. The PCR reaction for the detection of the *Listeria* genus targeting genus-specific gene, putative phosphoribosyl pyrophosphate synthetase (*prs*), and for the detection of *L. monocytogenes* species-specific gene (*isp*) was amplified.

The isolates showing characteristic biochemical properties were subjected to genus and species-specific multiplex PCR targeting *prs* (Genus specific) and *isp* (Species-specific) genes. The positive control used for PCR was *L. monocytogenes* 4b (MTCC 1143), one of the standard pathogenic strains (Rawool *et al*. 2016).

Table 3.5 Details of primers used for genus and species specific Multiplex PCR

Target gene	Primer sequence	Product size (bp)	Reference
prs	F= 5'AGCTGAAGAGATTCCGAAAGA 3' R= 5' TTCACCAAGAAGAGCTGCAA 3'	844	Rawool, *et al*. (2016)
isp	F = 5' TGCAGCGAATGCTCTTAGTG 3' R = 5' AGCCAAGCACGGCTACTTTA 3'	713	Rawool, *et al*. (2016)

Where, *prs*: gene used for identification of *Listeria* genus
isp: gene used for identification of *Listeria* species

The PCR reaction was performed in 25 µl of the reaction mixture for the detection of gene *prs* and *isp* genes. The conditions were adjusted for reaction as mentioned in Table 3.7. The details of components used for the PCR for *prs* and *isp* genes are given in Table 3.6. All the PCR reaction mixture was taken in 200 µl PCR tubes (Abdos, India) and amplification was performed in Thermocyler (Applied Biosystems, United States).

PCR tubes (200 µl) containing the reaction mixture were tapped thoroughly with a finger and then flash spun in a microcentrifuge to settle reactants at the bottom. The tubes were set onto the thermal cycler and the reaction was performed with a preheated lid adopting cycling conditions as illustrated in Table 3.6.

Table 3.6 Details of components used for species and genus-specific Multiplex PCR

Ingredients		Volume (in µl)
2X Master mix (Promega)		12.5
prs	Forward primer	0.75
	Reverse primer	0.75
isp	Forward primer	0.75
	Reverse primer	0.75
Nuclease free water		7.5
DNA Template		2
Total		**25 µl**

Table 3.7 Cycling conditions for genus and species specific Multiplex PCR :

Primers (Forward and reverse)	Cycling conditions				
	Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
prs; *isp*	95°C 5 min	95°C 30 sec	53°C 1 min	72°C 2 min	72°C 10 min
		Repeated for 40 Cycles			

3.9.3 PCR targeting virulence-associated genes of *Listeria monocytogenes*

Detection of *hlyA, actA, plc* and *iap* genes with 456 bp, 839 bp, 1484 bp, 131 bp respectively was done as per the protocol suggested by Rawool *et al*., (2007) The standard pathogenic strain of *L. monocytogenes* 4b (MTCC 1143) was used as the positive control for PCR. The details of primers of targeted genes, components used for the PCR for virulence-associated genes and the conditions that were adjusted for reaction are illustrated in Table 3.8, 3.9 & 3.10 respectively

Table 3.8 Details of primers of targeted genes for PCR targeting virulence-associated genes

Target gene	Primer sequence	Product size (bp)	Reference
*plc*A	**F** 5'CTGCTTGAGCGTTCATGTCTCATCCCCC3' **R** 5'CATGGGTTTCACTCTCCTTCTAC 3'	1484	Noterman *et al*.(1991)
*hly*A	**F** 5'GCAGTTGCAAGCGCTTGGAGTGAA 3' **R** 5'GCAACGTATCCTCCAGAGTGATCG 3'	456	Pazaik-Domanska*et al*. (1999)
*act*A	**F** 5'CGCCGCGGAAATTAAAAAAAGA 3' **R** 5'ACGAAGGAACCGGGCTGCTAG 3'	839	Suarez and Vazquez-Boland (2001)
iap	**F** 5'-ACA AGC TGC ACC TGT TGC AG-3' **R** 5'-TGA CAG CGT GTG TAG TAG CA-3'	131	Furrer *et al.* 1991

Table 3.9 Details of components used for virulence PCR

Ingredients		Volume (in µl)
2X Master mix (Promega)		12.5
plcA; hlyA; actA; iap	Forward primer	1
	Reverse primer	1
Nuclease free water		8.5
DNA Template		2
Total volume		**25**

Table 3.10 Cycling conditions for targeting virulence-associated genes PCR

Primers (Forward and reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
*hly*A; *plc*A; *act*A; *iap*	95°C 2 min	94°C 15 sec	60°C 30 sec	72°C 1 min 30 sec	72°C 10 min
		Repeated for 35 Cycles			

3.9.4 Multiplex PCR for the detection of Serotype of *L. monocytogenes*

Based on unique combination of somatic (O) and flagellar (H) antigen present in the strain, the strains of *Listeria monocytogenes* are allocated to 13 different serovars *viz*; 1/2a,1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d and 4e. The gene targeted for serotyping contains four DNA fragments *ORF2110, ORF2819, lmo1118, lmo0737.* The details of primers of targeted genes, components used for the PCR for serovars- specific genes and the conditions that were adjusted for reaction are mentioned in Table 3.11, Table 3.12 and Table 3.13, respectively.

Table 3.11 Details of primers of targeted genes for serotype PCR

Target Gene	Primer sequence	Product size (bp)	Serovar Specificity	Reference
lmo 0737	**F** 5'AGGGCTTCAAGGACTTACCC 3' **R** 5'ACGATTTCTGCTTGCCATTC 3'	691	*L. monocytogenes* serovars 1/2a, 1/2c, 3a and 3c	Doumith *et al*., (2004)
lmo 1118	**F** 5'AGGGGTCTTAAATCCTGGAA 3' **R** 5'CGGCTTGTTCGGCATACTTA 3'	906	*L. monocytogenes* serovars 1/2cand 3c	
ORF2819	**F** 5'AGCAAAATGCCAAAACTCGT3' **R** 5' CATCACTAAAGCCTCCCATTG3'	471	*L. monocytogenes* serovars 1/2b, 3b, 4b, 4d, and4e	
ORF2110	**F** 5'AGTGGACAATTGATTGGTGAA3' **R** 5'CATCCATCCCTTACTTTGGAC3'	597	*L. monocytogenes* serovars 4b, 4d,and 4e	

Table 3.12 Details of components used for *Listeria monocytogenes* serotype PCR

Ingredients		**Volume (in µl)**
2X Master mix (Promega)		12.5
Imo 0737	Forward primer	1
	Reverse primer	1
Imo1118	Forward primer	1
	Reverse primer	1
ORF2819	Forward primer	1
	Reverse primer	1
ORF2110	Forward primer	1
	Reverse primer	1
Nuclease free water		2.5
DNA Template		2
Total volume		**25**

Table 3.13 The cycling conditions for *Listeria monocytogenes* serotype PCR

Primers (Forward and reverse)	Cycling conditions				
	Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
*lmo*0737 *lmo*1118 ORF2819 ORF2110	95°C 5 min	94°C 30 sec	56°C 1 min	72°C 2 min	72°C 10 min
		Repeated for 40 Cycles			

3.10 Agarose gel electrophoresis

Horizontal gel electrophoresis was used to analyze the PCR product (Cleaver Scientific Ltd. UK).The gel casting tray was positioned on a flat platform, and the gel comb was placed across one end of the tray, 1mm above the gel casting tray's base. A 1.5% agarose gel was prepared by boiling molecular grade agarose (Ultrapure TM, USA) in 1X Tris Acetic acid-EDTA (TAE) buffer until it dissolves completely. The gel was allowed to cool to 45°C before adding ethidium bromide at a concentration of 0.5 g/ml. The mixture was then put onto the gel casting tray to solidify. The gel was transferred to an electrophoresis tank with the wells at the cathode end once it had solidified. The tank was then filled with 1X TAE buffer until the gel was entirely immersed and the wells are completely covered in the buffer. A 100 bp ladder (Promega, USA) was also loaded into one of the wells, along with 10µl of PCR products (samples), positive control and negative control. The mobility was seen by the migration of the dye fronts during electrophoresis at 80 V centimetre. The gel was taken out of the tank and visualized in the Gel documentation (BioRad, US) after electrophoresis was completed. The images were captured using software (Quantity one basic BioRad, US). Chemicals and reagents used for Agarose gel electrophoresis are elaborated in appendix II.

RESULTS AND DISCUSSION

India's varied climate guarantees access to an extensive variety of fresh vegetables and fruits. It holds the second position globally in vegetable and fruit production, following China. In the 2021-2022 period, India yielded 107.24 million metric tonnes of fruits and 204.84 million metric tonnes of vegetables (APEDA, 2022). Consequently, it is critical to regularly monitor the prevalence of foodborne pathogens, including *L. monocytogenes.* It can end up on vegetables during various stages like growing, harvesting, or handling. The issue is that people often eat these veggies raw. So, there's a significant chance of getting sick from *L. monocytogenes* when we consume them without cooking.

Considering the ubiquitous nature, *L. monocytogenes* can be recycled among humans, animals, vegetables, and faeces-contaminated soils. The capacity of this bacterium to modify its life cycle is intriguing. Although it is a saprophyte in the soil, it has the ability to enter human or animal cells and turn into a pathogenic life form. It possesses the ability to breach the placental and blood-brain barriers, intensifying the severity of the illness, unlike other foodborne pathogens primarily affecting the gastrointestinal tract. (Farber *et al*., 1991). In populations with weakened immune systems, the potential for a high fatality rate is a major concern.

In the present study, attempt has been made to detect potentially pathogenic *L. monocytogenes* among green leafy vegetables and its associated environment from in and around Nagpur region.

4.1 Isolation and Identification of *Listeria* spp.

For the isolation of *Listeria* spp. two step enrichment procedure as per ISO 11290-1:2017 followed by selective plating was followed. All the samples were enriched in Half Fraser broth at 30°C for 24 hours followed by Full Fraser broth at 37°C for 24 hours and then plated onto selective medium i.e. PALCAM agar as described by Soni *et al.* (2014). After 24 hrs incubation grayish green glistening colonies with diffuse zone of aesculin hydrolysis were picked up

and accordingly processed for morphological identification by gram staining and tumbling motility at 25℃. The isolates showing gram positive coccobacilli with tumbling motility were further processed for biochemical characterization for catalase-positive, oxidase-negative, MR and VP-positive and nitrate reduction-negative and sugar fermentation test. All the biochemically confirmed *Listeria* isolates were screened for *in vitro* pathogenicity tests including haemolysis on sheep blood agar, CAMP test, Phosphatidylinositol–specific Phospholipase C (PI - PLC) assay. For molecular confirmation of *Listeria monocytogenes* a multiplex PCR demonstrating amplification at 844 bp and 713 bp was done along with PCR for virulence gene profiling of *plcA, actA, hlyA, iap* followed by serotyping for *lmo1118, lmo0737, ORF2819,* and *ORF2110* genes.

4.2 Prevalence of *Listeria monocytogenes* in different samples

A cumulative sum of 425 samples consisting of 375 vegetables (75 each of spinach, fenugreek, dill, amaranth, coriander) and 50 associated environment (soil and water) were collected and processed for *Listeria* spp. isolation by using the ISO 11290-1:2017 protocol with suitable modifications. On microbiological screening, nine samples turned out to be *Listeria* spp. out of which four isolates (4/425) were *Listeria monocytogenes* depicting 0.94% total occurrence as illustrated in Table 4.1, Figure 4.1, 4.2. Amongst these, two isolates each were recovered from coriander and spinach.

Table 4.1 Overall prevalence of *Listeria monocytogenes*

Sr. No.	Sample	Total	Positive isolates	% Positivity
A) Local market samples				
1.	Coriander	68	2	2.94%
2.	Fenugreek	52	0	0%
3	Spinach	60	2	3.33%
4.	Dill	51	0	0%
5.	Amaranth	49	0	0%
Total		280	4	1.42%

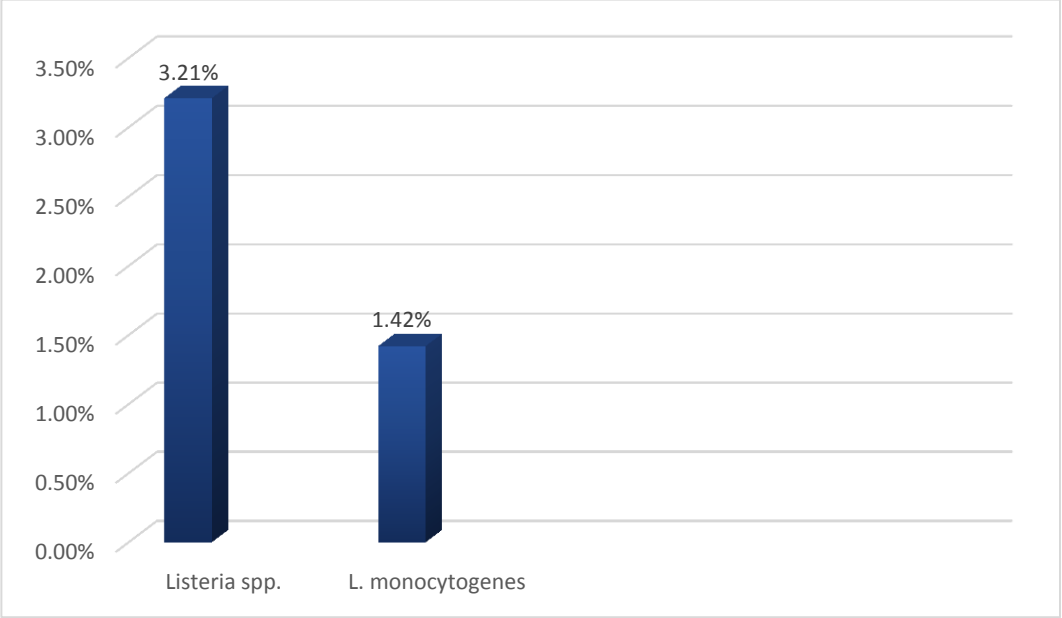

Fig 4.3 Prevalence of *Listeria* spp. and *L. monocytogenes* in local vegetable market

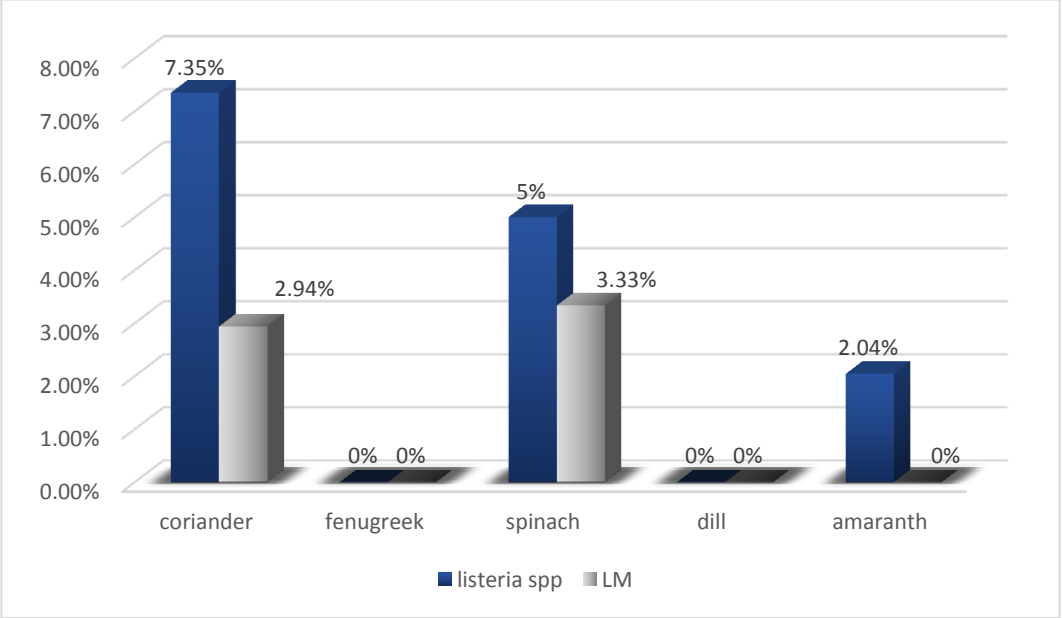

Figure 4.4 Sample wise prevalence of *Listeria* spp. and *L. monocytogenes* in local market vegetable

B) Organic farm samples				
1.	Coriander	4	0	0%
2.	Fenugreek	13	0	0%
3.	Spinach	10	0	0%
4.	Dill	15	0	0%
5.	Amaranth	16	0	0%
Total		58	0	0%
C) Nag river alongside samples				
1.	Coriander	3	0	0%
2.	Fenugreek	10	0	0%
3.	Spinach	5	0	0%
4.	Dill	9	0	0%
5.	Amaranth	10	0	0%
Total		37	0	0%
D) Environmental samples				
1.	Soil	25	0	0%
2.	Water	20	0	0%
3	Manure	5	0	0%
Total		50	0	0%
Grand Total		**425**	**4**	**0.94%**

4.2.1 Prevalence of *Listeria monocytogenes* in vegetables

4.2.1. a Local market vegetable

In the context of the present study, a comprehensive examination involved the collection and analysis of 280 vegetable samples collected from local markets in and around Nagpur to determine the presence of *L. monocytogenes*. Among the 280 samples including 68 coriander, 52 fenugreek, 60 spinach, 51 dill, and 49 amaranth samples, 59 displayed characteristic grayish-green colonies with diffuse black zone of aesculin hydrolysis when cultured on PALCAM agar (Plate 4.1).

After analyzing the 59 typical colonies, Gram staining was performed, revealing Gram-positive coccobacillary structures in 27 samples (with 12 from

coriander, 1 from fenugreek, 9 from spinach, 2 from dill, and 3 from amaranth). Among these, 9 samples (including 5 from coriander, 3 from spinach, and 1 from amaranth) exhibited tumbling motility at 25°C, prompting further exploration of their biochemical and sugar fermentation characteristics. Subsequent biochemical testing of these 9 isolates demonstrated that all were catalase-positive and oxidase-negative. Moreover, 5 coriander samples, 3 spinach samples, and 1 amaranth sample tested positive for MR-VP but negative for nitrate reduction. The isolates exhibited variable results in sugar fermentation tests. Earlier research conducted within the laboratory has also observed varied outcomes in sugar fermentation displayed by *Listeria monocytogenes*. This variability was documented in studies conducted by Sakhare (2014), Khawase (2015), Dhote (2016), and Kulesh (2017).

Table 4.2 Prevalence of *Listeria* spp. and *L. monocytogenes* in local market vegetable samples

Sr no.	Sample	No of sample	Prevalence of *Listeria* spp.	Prevalence of *L. monocytogenes*
1	Coriander	68	7.35% (5/68)	2.94% (2/68)
2	Fenugreek	52	0%	0%
3	Spinach	60	5% (3/60)	3.33% (2/60)
4	Dill	51	0%	0%
5	Amaranth	49	2.04% (1/49)	0%

Following the results of these biochemical tests and the sugar fermentation patterns, nine isolates were provisionally identified as *Listeria* spp. To confirm this identification, additional *in vitro* pathogenicity tests were conducted. Four out of the 9 isolates displayed an enhanced zone of hemolysis with *Staphylococcus aureus* in the CAMP test and also demonstrated hemolysis on sheep blood agar (SBA). Additionally, all four isolates exhibited light blue colonies on L.mono differential agar base, indicating a positive result for the phosphatidylinositol-specific phospholipase C (PI-PLC) assay.

Following isolation, these nine isolates underwent multiplex PCR targeting the genus specific *prs* and species specific *isp* genes. The subsequent

PCR analysis revealed the amplification of two distinct bands, measuring 844 bp and 713 bp, corresponding to the *prsA* and *isp* genes, respectively for four isolates (2 each from coriander and spinach). This outcome conclusively confirmed their classification as *Listeria monocytogenes*. Five isolates (C67, C54, S21, C59, A22) exhibited amplification at the 844 bp marker, thereby leading to their classification as belonging to the *Listeria* genus.

The overall prevalence of *L. monocytogenes* was identified as 1.42%, representing 4 positive: 2 each from coriander and spinach, out of the total 280 samples examined. The identification of *L. monocytogenes* was reported at a prevalence of 2.94% in coriander samples, 3.33% in spinach samples, and was absent (0%) in samples of amaranthus, dill, and fenugreek. The details are specified in the given Table 4.2, 4.3, Figure 4.3, 4.4.

The findings of our research align with those of Roth *et al*. (2018), who observed a 3.9% prevalence of *L. monocytogenes* in spinach and 2.6% prevalence in leafy greens. In a similar fashion, Hosein *et al.* (2008) conducted an analysis of 71 vegetables in Trinidad, revealing that only a single sample of lettuce was detected positive for *L. monocytogenes*, exhibiting a prevalence rate of 1.7%. In the United States, Lin *et al*. (1996) documented 1.58% prevalence (1 out of 63) in the salad samples. De Silva, *et al.* (2013) observed 2% incidence of contamination in vegetables and highlighted that leafy vegetables are particularly susceptible to soil contamination due to their larger surface area, facilitating bacterial colonization. Roth, *et al*. (2018) detected *L. monocytogenes* in 3.9% (2 of 52) and 2.6% (2 of 77) of farmers' market spinach and leafy greens, respectively. Sant' Ana *et al*. (2012) detected 3.1% prevalence and Mritunjay and Kumar (2017) detected 3.5%.

However, Satish (2015) found a lower prevalence of *L. monocytogenes*, specifically 0.277%, in their examination of 360 samples consisting of six varieties of raw vegetables in Kerala. Likewise, Ajaykumar (2014) highlighted that the presence of *Listeria monocytogenes* in cabbage (40) and leafy vegetables

Plate 4.1 Typical greenish-yellow, glistening, and pointed colonies surrounded by a diffuse black zone of aesculin hydrolysis on PALCAM agar

Table 4.3 Details of cultural, morphological, biochemical profiles of *Listeria* isolates from local vegetable market

Sample	No.	Morphological and biochemical test								Sugars			CAMP positive Against SA	Confirmed as
		AH +	GS	TM	Catalase +	Oxidase –	MR +	VP +	Nitrate –	L-Rhamnose	D-Xylose	α –methyl D mannioside		
Coriander	68	25	12	05	05	05	05	05	-	V	V	V	2	*Listeria monocytogenes*
Fenugreek	52	03	01	-	-	-	-	-	-	V	V	V	-	-
Spinach	60	19	09	03	03	03	03	03	-	V	V	V	2	*Listeria monocytogenes*
Dill	51	05	02	-	-	-	-	-	-	V	V	V	-	-
Amaranth	49	07	03	01	01	01	01	01	-	V	V	V	-	-

AH- Aesculin hydrolysis GS- Gram staining (Gram positive coccobacillary) V- Variable
TM- Tumbling motility at 25℃ SA- *Staphylococcus aureus* MR- Methyl red VP- Voges proskeuar

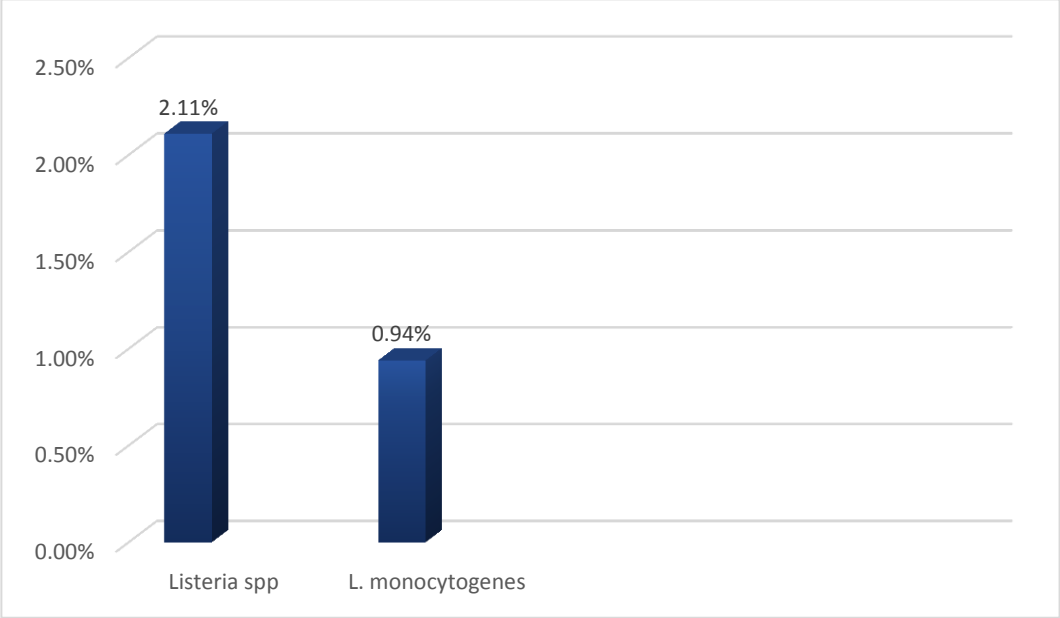

Fig 4.1 Overall prevalence of *Listeria* spp. and *L. monocytogenes*

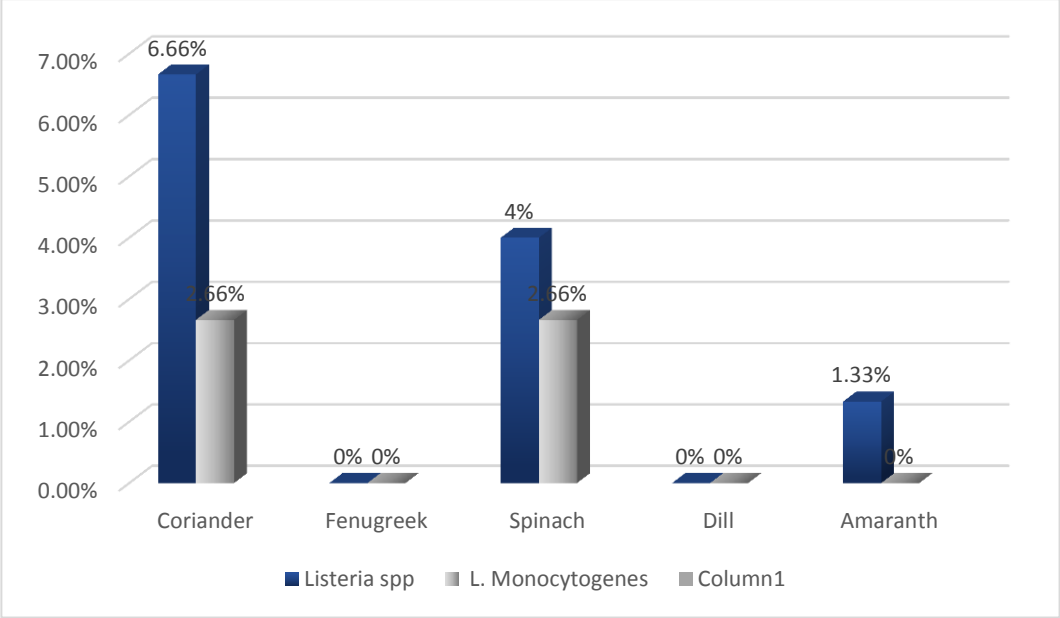

Figure 4.2 Overall sample wise prevalence of *Listeria* spp. and *L. monocytogenes*

(70) obtained from Thrissur was found to be 0.9%. *L. innocua* was detected in 4.5% of the samples. Also Fröder *et al.* (2007) reported *L. monocytogenes* in only 1 (0.6%) of the 181 leafy salad samples examined in the city of Sao Paulo, Brazil. Guévremont *et al.* (2017) suggested abstaining irrigation for at least three days prior to harvesting as a measure to potentially lower the risk of *L. monocytogenes* contamination in produce. Moreover, the transfer of foodborne pathogens to vegetables and their behavior in the preharvest environment are influenced by multiple factors, including abiotic elements, farming techniques, and the properties of the bacteria themselves. Lower prevalence was also reported by Li, *et al*. (2017)

In contrast to the present study highest prevalence of 50% was found in coriander and spinach, followed by 25% in cabbage and 11% in tomatoes as reported by Pingulkar *et al*. (2001) in Mumbai, India. Similarly higher prevalence of 65% in green leaves was reported by Gunasena, (1995) in Sri lanka. Dhanashree *et al*. (2003) reported that 10% of the spinach leaves and 30% of the coriander leaf samples tested positive for *L. innocua*. Arumugaswamy *et al*. (1994) documented a prevalence of 22% for *L. monocytogenes* in leafy vegetables sourced from Kajang, Serdang in Selangor and Kuala Lumpur. In Welshiemer (1960) view the presence of abundant water in low-lying regions where green leaves thrive might create conditions favorable for the contamination of *L. monocytogenes*. Similarly higher prevalence was noted by Heisick et al. (1989), Guansena, (1995), Cordano *et al.* (2009), Ponniah *et al*. (2010), Jamali *et al*. (2012), Moreno *et al*. (2012), Soni *et al*. (2014), Verma and Singh (2022), Kayode *et al*. (2022)

Cheong and Zainuldin (1994) made the observation that root crops, including radishes and potatoes, displayed a significant presence of *Listeria* contamination when compared to other vegetables like broccoli, cauliflower, and tomatoes. The reduced contamination in these vegetables could be attributed to less soil contact during their cultivation. Employing proper washing techniques can reduce the occurrence and proliferation of harmful bacteria. According to Houang *et al*. (1991), rinsing salad ingredients by vigorously shaking them in a

colander under running tap water for 2 minutes resulted in a significant decrease in bacterial counts.

4.2.1.b Organic farm vegetable

In the current study 58 leafy vegetable samples from organic farms in and around Nagpur were screened for isolation and identification of *Listeria monocytogenes.*

Total 58 organic leafy vegetable samples (4 coriander, 13 fenugreek, 10 spinach, 15 dill, 16 amaranth) processed for isolation of *L. monocytogenes* 17 showed typical greenish yellow aesculin hydrolyzing colonies which were further screened for tumbling motility revealed 2 positive. Processing of present isolates for biochemical characterization, sugar fermentation tests and CAMP test revealed that none of the isolate could be characterized as *L. monocytogenes*. The details are given in the Table 4.4 and 4.5.

Table 4.4 Prevalence of *Listeria* spp. and *L. monocytogenes* in organic farm samples

Sr no.	Sample	No of samples	Prevalence of *Listeria* spp.	Prevalence of *L. monocytogenes*
1	Coriander	4	0%	0%
2	Fenugreek	13	0%	0%
3	Spinach	10	0%	0%
4	Dill	15	0%	0%
5	Amaranth	16	0%	0%

Out of 58 samples from organic farms screened for *Listeria monocytogenes* none of them turned positive indicating 0% prevalence agreeing with McMahon and Wilson (2001), Oliveira *et al*. (2010), Park *et al.* (2014), Xu, *et al*. (2015), Richa Routela (2018) and Ueda and Kuwabara (2002) who screened organic farms and reported absence of *Listeria* spp. On contrary, Loncarevic *et al*. (2005) reported a prevalence of 1.11% in organically grown lettuce in Norway. Also Tango *et al*. (2014) reported a higher prevalence

Table 4.5 Details of cultural, morphological, biochemical profiles of *Listeria* isolates from organic farm

Sample	No.	Morphological and biochemical test								Sugars			CAMP positive Against SA	Confirmed as
		AH +	GS	TM	Catalase +	Oxidase –	MR +	VP +	Nitrate –	L-Rhamnose	D-Xylose	α –methyl D mannioside		
Coriander	4	1	-	-			-	-	-	V	V	V	-	-
Fenugreek	13	4	2	-	-	-	-	-	-	V	V	V	-	-
Spinach	10	3	1	1	1	1	1	1	-	V	V	V	-	-
Dill	15	6	2	1	1	1	1	1	-	V	V	V	-	-
Amaranth	16	3	-	-	-	-	-	-	-	V	V	V	-	-

of 6.4% each in romain lettuce and spinach screened at Korea. There are many studies that reported higher prevalence of *Listeria monocytogenes* in organic farms as compared to conventional farms (Tango *et al*. 2014, Kuan *et al*. 2017) which relates the use of manure from animal as organic fertilizer. But there hasn't been a general agreement on which is superior, other than the fact that both kinds of farms are apparently not entirely safe. Also Marine *et al*. (2015) reported that presence of bacterial pathogen depends on growing season and not on farming type.

4.2.1.c Vegetables cultivated alongside Nag River

In the current study 37 leafy vegetable samples cultivated alongside Nag river which is highly sewage contaminated were screened for isolation and identification of *Listeria monocytogenes.* The study reported 0% prevalence of *L. monocytogenes*. The details are elaborated in the table 4.6 and 4.7.

Total 37 leafy vegetable samples (3 coriander, 10 fenugreek, 5 spinach, 9 dill, 10 amaranth) were processed for isolation of *L. monocytogenes* 13 showed typical greenish yellow aesculin hydrolyzing colonies which were further screened for tumbling motility revealed 1 positive. Processing of these isolates for biochemical characterization, sugar fermentation tests and CAMP test revealed that none of the isolate could be characterized as *L. monocytogenes*.

Table 4.6 Prevalence of *Listeria* spp. and *L. monocytogenes* in vegetables cultivated alongside Nag river

Sr no.	Sample	No of sample	Prevalence of *Listeria* spp.	Prevalence of *L. monocytogenes*
1	Coriander	3	0%	0%
2	Fenugreek	10	0%	0%
3	Spinach	5	0%	0%
4	Dill	9	0%	0%
5	Amaranth	10	0%	0%

The results of our study of 0% prevalence of *L. monocytogenes* in leafy vegetables grown on sewage and waste water contaminated Nag river aligns with

Table 4.7 Details of cultural, morphological, biochemical profiles of *Listeria* isolates vegetables cultivated alongside Nag river

Sample	No.	Morphological and biochemical test								Sugars			CAMP positive Against SA	Confirmed as
		AH +	GS	TM	Catalase +	Oxidase –	MR +	VP +	Nitrate –	L-Rhamnose	D-Xylose	α –methyl D mannioside		
Coriander	3	1	-	-	-	-	-	-	-	V	V	V	-	-
Fenugreek	10	4	2	1	1	1	1	1	-	V	V	V	-	-
Spinach	5	3	1	-	-	-	-	-	-	V	V	V	-	-
Dill	9	2	-	-	-	-	-	-	-	V	V	V	-	-
Amaranth	10	3	2	-	-	-	-	-	-	V	V	V	-	-

the work of Gholipur *et al*. (2020) who also reported 0% prevalence in wastewater-irrigated crops in Iran. The absence of *L. monocytogenes* in the samples could be attributed to either the lack of presence or the minimal levels of bacteria in the effluent samples utilized for irrigation purposes. Rodriguez *et al*. (1984) have discussed the challenge of isolating *L. monocytogenes* from highly contaminated environments due to the dominance of other microbes and the morphological similarities and cross-reactions with enterococci.

On contrary, Guévremont *et al.* (2017) found *L. monocytogenes* in one sample of manure-contaminated irrigation water (n = 27) and one lettuce sample (n = 288) irrigated with the same water. There has been a reported outbreak of *Listeria* infection linked to the consumption of coleslaw that was produced using cabbage fertilized with sheep manure (Newell *et al*. 2010).

4.2.2 Prevalence of *Listeria monocytogenes* in environment samples

In the present study total 50 environment samples (25 soil, 20 water, 5 manure) were evaluated for *Listeria monocytogenes*, out of which none of them turned positive indicating complete absence of the pathogen in samples. The results are mentioned in the given Tables 4.8 and 4.9.

Out of the 50 environmental samples which were screened for isolation of *L. monocytogenes*, 4 from soil, 1 from water and 3 from manure showed typical aesculin hydolyzing colonies on PALCAM agar. On further processing of these colonies revealed 3 colonies with gram positive coccobacillary appearance (2 soil, 0 water, 1 manure) and 2 showed tumbling motility at 40X (1 from soil, 0 from water, 1 from manure). By further biochemical and sugar fermentation tests were no isolate was identified as *L. monocytogenes.*

Table 4.8 Prevalence of *Listeria* spp. and *L. monocytogenes* in environmental samples

Sr no.	Sample	No of sample	Prevalence of *Listeria* spp.	Prevalence of *L. monocytogenes*
1	Soil	25	0%	0%
2	Water	20	0%	0%
3	Manure	5	0%	0%

Table 4.9 Details of cultural, morphological, biochemical profiles of *Listeria* isolates from Environmental samples

Sample	No.	Morphological and biochemical test								Sugars			CAMP positive Against SA	Confirmed as
		AH +	GS	TM	Catalase +	Oxidase _	MR +	VP +	Nitrate _	L-Rhamnose	D-Xylose	α –methyl D mannioside		
Soil	25	4	2	1	1	1	1	1	-	V	V	V	-	-
Water	20	1	-	-	-	-	-	-	-	V	V	V	-	-
Manure	5	3	1	1	1	1	1	1	-	V	V	V	-	-

4.2.2.a Soil

Out of 25 soil samples screened for *Listeria* spp. isolation none of them turned positive. Similarly Raorane *et al*. (2013) reported 0% prevalence of *L. monocytogenes* in soil & floor swab samples of animal environment. According to Buchanan *et al*. (2017) the survival of microbes in soil depend upon the soil type and conditions where they survive better in moist, organic soils than dry low organic soil. Here one more reason behind the 0% prevalence of *Listeria* spp. in soil may be long time interval between application of sewage sludge, organic manure and crop harvesting.

The results in the current study deviates from Ikeh *et al*. (2010) who noted 100% occurrence of *Listeria* spp. in soil samples taken from areas where cows and pigs were housed before slaughter, indicating the likelihood of faecal carriage of *Listeria* spp. by these animals. This tends to support Bockserman's (2000) assertion that *L. monocytogenes* primarily resides in the intestinal tracts of mammals and birds, serving as the source of entry into the soil through animal droppings. Higher prevalence was also noted by Moshtagi *et al.* (2003), Linke, *et al*. (2014), Nassirabady *et al*. (2015), Sarker and Ahmed (2015).

Also Tahir *et al*, (2022) reported 1.7% prevalence by screening 970 soil samples around Punjab province and 14% was reported (Sunitha *et al.* 2016) in Kerala and 5% (Sarangi and Panda, 2012) in Odisha. Shantha and Shubha (2014) detected 1% prevalence of *L. ivanovii* in environmental from Mysore city. One of the reason behind varying results might be the varying environmental factors including humidity, ambient temperature, moisture content of the soil, ultraviolet radiation rate which might influence the survivability of pathogenic microbes in soil (Gholipur *et al*. 2020).

4.2.2.b Water

No *Listeria* spp. were isolated from 20 water samples screened in the current study which goes in concurrence with Sarangi and Panda (2012) and Kulesh (2017) who reported no isolation of *L. monocytogenes* in water samples screened in Odisha and Nagpur, respectively. However, *L. monocytogenes* has

been reported by Nightingale *et al*. (2004) in animal farm environment which may be due to improper sewage water disposal and also by Strawn, *et al*. (2013), Soni *et al*. (2013).

4.2.2.c Manure

Listeria spp. was not detected in any of the 5 samples of manure screened in the current study. The results are similar to study of Van Renterghem *et al*. (1991) in which researchers examined manure samples and reported 0% prevalence of *Listeria* spp. This might be due to destruction of microbes or desiccation of faecal matter. Although, researches have reported that animal origin organic fertilizers may contribute in contaminating the soil with *L. monocytogenes* (Al·Ghazali and Al-Azawi, 1990: Watkins and Sleath, 1981). Also Gholipour *et al*. (2020) in Iran reported 8% prevalence of the organism which was on higher side as compared to current study.

4.3 *In vitro* pathogenicity test

In the current study in order to study the *in vitro* pathogenecity test the recovered 4 isolates (2 coriander, 2 spinach) were subjected to haemolysis on sheep blood agar (SBA), Christie, Atkins, Munch-Petersen (CAMP) test and Phosphatidylinositol-specific phospholipase C (PI-PLC) Assay.

4.3.1 Haemolysis on Sheep Blood Agar

Haemolysin production was studied in the present study by streaking the recovered 9 isolates on 7% sheep blood agar and then incubating it at 37℃ for 24 hrs and then zone of haemolysis around the colonies was observed. On the basis of this test *Listeria* spp. can be characterized as pathogenic and non-pathogenic. All the 4 isolates of *L. monocytogenes* showed typical beta haemolysis (Plate 4.2) thus conferring it pathogenic.

The results in the current study are in conformity with Barbuddhe *et al.*, (2000), Chaudhari *et al*. (2004), Kalorey *et al*. (2006), Rawool *et al*., (2007), Yadav *et al*. (2010), Suryawanshi (2012) and Sakhare (2014), Khawase (2015)

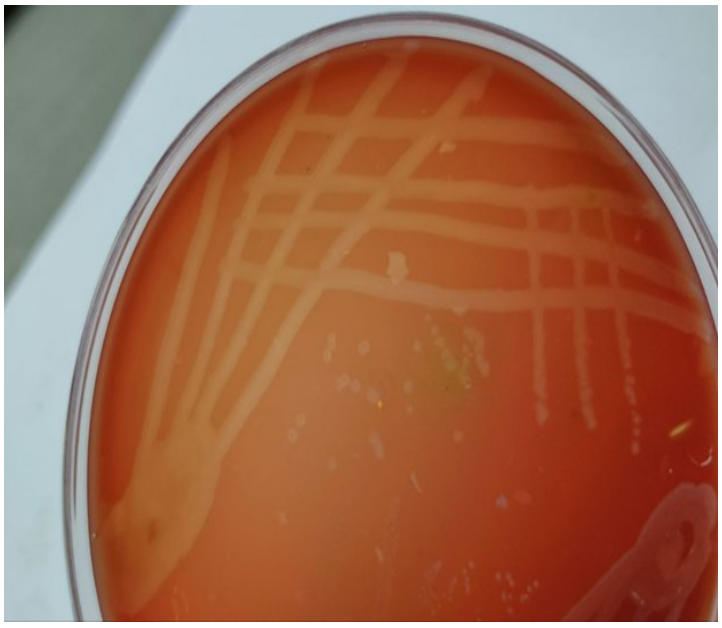

Plate 4.2 Haemolysis on sheep blood agar by *Listeria* spp

Yadav *et al*. (2010), Suryawanshi (2012) and Sakhare (2014), Khawase (2015) Dhote (2016) and Kulesh (2017) who reported haemolytic activity showed by *L. monocytogenes* isolates.

Varying degree of haemolysis was reported by Vaidya *et al*. (2018) by analyzing 17 isolates of *L. monocytogenes*. All the isolates were concluded pathogenic as they showed haemolytic activity. Strong haemolytic activity was reported for 6 isolates from pig, moderate by 6 isolates of faecal origin of goat and porcine weak by 5 isolates of goat.

4.3.2 Christie, Atkins, Munch-Petersen (CAMP) test

McKellar (1994) and Vazquez-Boland, *et al* (1989) discussed the factors related to the combined breakdown of red blood cells (RBC) and the use of the CAMP test to identify *L. monocytogenes* and *L. ivanovii* isolates. In case of *L. monocytogenes*, a 58-KDa protein called LLO, which is secreted by the bacteria, causes strong hemolysis when combined with *Staphylococcus aureus* culture but not with *Rhodococcus equi*. On the other hand, *L. ivanovii* secretes a 24-27 kDa protein called Sphingomylinase C, which causes strong hemolysis when combined with *R. equi* but not with *S. aureus*. This protein is responsible for the characteristic CAMP reaction in *L. ivanovii* (Farber and Peterkin, 1991).

All nine *Listeria* isolates in the current study were tested for CAMP test in order to differentiate and study its pathogenicity using *Staphylococcus aureus* (ATCC 12600) and *Rhodococcus equi* (ATCC 6939). Out of 9, 4 isolates (C47, C31, S69, S48) showed a typical zone of haemolysis in the direction of *Staphylococcus aureus* and thus they were considered as *L. monocytogenes* (Plate 4.3). The remaining eight isolates did not exhibit enhanced zones of haemolysis towards *S. aureus* and *R. equi*.

The results in the present study aligns with, Vaz-VeIho *et al*. (2000), Chaudhari *et al*. (2004) and Rawool *et al*. (2007), Raorane *et al*. (2013) who carried out confirmation of *L. monocytogenes* by CAMP test.

The findings of this study are consistent with those of previous

investigations carried out in this laboratory by, Suryawanshi (2012), Thomas (2013), Vaidya (2018) Sakhare (2014), Khawse (2015), Dhote (2016), Kulesh (2017), all of whom confirmed the presence of *L. monocytogenes* through the CAMP test.

4.3.3 Phosphatidylinositol-specific phospholipase C (PI-PLC)

Another virulence indicator, phosphatidylinositol-specific phospholipase (PI-PLC), has been discovered in pathogenic strains, specifically within *L. monocytogenes,* as documented by Notermans *et al*. in 1991. In the current study, in order to evaluate the PI-PLC activity, all 9 isolates were freshly grown on 7% SBA and then streaked on L.mono differential agar base, incubated at 37℃ for 24 hrs. All 5 isolates of *Listeria* spp. (C67, C54, S21, C59, A22) and 4 isolates of *L. monocytogenes* (C31, C47, S48, S48) produced light blue colonies surrounded by halo which was considered as positive (Plate 4.4).

The findings in the present study are in conformity with Thomas *et al*. (2013) and Raorane *et al*. (2013) who employed a PI-PLC assay to detect pathogenicity in 18 and 11 isolates of *L. monocytogenes*, respectively. The isolates were sourced from food animals slaughtered in the Nagpur region and from samples of pigs, sheep, and goats. The researchers observed that all isolates tested positive in the PI-PLC assay, leading to the classification of these isolates as pathogenic. Also the findings of this present investigation are consistent with the results reported in various studies conducted by Shelke (2011), Suryawanshi (2012), Yadav (2011), Bharate (2012), Thomas (2013), Sakhare (2014), Khawse (2015), Kulesh (2017), Vaidya (2018).

The complete positivity observed in this study is notably higher than the findings of Pimenta *et al.* (1999), who reported a positivity rate of 41.30% in *L. monocytogenes* isolated from various food samples. Furthermore, Kalorey *et al*. (2008) documented that 4.36% of the *L. monocytogenes* strains tested positive for PI-PLC assay. This conclusion was drawn from the examination of 105 *L. monocytogenes* strains recovered from 1060 raw milk samples collected from buffaloes in the Vidharbh region.

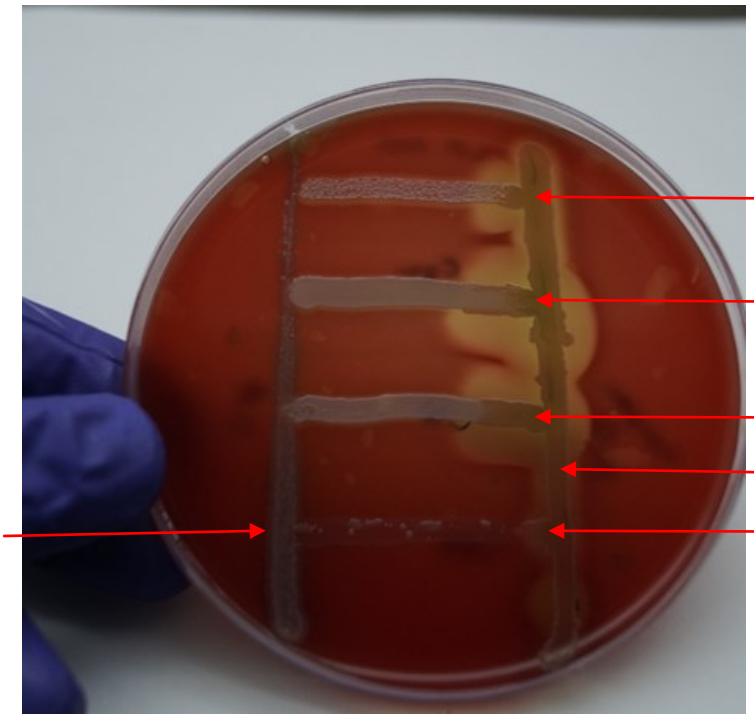

Plate 4.3 The characteristic CAMP reaction by *Listeria monocytogenes*

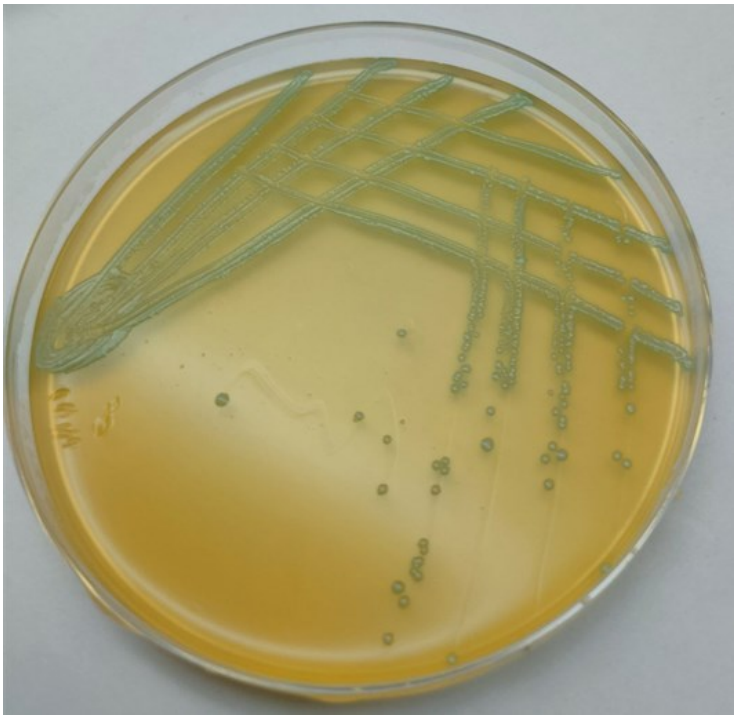

Plate 4.4 PI-PLC Assay by *Listeria* spp.

In the course of this investigation, the identification of haemolytic activity and consistent CAMP positivity in all four isolates serves as compelling evidence confirming the pathogenic nature of these recovered strains. These tests are essential, as there are non-pathogenic strains in nature that cannot be distinguished solely through cultural and biochemical tests.

Table 4.10 Pathogenicity profile of *L. monocytogenes* isolates

Isolate No.	Source	Haemolysis on SBA	CAMP		PI-PLC Production assay	Confirmed as
			Staphylococcus aureus	*Rhodococcus equi*		
C47	Coriander	+	+	-	+	*L. monocytogenes*
C31	Coriander	+	+	-	+	*L. monocytogenes*
S69	Spinach	+	+	-	+	*L. monocytogenes*
S48	Spinach	+	+	-	+	*L. monocytogenes*

4.4 Characterization of genus and species specific genes by Polymerase Chain Reaction

The nine isolates of *Listeria* spp. obtained in this research work were examined to detect the presence of two specific genes: the genus-specific *prs* gene (844 bp) and the species-specific *isp* gene (713 bp). The sequences of these genes used in the study are documented in Table 3.5. A reaction volume of 25 µl was prepared for each sample DNA, as indicated in Table 3.6. PCR conditions were optimized as outlined in Table 3.7.

Table 4.11 Genus and species-specific genes in *Listeria* isolates

Isolate ID	Source	*prs* 844 bp	*isp* 713 bp
C47	Coriander	+	+
C31	Coriander	+	+
S69	Spinach	+	+
S48	Spinach	+	+

C67	Coriander	+	-
C54	Coriander	+	-
S21	Spinach	+	-
C59	Coriander	+	-
A22	Amaranth	+	-

Out of the 9 isolates examined, four exhibited amplification at both 844bp and 713bp, conclusively identifying them as *Listeria monocytogenes*. Meanwhile, the remaining five isolates only displayed amplification at the genus-specific *prs* 844bp and not the species-specific *isp* 713bp, indicating them as *Listeria* spp. (Table 4.11, Plate 4.5.a, 4.5.b).

The findings align with those of Rawool *et al*. (2016), who investigated the molecular characterization of *Listeria* genus and species using multiplex PCR targeting the *prs* gene (844 bp) and the *isp* gene (713 bp). Our findings are also in consistent with the results documented by Suryawanshi, *et al*. (2023). In his study, the researchers validated all three biochemically positive *Listeria* isolates using multiplex PCR, in which all three turned out positive for both the genes; *prs* 844 bp and *isp* 713 bp, endorsing their identification as *Listeria monocytogenes*.

4.5 Assessment of *hlyA, actA, plcA*, and *iap* virulent genes by PCR

In *L. monocytogenes* pathogenesis, various key virulence factors, such as haemolysin (*hly*A), actin polymerization protein (*act*A), phosphatidylinositol phospholipase C (*plc*A), and invasive associated protein (*iap*), play crucial roles. Consequently, relying on the detection of a single gene, even one as significant as *hly*A, falls short in confirming the presence of a virulent *L. monocytogenes* strain. As a result, researchers have endeavored to enhance the accuracy by simultaneously detecting multiple virulent genes through the application of multiplex PCR techniques.

In the present study four virulent markers *hlyA* (456 bp), *actA* (839 bp), *plcA* (1484 bp) and *iap* (131 bp) were targeted for the PCR amplification. All the four isolates of *L. monocytogenes* (C47, C31, S48, S69) amplified and showed positivity for the four genes (Table 4.12, Plate 4.6, 4.7, 4.8, 4.9).

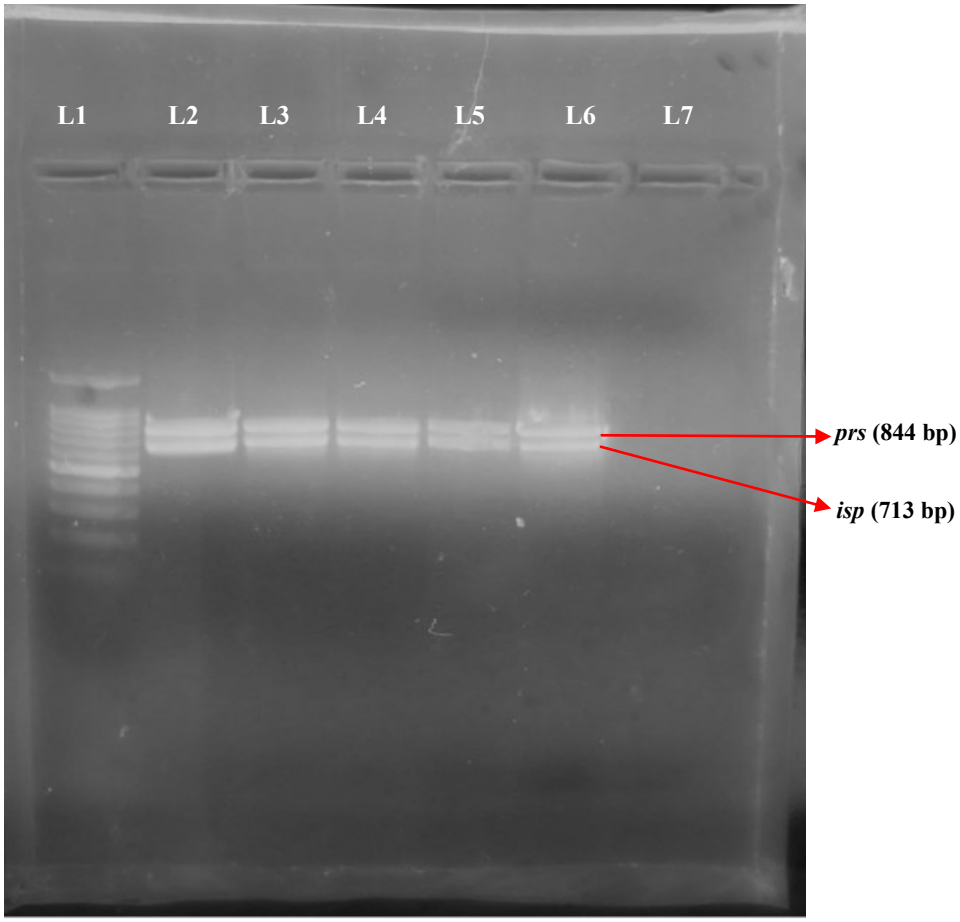

Plate 4.5.a PCR profile of genus (*prs*) and species (*isp*) specific genes of *Listeria monocytogenes*

Lane 1 100 bp Ladder

Lane 2 Positive isolate C47 from Coriander

Lane 3 Positive isolate C31 from Coriander

Lane 4 Positive isolate S69 from Spinach

Lane 5 Positive isolate S48 from Spinach

Lane 6 Positive control template (*L. monocytogenes* MTCC 1143)

Lane 7 Negative control

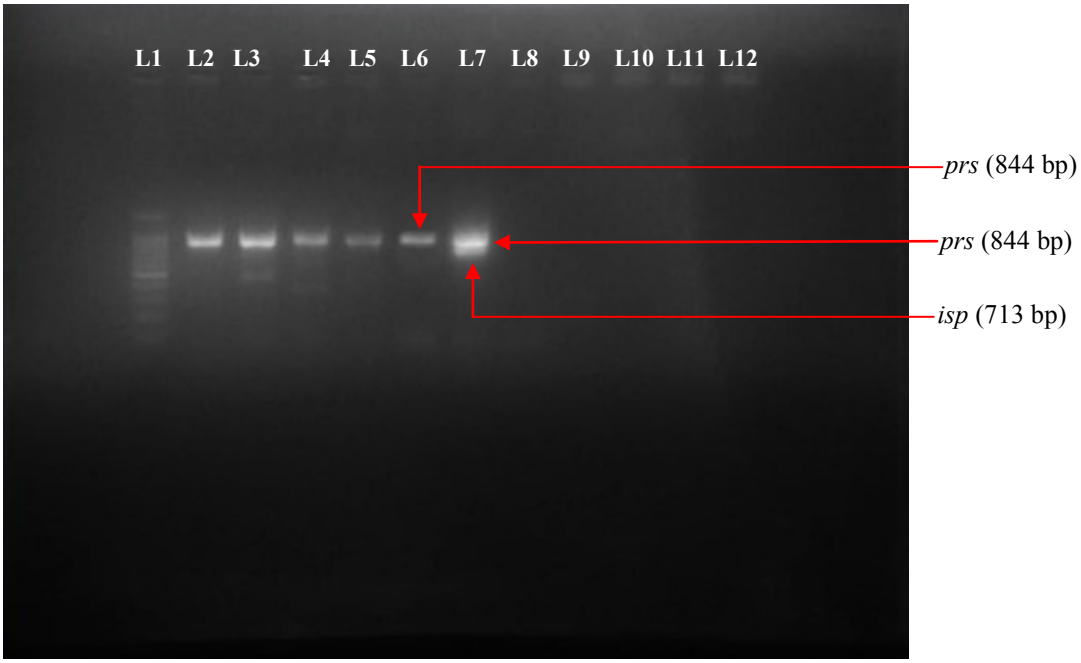

Plate 4.5.b PCR profile of genus (*prs*) and species (*isp*) specific genes of *Listeria monocytogenes*

Lane 1 100 bp Ladder

Lane 2 Positive isolate C59 from Coriander

Lane 3 Positive isolate C54 from Coriander

Lane 4 Positive isolate C 67 from Coriander

Lane 5 Positive isolate S21 from Spinach

Lane 6 Positive isolate A22 from Amaranth

Lane 7 Positive control template (*L. monocytogenes* MTCC 1143)

Lane 8 Negative control

Lane 9 Not related with present work

Lane 10 Not related with present work

Lane 11 Not related with present work

Lane 12 Not related with present work

Table 4.12 Details of virulence-associated genes in *L. monocytogenes* isolates

IsolateID	Source	Virulence specific genes			
		plc A (1484bp)	*hly* A (456bp)	*act* A (839bp)	*iap* (131bp)
C47	Coriander	+	+	+	+
C31	Coriander	+	+	+	+
S69	Spinach	+	+	+	+
S48	Spinach	+	+	+	+

The results in the current study of four isolates possess all four genes are in conformity with Yadav *et al*. (2010) who conducted a genotypic characterization of three *L. monocytogenes* isolates obtained from 85 mastitic milk samples. The investigation targeted five specific genes—*plcA*, *prfA*, *actA*, *hly*A, and *iap*. The findings revealed that all three isolates exhibited the presence of these five virulence-associated genes. Similarly all 18 isolates screened by Sunil *et al*, (2012) showed positivity for *iap, hlyA, actA, prfA, plcA* and *inlA* genes thus confirming its virulent nature. Also 4 isolates of *L. monocytogenes* screened by Jagtap *et al*. (2017) showed positivity for *plc*A, *hly*A, *act*A and *iap* genes. Results also align with Raorane *et al*. (2015)

On contrary, Kaur *et al*. (2007) have documented a variability during the screening of virulence genes. They found that two *L. monocytogenes* isolates exhibited the presence of all five genes, while one isolates did not amplify *prf*A and *plc*A genes. It is worth noting that certain strains of *L. monocytogenes* might lack one or more virulence factors due to genetic mutations, as suggested by Cooray *et al*. (1994). Also Negi *et al*. (2015) during the screening of virulence genes documented that 22 out of 36 *L. monocytogenes* isolates exhibited the presence of all four genes, while the remaining 14 isolates either did not amplify the *prfA* and/or *actA* genes or both. Similarly, Kulesh (2017) reported variable results.

The PCR method, standardized for detecting virulence-associated genes of *L. monocytogenes*, has been confirmed to efficiently amplify all genes, as evidenced by the positive control, the *Listeria monocytogenes* (MTCC) standard

strain. Generally, there was a notable consistency observed between *in vitro* tests such as haemolysin production, CAMP, and the detection of virulent genes in the isolated strains.

4.6 Assessment of *L. monocytogenes* serotype by multiplex PCR

L. monocytogenes comprises 13 serovars based on their combination of flagellar (H) and somatic (O) antigen, of which the prevalent strains isolated from food and patients, constituting a minimum of 95%, are those belonging to serovars 1/2a, 1/2b, 1/2c, and 4b.

In the present study, serotyping of all four isolates was conducted by targeting four different genes: *lmo1118*, *lmo0737*, *ORF2110*, and *ORF2819*. These genes have molecular sizes of 906 bp, 691 bp, 597 bp, and 471 bp, respectively. Each of these genes corresponds to distinct serotypes, with *lmo1118* representing serotypes 1/2c and 3c, *lmo0737* representing serotypes 1/2a, 1/2c, 3a, and 3d, *ORF2110* representing serotypes 4b, 4d, and 4e, and *ORF2819* representing serotypes 1/2b, 3b, and 4b. Serotype 1/2a is commonly found in food, whereas serotype 4b is responsible for the majority of human epidemics (Gilot *et al*. 1996).

The multiplex PCR technique was standardized using a 25 µl reaction mixture, as outlined in Table 3.12, and optimized conditions as detailed in Table 3.13 were employed.

Table 4.13 Details of Serotyping of *Listeria monocytogenes* isolates

IsolateID	*Imo1118* (906 bp)	*lmo*0737 (691 bp)	*Orf 2110* (597bp)	*Orf2819* (471bp)	Serotype
C47	-	-	-	+	1/2b, 3b, 4b,
C31	-	-	+	+	4b, 4d, 4e and 1/2b, 3b, 4b,
S69	-	-	-	+	1/2b, 3b, 4b,
S48	-	-	-	+	1/2b, 3b, 4b,

All four recovered isolates (C47, C31, S48, S69) exhibited positivity for the *ORF2819* gene (471bp), which is responsible for encoding a putative

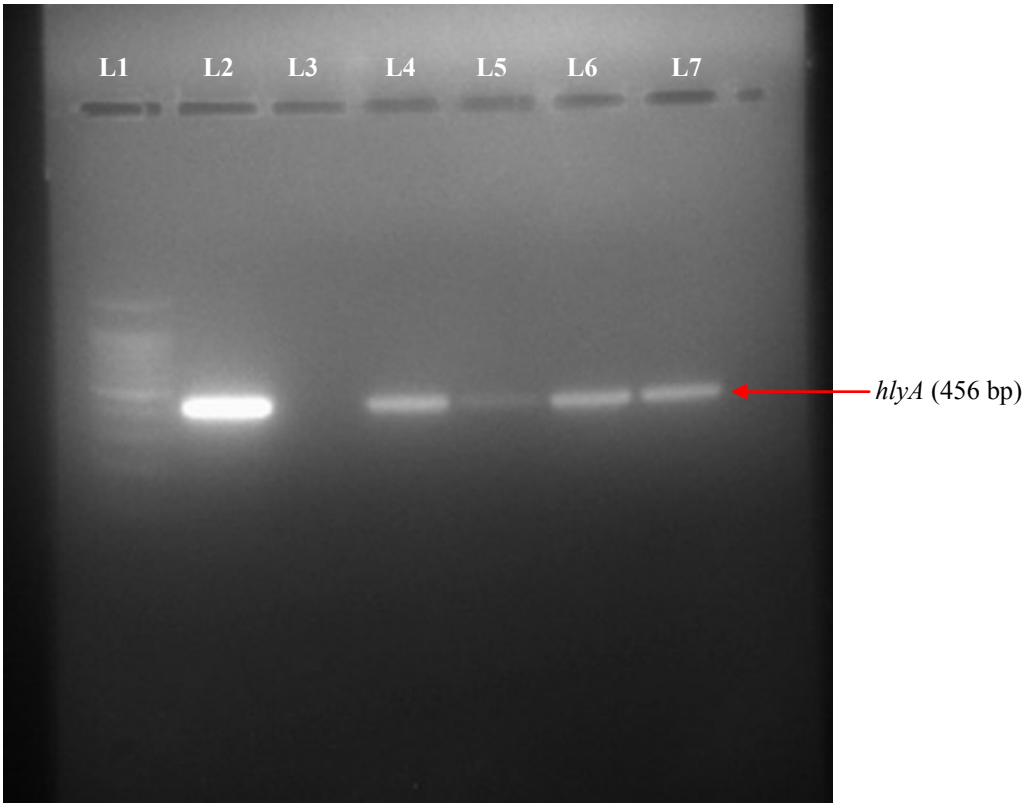

Plate 4.6 PCR profile of *L. monocytogenes* targeting virulence associated gene *hlyA*

Lane 1 100 bp Ladder

Lane 2 Positive control template (*L. monocytogenes* MTCC 1143)

Lane 3 Negative control

Lane 4 Positive isolate S69 from Spinach

Lane 5 Positive isolate S48 from Spinach

Lane 6 Positive isolate C47 from Coriander

Lane 7 Positive isolate C31 from Coriander

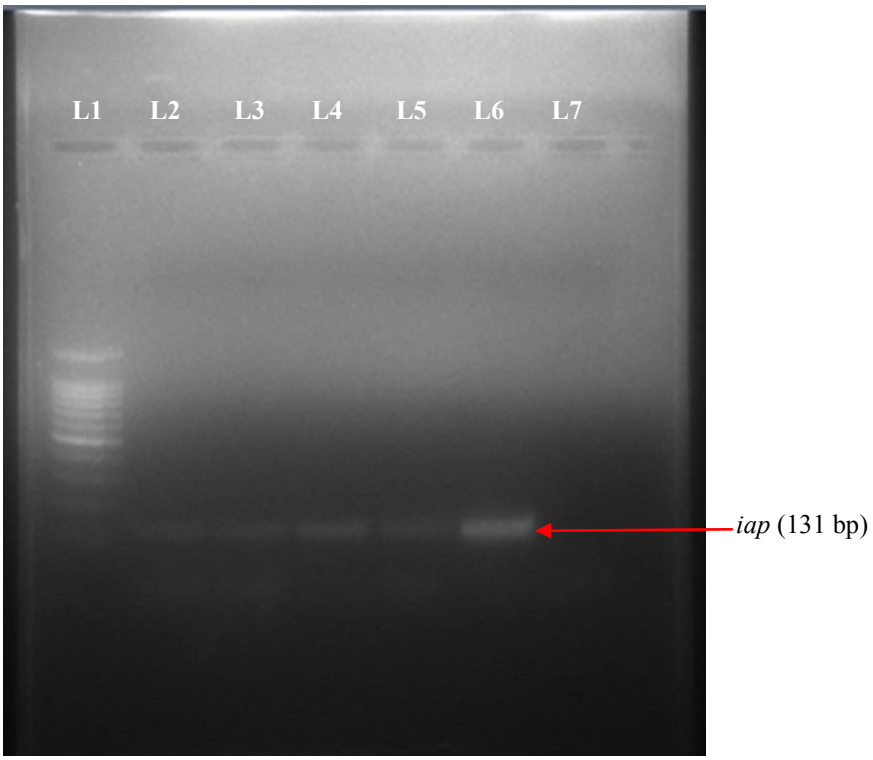

Plate 4.7 PCR profile of *L. monocytogenes* targeting virulence associated gene *iap*

Lane 1 100 bp Ladder

Lane 2 Positive isolate C47 from Coriander

Lane 3 Positive isolate C31 from Coriander

Lane 4 Positive isolate S69 from Spinach

Lane 5 Positive isolate S48 from Spinach

Lane 6 Positive control template (*L. monocytogenes* MTCC 1143)

Lane 7 Negative control

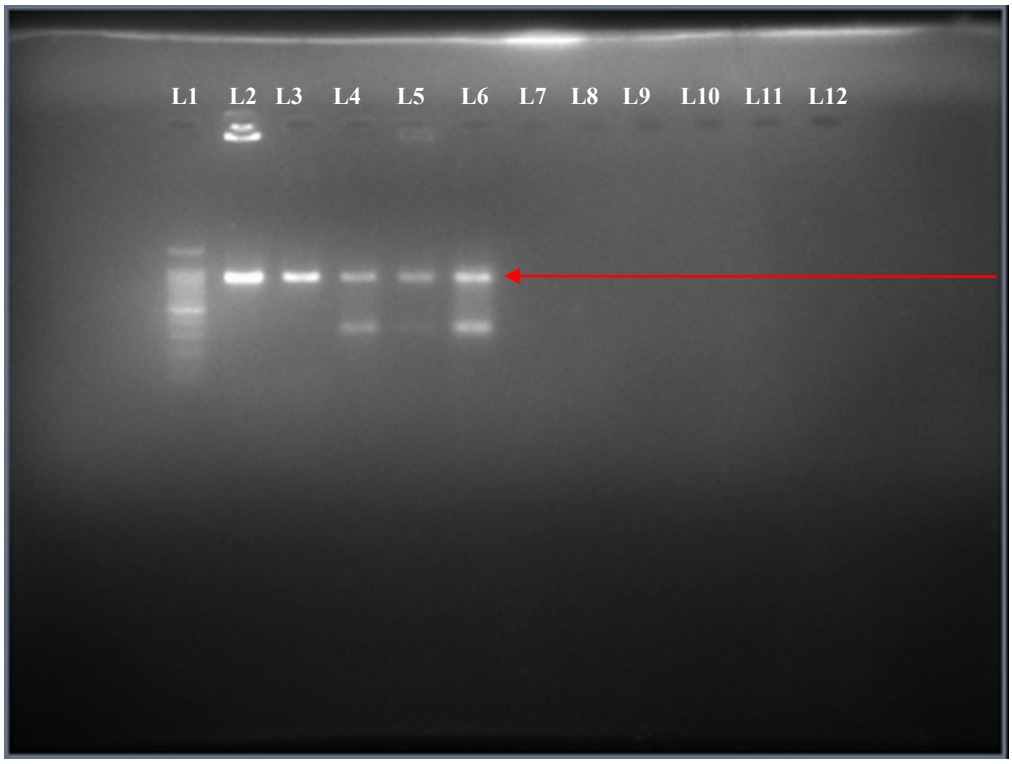

Plate 4.8 PCR profile of *L. monocytogenes* targeting virulence associated gene *actA*

Lane 1 100 bp Ladder

Lane 2 Positive control template (*L. monocytogenes* MTCC 1143)

Lane 3 Positive isolate S48 from Spinach

Lane 4 Positive isolate S69 from Spinach

Lane 5 Positive isolate C31 from Coriander

Lane 6 Positive isolate C47 from Coriander

Lane 7 Negative control

Lane 8 Not related with present work

Lane 9 Not related with present work

Lane 10 Not related with present work

Lane 11 Not related with present work

Lane 12 Not related with present work

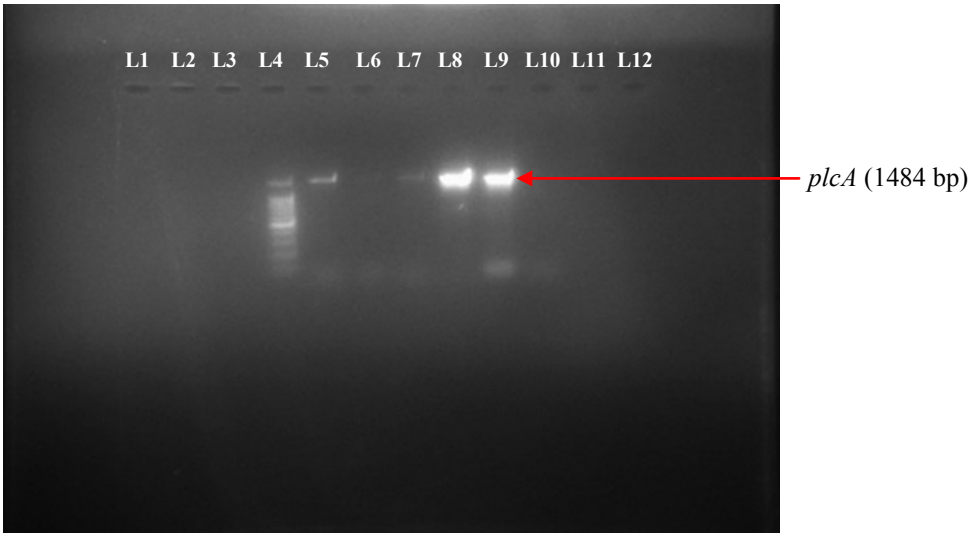

Plate 4.9 PCR profile of *L. monocytogenes* targeting virulence associated gene *plcA*

Lane 1 Not related with present work

Lane 2 Not related with present work

Lane 3 Positive isolate S48 from Spinach

Lane 4 100 bp Ladder

Lane 5 Positive isolate C31 from Coriander

Lane 6 Positive isolate C47 from Coriander

Lane 7 Positive isolate S48 from Spinach

Lane 8 Positive isolate S69 from Spinach

Lane 9 Positive control template (*L. monocytogenes* MTCC 1143)

Lane 10 Negative control

Lane 11 Not related with present work

Lane 12 Not related with present work

transcriptional regulator protein. Additionally, one isolate (C31) was found to be positive for *ORF2110* (597 bp), which encodes a putative secreted protein. The most common serotype found in the isolates was 1/2b, followed by 4b. The results are mentioned in table 4.13 and Plate 4.10.

The current findings can be compared to those of Wang *et al*. (2015) who conducted a study on 628 samples, comprising ready-to-eat (RTE) products sourced from both supermarkets and open-air markets in Nanjing, China, and subjected the isolated obtained analyses for serogroup, virulence genes, genotype, and antibiotic resistance. Their findings indicated that among the isolates, 48.5% belonged to serogroup 1/2b, 3b; 45.5% belonged to serogroup 1/2a, 3a; and 6.1% were classified into serogroup 1/2c, 3c. Also, Singh *et al*. (2016), investigated 29 *L. monocytogenes* isolates collected from milk, meat products, vegetables, and clinical cases in Nagpur, Maharashtra state. Their study revealed the highest occurrence of serotypes 1/2b and 4b.

Okwumabua *et al*. (2005) screened a total of 21 isolates of *L. monocytogenes* originating from both food sources and clinical cases in animals and their serotyping analysis indicated that five of the isolates were classified as serotype 1/2a, six as 1/2b, nine as 4b, and one isolate could not be assigned a serotype.

However, Barbuddhe *et al*. (2016) in their investigation, reported that 60.35% of isolates were serotype 4b (239/396), 27.77% were serotype 1/2a (110/396), and 11.86% were serotype 1/2b (47/396) across India from 2000 to 2014. Their study observed the highest recovery of 4b serotype, followed by 1/2a and 1/2b serotypes. Terzi *et al*. (2015) confirmed the presence of four *Listeria monocytogenes* isolates among 100 ready-to-eat food products. Upon serotyping, two of these isolates were identified as serotype 4b (or 4d, 4e), while the remaining two were classified as serotype 1/2a (or 3a).

As *Listeria* is prevalent, long-lasting, and diverse in both natural surroundings and outdoor production environments, it is essential for producers to

rigorously follow Good Agricultural Practices (GAPs) and Good Handling Practices (GHPs). This precautionary step is vital to avoid the transmission of *Listeria* onto fresh produce from different origins such as agricultural water, soil, personnel, and equipment, during harvesting and packaging phases.

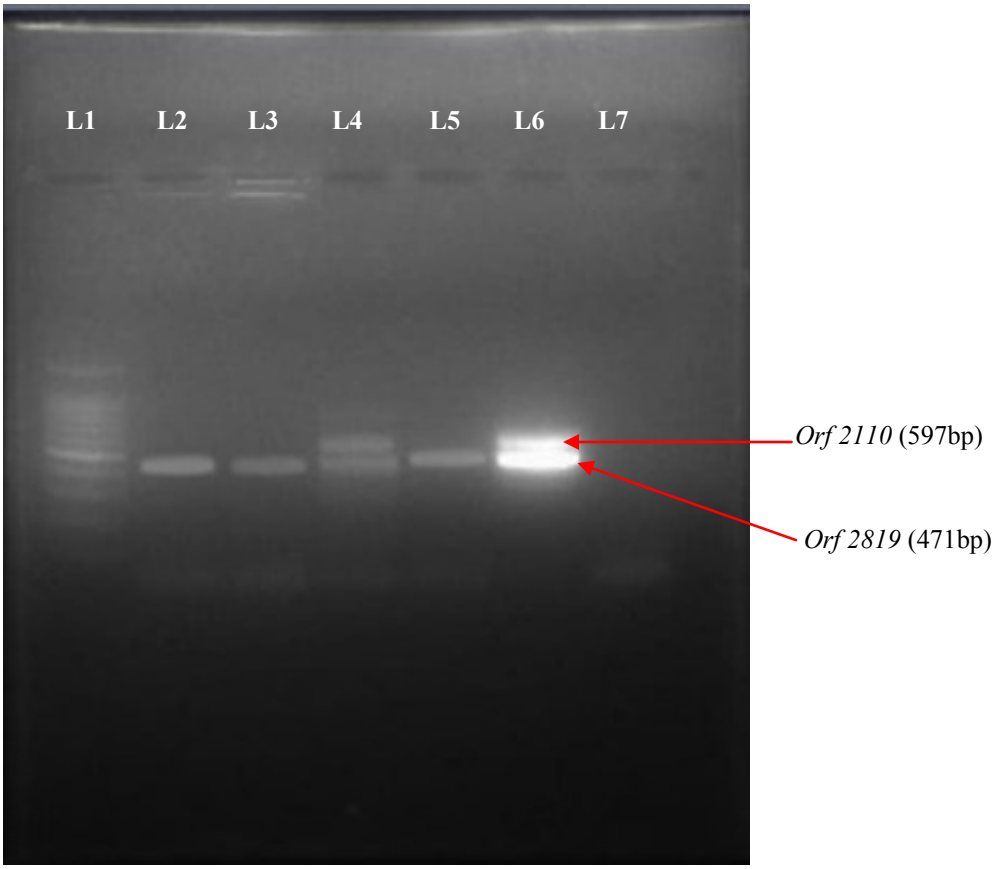

Plate 4.10 Multiplex PCR profile of *L. monocytogenes* serovars-associated genes

Lane 1 100 bp Ladder

Lane 2 C 47 from Coriander

Lane 3 S 69 from Spinach

Lane 4 C 31 from Coriander

Lane 5 S 48 from Spinach

Lane 6 Positive control template (*L. monocytogenes* MTCC 1143)

Lane 7 Negative control

SUMMARY & CONCLUSIONS

The present study involved an extensive assessment of *Listeria monocytogenes* as a potential contaminant in green leafy vegetables and their surrounding environments. It included a comprehensive sampling strategy, covering various types of green leafy vegetables such as coriander (75), fenugreek (75) , amaranth (75), spinach (75), dill (75) sold in the local market, grown in Nag river basin and organic farms. Further the environmental samples including soil (25), water (20) and manure (5) were also screened. Thus the overall sample size included was 425.

The samples were processed for isolation of *L. monocytogenes*, utilizing a two-step enrichment method involving Half Fraser Broth and Full Fraser Broth, followed by streaking onto PALCAM medium, which served as a selective agar. All the presumptive isolates were subjected to biochemical and sugar fermentation tests. In the present study, nine isolates (5 from coriander, 3 from spinach, 1 from amaranth) were identified as *Listeria* spp. and the prevalence was 2.11% (9 out of 425 samples), while *L. monocytogenes* specifically accounted for 0.94% (4 out of 425 samples).

After being confirmed microbiologically, all four isolates of *L. monocytogenes* were subjected to *in-vitro* pathogenicity testing. These tests assessed hemolysis on 7% Sheep Blood Agar (SBA), Christie, Atkins, Munch-Petersen (CAMP) test, and the Phosphatidylinositol-specific phospholipase C (PI-PLC) assay to further characterize their phenotypic pathogenicity. All *Listeria* spp. and *L. monocytogenes* isolates demonstrated distinct β-haemolysis on SBA and positivity for the PI-PLC assay; additionally *L. monocytogenes* isolates showed positivity for CAMP test, confirming their pathogenic nature.

The isolates showing distinct biochemical and sugar fermentative characteristics were then analyzed using multiplex PCR, targeting the *prs* (844bp) gene for genus-specific identification and the *isp* (713bp) gene for species-specific identification. Out of nine, four isolates two each from coriander and spinach were confirmed as *Listeria monocytogenes* using PCR analysis. The presence of bands

with product sizes of 844 bp and 713 bp, respectively, validated this identification. While, the remaining five isolates, including three from coriander, one from spinach, and one from amaranth, tested positive only for the genus-specific gene (*prs*) and negative for the species-specific gene (*isp*), thus indicating their classification as *Listeria* spp.

Furthermore, all four strains of *Listeria monocytogenes* were subjected to PCR analysis to identify specific virulence-associated genes, including listeriolysin O (*hly*A), phosphatidylinositol-specific phospholipase C (*plc*A), Actin filament protein (*act*A), and invasion-associated protein p60 (*iap*). All four isolates of *L. monocytogenes* tested positive for all four genes investigated in the study. The serotyping analysis of isolates, using four different genes (namely *lmo1118, lmo0737, ORF2110,* and *ORF2819*), demonstrated the presence of the *ORF2819* gene in four isolates and the *ORF2110* gene in one isolate. This confirms that the ½ b serotypes are more common, followed by 4b.

Based on the research findings, the following conclusions were derived:

1. *Listeria monocytogenes* isolation was done using a two-step enrichment process using Fraser Broth followed by plating on PALCAM media. This method proved to be effective for the isolation of *L. monocytogenes* from vegetables and environment samples.
2. The total prevalence noted for *Listeria* spp. was 2.11% (9 out of 425 samples) and *L. monocytogenes* was 0.94% (4 out of 425 samples). Specifically, two *L. monocytogenes* isolates were recovered from coriander, and another two were identified from spinach from local market.
3. All *L. monocytogenes* isolates displayed positive results for hemolysin on sheep blood agar (SBA), phosphatidylinositol-specific phospholipase C (PI-PLC) tests and CAMP test; thus confirming them virulent.
4. As per the *in-vitro* pathogenicity assays, four identified pathogenic isolates of *L. monocytogenes* were found to possess all the four targeted virulence-associated genes (*plcA, iap, hlyA,* and *actA*) as detected through PCR analysis. The results demonstrated a strong agreement between the results obtained from phenotypic and genotypic assays.

5. The molecular serotyping of *Listeria monocytogenes* revealed the presence of *ORF 2819* in four isolates and *ORF 2110* in one isolate, thereby confirming the predominance of the 1/2b serotype, followed by 4b.
6. The identification of *L. monocytogenes* being prevalent in leafy vegetables, particularly coriander leaves, holds noteworthy implications for public health because these leaves are traditionally utilized as garnishes not just in salads but also in a range of cooked dishes.
7. Despite the limited number of vegetable samples in the present study, this information is crucial for mitigating the risk of illness associated with the consumption of raw produce. In the near future, we can expect the commercialization of pre-cut vegetables and fruits in India. This research highlights the significance of focusing on how vegetables are grown, harvested, handled, processed, and distributed locally or regionally within the country. It emphasizes the need for identifying and controlling microbiological risks, and strongly recommends enforcing the application of Hazard Analysis Critical Control Points (HACCP) to ensure safe practices in these processes.

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APPENDIX-I

1) Fraser broth base Medium

A. Half Fraser broth Base

Broth base	: 54.92 gm
Distilled water	: 1000ml
pH	: 7.4 ± 0.2

B. Full Fraser broth base

Broth base	: 54.92 gm
Distilled water	: 500ml
pH	: 7.4 ± 0.

Dissolved ingredients completely and sterilize by autoclaving at 15lbs pressure (121˚C) for 15 min. Cool to around 45-50˚C and aseptically add rehydrated contents of one vial of *Listeria* selective supplements.

C. Complete medium

a. For Half Fraser broth base

Allow the base to cool and then aseptically add rehydrated contents of 1 vial of Fraser Selective Supplement (FD1251) and 2 vials of Fraser Selective Supplement (FD141) to 1000 ml medium.

b. For Full Fraser broth base

To the base, add rehydrated contents of 1 vial of Fraser Selective Supplement (FD1251) and 1 vial of Fraser Selective Supplement (FD141) to 500 ml medium.

c. PALCAM Agar

***Listeria* identification agar base**

Agar base	: 34.5 gm
Distilled water	: 500 ml
pH	: 7.0 ± 0.2

Dissolved ingredients completely and sterilize by autoclaving at 15lbs pressure (121˚C) for 15 min. Cool to around 45-50˚C and aseptically add rehydrated contents of one vial of *Listeria* selective supplements (PALCAM-FD061). Mix well and pour into sterile petri plates.

d. Brain heart infusion (BHI) Broth

BHI broth powder	: 37.0 gm
Distilled water	: 1000 ml
pH	: 7.2

Autoclaved at 121^0C for 20 min

e. Brain heart infusion (BHI) Agar

BHI agar powder	: 47.0 gm
Distilled water	: 1000ml
pH	: 7.2

Autoclaved at 121^0C for 20 min

f. Sheep blood agar (SBA)

A. Base

Nutrient agar base	
Peptone	: 10.0 mg
Beef extract	: 10.0 mg
Sodium chloride	: 5.0 mg
Adjusted pH to 7.2 ± 0.2	
Agar	: 20.0 mg
Distilled water	: 1000 ml
Dissolved the ingredients completely and autoclaved at 121^0C for 20 min.	

B. Complete Medium

Cooled the autoclaved base to 46-48^0C and aseptically added 50 ml of defibrinated sheep blood mixed properly and then poured into Petri dishes.

g. Sugar fermentation Test

A. Peptone water base

Peptone bacteriological	: 10 gm
NaCl	: 5 gm
Distilled water	: 1000 ml

B. Indicator Solution

Bromocresol purple (0.2%)	: 25 ml
Test compound	: 50 ml

C. Sugar Solution (10%)

Sugar	: 1 gm
Distilled water	: 10 ml
Filtered by passing solution through cellulose Acetate 0.20 µm	

D. Complete media

Peptone water base (A)	: 950ml
Indicator solution (B)	: 25ml
Adjusted pH to	: 7.2 to7.3
Sterilized by autoclaving at 121^0C for 20 min. cooled and poured into test tube @ 5ml/ tube.	
Add 256µl of sugar (10%) per 5ml of peptone media	

The sugars used were L-Rhamnose,D-Xylose, and α- meythyl-D-mannoside.

h. Methyl Red test

A. Medium (Glucose phosphate peptone water)

Peptone	: 5.0gm
Dipotassium hydrogen phosphate	: 5.0gm
Distilled water	: 1000ml
Glucose(10% solution)	: 50 ml
pH	: 7.6 ± 0.2

Autoclaved at 121^{0}C with 15 lbs for 15 minutes

Dissolved ingredients completely and sterilize by autoclaving at 15lbs pressure (121˚C) for 15 min.

B. Methyl Red Indicator Solution

Methyl red	: 0.1 gm
Ethanol (95%)	: 300 ml
Distilled water	: 200 ml

i. Voges- Proskauer Test

Media Contents are same as Methyl Red Test

A. Voges- Proskauer indicator solution

a. 40% potassium hydroxide solution

Potassium hydroxide	: 40.0 gm
Distilled water	: 100 ml

b. 5% α-Napthol solution

α-Napthol	: 5.0gm
Absolute ethanol	: 100 ml

c. Nitrate Reduction Test

A. Nitrate broth

Potassium nitrate	: 1.0gm
Beef extract	: 10.0 gm
Peptone	: 10.0 gm
Sodium chloride	: 5.0 gm
Distilled water	: 1000ml
Ph	: 7.2 ± 0.2
Autoclaved at 121 ^{0}C at 15 lbs for 15 minutes	

B. Test Reagent

a. Solution A

0.8% Sulphanilic acid in 5 N acetic acid(Dissolved by gentle heating)

b. Solution B

0.6% dimethyl- α- napthylamine in 5N acetic acid(Dissolved by gentle heating)

Sterile nitrate broth 5 ml was inoculated with a heavy growth of the test organism and incubated at 37^0C for 24hrs. Subsequently add 0.1 ml of test reagent to the culture in broth. The development of red color with in on minute was taken as positive reaction.

APPENDIX- II

1) Reagents for agarose gel electrophoresis:

A) Tris-glacial acetic acid EDTA (TAE) buffer (5X stock solution):

Tris base/Tris buffer	292 gm
Glacial acetic acid	57.1 gm
0.5M EDTA (pH 8.0)	100 ml
Distilled water	upto 1000 ml
For working solution, dilute 1:50 (1X TAE) for agarose gel electrophoresis	
50X TAE Buffer	20 ml
Distilled water	980 ml
Total volume	1000 ml
Store at room temperature	

B) Gel loading dye:

Blue/ Orange 6X Loading Dye	1 µl
100bp DNA ladder	5 µl
Total volume	6 µl
Store at -20°C.	

C) Ethidium bromide solution (10mg/ml):

Ethidium bromide	0.1 gm
Distilled water	10 ml
Store at 4°C	

VITA

The author, **Dr. KARPE SHIVANI SHIVAJI**, born on 7th of October 1997 in Ahmednagar District of Maharashtra. She completed her matriculation with 95.0% percentage in year 2014 and completed Higher Secondary Examination with 77.54% in the year 2016.

She has completed her undergraduate degree course (B. V. Sc. & AH) from Nagpur Veterinary College under MAFSU, Nagpur, Maharashtra, in the year 2022 securing an OGPA of 7.95 in First Class. She took admission in Nagpur Veterinary College, Nagpur under Maharashtra Animal and Fishery Science University, Nagpur for Post-graduation in Veterinary Public Health and Epidemiology discipline. She has attended the training programme held at NMRI, Hyderabad to flourish the skills of Public Health and epidemiology.

THESIS ABSTRACT

a)	Title of the thesis	:	**ASSESSMENT OF *Listeria monocytogenes* AS PROBABLE CONTAMINANT OF GREEN LEAFY VEGETABLES AND IT'S ASSOCIATED ENVIRONMENT**
b)	Full name of student	:	**Karpe Shivani Shivaji**
c)	Name and address of Major Advisor	:	(**Dr. S.P.Chaudhari)** Signature of Advisor/Guide Professor & Head Department of Veterinary Public Health
d)	Degree to be awarded	:	**Master of Veterinary Science**
e)	Year of award of degree	:	**2024**
f)	Major subject	:	**Veterinary Public Health**
g)	Total number of pages in the thesis	:	**78**
h)	Number of words in the abstract	:	**293**
i)	Signature of Student	:	
j)	Signature, Name and address of forwarding authority	:	

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ABSTRACT

In the present study, a comprehensive analysis was conducted for the assessment of *Listeria monocytogenes* as probable contaminant of green leafy vegetables and its associated environment. A total of 425 samples which included 375 samples of green leafy vegetables, comprising 75 samples each of coriander,

spinach, dill, amaranth, and fenugreek, as well as 50 environmental samples consisting of 25 soil samples, 20 water samples, and 5 manure samples were screened for isolation and identification of *Listeria monocytogenes*. Out of 375, 280 were from local market, 58 from organic farm, 37 grown alongside Nag river.

All samples underwent a dual-phase enrichment procedure using Fraser Broth (Half and Full Fraser Broth), followed by streaking onto Polymyxin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM) agar plates. Biochemical characterization revealed nine presumptive *Listeria* isolates which on further molecular characterization targeting genus specific *prs* gene and species specific *isp* gene concluded 4 (2 each from coriander and spinach) as *Listeria monocytogenes*. The remaining five isolates (3 from coriander, 1 from spinach, 1 from amaranth) amplified genus specific *prs* gene only thus concluding them to belonging to *Listeria* spp. The overall prevalence of *Listeria* spp. was noted 2.11% (9/425) while for *Listeria monocytogenes* was 0.94%. All the isolates recovered were from local market in and around Nagpur. None of the samples from organic farm, alongside Nag river cultivation and environment turned positive for *Listeria* spp. indicating its zero prevalence.

All four *L. monocytogenes* isolates displayed evident positive reactions for β-haemolysis on sheep blood agar, the CAMP test, and the PI-PLC assay, confirming their pathogenic characteristics. Additionally molecular virulence characterization of these four isolates revealed amplification of *hlyA, actA, iap, plc* gene in all 4 isolates of *L. monocytogenes*. The molecular serotyping revealed dominance of 1/2b serotype, followed by 4b.

प्रबंध सारांश

अ	**प्रबंधाचे शिर्षक**	:	**हिरव्या पालेभाज्या तसेच निगडीत वातावरणास प्रदुषित करणाऱ्या संभाव्य लिस्टेरिया मोनोसायटोजेन्स जीवाणूंचे विश्लेषण**
ब	**विद्यार्थ्याचे पूर्ण नाव**	:	**कर्पे शिवानी शिवाजी**
क	**मार्गदर्शकाचे नाव व पत्ता**	:	डॉ. सं. प्र. चौधरी प्राध्यापक व विभागप्रमुख पशुवैद्यक सामुहिक स्वास्थ्यविभाग नागपूर पशुवैद्यक महाविद्यालय, नागपूर
ड	**प्रदान करण्यात येणारी पदवी**	:	एम. व्हि. एस्सी.
इ	**पदवी प्रदान करण्याचे वर्ष**	:	२०२४
ई	**मुख्य विषय**	:	पशुवैद्यक सामुहिक स्वास्थ्य
फ	**प्रबंधातील एकूण पृष्ठ**	:	७८
ग	**सरांशातील एकूण शब्द**	:	२६२
ह	**विद्यार्थ्याची सही**	:	
ज	**अग्रेषित करणाऱ्या अधिकाऱ्याची सही, नाव आणि पत्ता**	:	

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प्रबंध सारांश

प्रस्तुत संशोधनात हिरव्या पालेभाज्या तसेच निगडीत वातावरणास प्रदुषित करणाऱ्या संभाव्य लिस्टेरिया मोनोसायटोजेन्स जीवाणूंचे

विश्लेषण करण्याच्या उद्देशाने एकत्रित अभ्यास करण्यात आला. याकरिता विविध ४२५ नमुने संकलित करण्यात आले. ज्यामध्ये ३७५ हिरव्या पालेभाज्यामध्ये प्रत्येकी ७५ कोथिंबीर,पालक,शेपू,राजगीरा तसेच मेथी भाजीचा समावेश होता. तसेच वातावरणाशी निगडीत संकलित केलेल्या नमुन्यांमध्ये २५ माती, २० पाण्याचे नमुने तसेच ५ खताच्या नमुन्यांचे समावेश होता. उपरोक्त संकलित नमुन्यांव्दारे लिस्टेरिया मोनोसायटोजेन्स जीवाणूंचे पृथ:करण व ओळख पटविण्यास प्रयोगशाळेत तपासण्यात आले.

संकलित सर्व नमुन्यांचे फ्रेजर ब्रॉथ (हाफ ॲड फुल फ्रेजर ब्रॉथ) असे व्दिस्तरीय संवर्धन करण्यात येऊन तद्नंतर पॉलीमिक्सिन ॲक्रिफलेविन लिथीयम क्लोराईड सेफ्टाझिडिम एस्कुलिन मॅनिटॉल(पाल्कम) अगार माध्यमावर स्ट्रिकिंग करण्यात आली. जैवरासायनीक चाचण्यांमध्ये एकुण ०९ संभाव्य लिस्टेरिया जीवाणूंचे पुष्टिकरण होऊन सदर जीवाणूंच्या विशिष्ट जीनस पातळीवर पीआरस जनुके तसेच विशिष्ट प्रजाती पातळीवर आयएसपी जनुक करिता आणिविक विश्लेषण करण्यात आले. यामध्ये कोथिंबीर,पालक च्या एकुण ०४ नमुन्यातील प्रत्येकी ०२ नमुन्यांव्दारे लिस्टेरिया मोनोसायटोजेन्स जीवाणूंची ओळख पटविण्यात आली. तसेच उर्वरित ०५ नमुन्यांपैकी ०३ कोथिंबीर,०१ पालक तसेच ०१ राजगीरा भाजीच्या नमुन्यांव्दारे जीनस पातळीवर पीआरस जनुकांनी सकारात्मकता दर्शविल्याने सदरील नमुने लिस्टेरिया प्रजातींचे जीवाणूंचे पुष्टिकरण झाले. अशाप्रकारे जीवाणूंचा प्रादुर्भाव हा २.११ टक्के (९/४२५)तसेच लिस्टेरिया मोनोसायटोजेन्स जीवाणूंचा प्रादुर्भाव ०.९४ टक्के असल्याचे निदर्शनास आले.सेद्रिय शेती व प्रक्षेत्र तसेच नाग नदीच्या काठालगतच्या शेतीतून तसेच सभोवतालच्या परिसरातून संकलित नमुन्यांव्दारे लिस्टेरिया प्रजातीच्या जीवाणूंकरिता प्रादुर्भाव नोंदविण्यात आलेला नाही.

लिस्टेरिया मोनोसायटोजेन्स जीवाणूंचे ०४ पृथीकृत नमुन्यांची शिप ब्लड अगार वापरून कॅम्प टेस्ट केली असता सदर माध्यमावर बिटा हिमोलायसिस करिता तसेच पीआय-पीएलसी चाचणीत सुध्दा सकारात्मकता दर्शविल्याने त्यांची रोगकारक वैशिष्टयतेची पुष्टि झाली. या व्यतिरिक्त वरील लिस्टेरिया मोनोसायटोजेन्स च्या ०४ पृथीकृत नमुन्यांचे एचएलवाय,ॲक्ट ए, आयएपी व पीएलसी जनुकांकरिता आण्विक वैशिष्टीकरण करण्यात आले. आण्विक सीरोटायपिंग मध्ये १/२बी सीरोटाईप हा ४बी सीरोटाईप पेक्षा प्रबळ असल्याचे सिध्द झाले.