

CHARACTERIZING *Listeria* species of DAIRY ENVIRONMENTS: PHENOTYPIC INSIGHTS

ABSTRACT

Aim: Genus *Listeria* include food borne pathogens. They contaminate the milk through soil, sewage and other sources in the dairy farm. In the present study, attempt was made to collect dairy environmental samples and carried out enumeration, isolation as well as phenotypic characterization of the *Listeria* isolates obtained.

Study design: The dairy environmental samples were collected from ILFC, Veterinary college, KVAFSU, Bengaluru, Karnataka and subjected for enumeration of *Listeria* and total bacterial counts. The *Listeria* isoates obtained were characterized for morphology, Gram's reaction and other biochemical tests. Identification key for *Listeria* isolates were prepared based on recent edition of Bergey's manual of systematic Bacteriology and based on the activities they were identified up to species level.

Place and Duration of Study: The work was carried out in the Department of Dairy Microbiology, Dairy Science College, KVAFSU, Hebbal, Bengaluru, Karnataka from June.2021 to June 2022.

Methodology: The dairy environmental samples were collected aseptically and enumerated for *Listeria* and total bacterial counts using PALCAM agar and Plate Count agar, respectively. The *Listeria* isolates were coded and subjected for Gram's staining, catalase test, oxidase test, motility test, methyl red and nitrate reduction to confirm the genus and specific biochemical tests like growth at 10% sodium chloride, sugar fermentation tests, CAMP test, coagulase, haemolysin and lecithinase activities on particular media. The results obtained for the tests conducted were compared with the standard identification key and declared the species of *Listeria*.

Result: "*Listeria* count in soil, fodder, dung and feed samples ranged from 2.30 to 3.14 log₁₀cfu/g, in which fodder showed more followed by feed. Udder swabs, pail rinse, pail milk and can milk had *Listeria* count of 0.30 log count while 0.60 log count was observed in chilled milk whereas water, swabs of hand, udder and equipment, pail and can rinses, air from dairy farm, aseptic milk and pasteurized milk did not show the presence of *Listeria*. The phenotypic identity of the 23 isolates of *Listeria* obtained from dairy environmental samples revealed that *Listeria monocytogenes* predominated (56.50) followed by *L. innocua* (17.39), *L. ivanovii* (8.70); *L. seeligeri* (4.35) and 13.06 percent of unidentified *Listeria* spp. All the 23 *Listeria* isolates, except one isolate of *L.monocytogenes* (L16) grew well at 5°C indicating psychrotrophic nature. At deep freeze temperature (-18°C), only 9 out of 23 *Listeria* isolates showed growth specifying their psychrophilic nature. Among 23 *Listeria* isolates, 14 showed haemolysis, phospholipase and coagulase activities indicating their pathogenic nature. "

Conclusion: Dairy fodder, soil, dung, feed showed high *Listeria* count among the dairy environmental samples. Among the twenty-three species of *Listeria*, *Listeria monocytogenes* predominated, majority were psychrotrophs, while only a few were psychrophiles and pathogens indicating the entry of pathogen from dairy farm samples to milk. Introducing strict hygienic practices at dairy farm level during milking and storage as well in processing of milk may reduce the entry of pathogenic species of *Listeria*.

Keywords: *Listeria* spp.; Dairy environmental samples; Haemolysis; Psychrophile; Lecithinase

INTRODUCTION

"Food-borne pathogens are a major threat to food safety. Most food-borne illness is caused by infection by microbial pathogens that have entered the food chain at some point from farm to fork". NIAID (2014) classified the pathogens under category A, B, C based on their transmittance and effect on human beings. *Listeria monocytogenes* is placed in category B which is easily disseminated leading to moderate morbidity and low mortality. *Listeria* is a genus consisting of Gram positive, rod shaped bacteria occurring singly or in short chains that can grow under either facultative anaerobic or aerobic condition. They are psychrophilic in nature and can tolerate the temperature range of less than 0°C to 40°C. "The species of *Listeria* are widely distributed in nature and have been isolated from soil, vegetation, sewage water, animal feed, fresh and frozen poultry raw foods, silage, slaughter-house waste and healthy human and animal carrier. *Listeria monocytogenes* is an intracellular, foodborne pathogen responsible for outbreaks of listeriosis. Listeriosis has emerged as a typical foodborne illness of major public health concern (because of the severity of the disease (meningitis, septicemia, and abortion), the high case fatality rate of 20–30% of cases " (WHO, 2014). Listeriosis predominantly affect pregnant women, neonates, elderly or immunocompromised people. "It manifests as abortion, septicaemia, meningitis and meningoencephalitis and potentially life threatening because of the mortality rate (20 – 30 percent) and hospitalization (91 percent) following

infection. The epidemiological data on listeriosis in India available to date are not adequate for assessing the extent of disease. The disease largely remains undiagnosed because of the lack of a suitable and rapid detection test due to confusion of the disease symptoms with meningitis and hepatitis. Human infections with *Listeria* arises mainly from the consumption of contaminated food like milk, icecream, contaminated water, meat products, ready to eat foods and so on. *Listeria monocytogenes* is the only *Listeria* species of public health significance as a foodborne pathogen. *Listeria ivanovii*, widely recognized as pathogenic to domestic livestock only rarely infects humans as is also true for *L. seeligeri*. *Listeria innocua*, the most commonly isolated species, is nonpathogenic as is also generally true for *L. welshimeri* and *L. grayi* " (de Noordhout *et al.*, 2014).

"*Listeria* could be a common contaminant in the dairy environment, both on the farm and in the processing plant. On the farm, important sources include manure and improperly fermented silage. It was most frequently found in moist environments or areas with condensed or standing water or milk, including drains, floors, coolers, conveyors and case washing areas" (Usman *et al.*, 2016). Korasapati *et al.* (2016) reported that *Listeria monocytogenes* survived in dahi or buttermilk and it is essential that extreme care is required to avoid the post-heating contamination of milk by adopting good hygienic practices during manufacture of dahi and butter milk. *L. monocytogenes* was found to survive for 3 months in sheep feces, 7 months in dry straw. 11.5 months in damp soil, 16.5 months in cattle feces and up to 2 years in dry feces, 2 years in dry soil, 12 years in silage, 13 years in milk and 16 years in brain sample(Hagos, 2017). Chen *et al.* (2017) disclosed that the pathogen produces toxin called Listeriolysin O (LLO) which is mainly responsible for the cause of disease coded by the gene hly A.

Once, pathogenic species of *Listeria* such as *L.monocytogenes* and *L.ivanovii* enter into processed milk and milk products, they survive even in unfavourable condition and bring about disease conditions in human beings after the ingestion of such contaminated products. "Samples of raw milk and flavoured milk were 100% contaminated by *L. monocytogenes* followed by branded milk (65.9%), cheese (62.5%), icecream (49.2%), milk powder (26.6%), milk sweets (20%), ghee and paneer (13.3%). Conversely, curd and butter were free from *L. monocytogenes*. The samples were enriched and then plated on PALCAM agar medium, the grey coloured colonies with black hallow zone were confirmed as *L. monocytogenes*" (Mary and Dandapani, 2017). "A study conducted by Chow *et al.* (2021) strikingly revealed high incidence of *Listeria monocytogenes* shedding in 90% of fecal samples from 20 lactating dairy cows in one Wisconsin farm over a 29-d period. Samples of bulk-tank milk from 444 small-ruminant farms (sheep and goat) around Greece were collected and examined by standard microbiological techniques for *Listeria* spp by enrichment and streaking on PALCAM agar medium (ISO 11290-1:2017) and found the presence of *Listeria* at the rate of 1.2 %" (Lianou *et al.*, 2022). "*Listeria* was found in 10% of pasteurized milk samples collected from retail markets across Sharkia Governorate, Egypt which were pre-enriched and streaked onto Himedia agar named *Listeria* Ottaviani Agosti agar medium" (Abou Elez *et al.*, 2023). Kananub *et al.* (2024) "conducted a study to survey *Listeria* contamination at the farm and retailer levels. Milk samples were collected from milk-collecting centers and small-scale retailers. One out of 99 farms presented *Listeria* contamination, confirmed to *L. marthii*". No *Listeria* spp but species of *Staphylococcus* and *Bacillus* were identified in the processed milk. To assess the efficacy of the processing methods, a trial using *L.*

monocytogenes as a reference strain was conducted. Interestingly, no *L. monocytogenes* was detected after sample treatment. The present study was aimed to find the occurrence of species of *Listeria* in dairy environmental samples followed by their isolation and phenotypic characterization of the isolates.

MATERIAL AND METHODS

- **Collection of sample and enumeration of *Listeria***

Solid samples such as soil, dung, feed, fodder; air & liquid samples like water, milch animal udder swab, milker's hand swab, pail rinse, can rinse, equipment swab and milk samples such as aseptic milk, pail milk, can milk, chilled milk and pasteurized milk were collected in sterile conditions from ILFC, Veterinary college, KVAFSU, Hebbal, Bangalore as per the standard procedure mentioned by Harrigan (1998). All the four teats, outer surface and inner surface of udder of milch animal were swabbed using swab (50mm thick cotton of 5 g wound around the notched end of stainless steel wire over 150 mm length and secured with thread) dipped in 25ml sterile quarter strength Ringer's solution. The outer surface and inner surface as well between fingers of both the palms of milker's hand was swabbed and collected in 25 ml of sterile quarter strength Ringer's solution. Swab of float balance tank of the pasteurizer plant was collected at nine spots using 10x10 cm² sterile square metal mask equivalent to 900 cm² area in 25ml sterile quarter strength Ringer's solution. Before carrying out the serial dilution of swab samples, swabs were rotated thoroughly and pressed above the diluent to remove all the liquid from the swabs and then discarded, the diluent was mixed and that became the swab sample. Dairy environmental samples collected were subjected for enumeration of *Listeria* species as per the procedure of

ISO 11290-1:2017. Dairy environmental samples (solid or liquid) of 11 g (solid) or ml (liquid) were weighed (for solid triturate using pestle and mortar) and transferred to the sterile 99 ml flask containing physiological saline to make the 1st dilution. Further required dilutions were prepared serially using 1st dilution. Serially diluted samples were transferred to labelled sterile petri plates for the enumeration of total bacteria and *Listeria* using sterile pipettes. Sterile molten Standard Plate Count agar (SPCA) and Polymyxin Acriflavine Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) agar (HiMedia, 1998) maintained at 55°C water bath was poured to labelled plates containing 1 ml of dilution and mixed thoroughly without spilling the medium. Later the poured agar plates were allowed to solidify. All the poured plates were incubated at 37°C/24-48 h by inverting the plate. All the colonies on SPCA while black or black green colony with a black halo and black sunken center colonies on PALCAM agar were taken as positive for *Listeria* species and counted. Average count was expressed as log₁₀ cfu/ g or ml of the sample.

2.2 Isolation and maintenance of *Listeria* isolates

The colonies of *Listeria* from PALCAM were selected based on colony morphology. Each colony selected was considered as *Listeria* and coded accordingly. These isolates were purified by streaking 3 times onto poured nutrient agar plates. After the third streak, the discrete colonies were selected and maintained on sterile PALCAM agar slants as stock cultures. Working cultures were prepared by inoculating the isolate on the slant to sterile nutrient broth tubes.

2.3 Characterization of *Listeria* species

Isolates of *Listeria* were subjected for preliminary identification and specific tests
in

order to phenotype the isolate upto species level.

2.3.1 Preliminary identification of isolates of *Listeria* species

Preliminary tests of *Listeria* isolates included Gram's staining, catalase test, oxidase test, motility test, methyl red and nitrate reduction to confirm the genus. The procedures followed for the above mentioned tests are as given by Harrigan (1998).

2.3.2 Specific tests

Growth at 10% NaCl; carbohydrate fermentation tests (lactose, L-Rhamnose, D-mannitol and D-xylose); CAMP(Christie-Atkins-Munch-Peterson); coagulase; haemolysin; lecithinase or phospholipase activity (Harrigan, 1998) were the specific tests carried out to speciate *Listeria* isolates.

2.3.2.1 List 1 : Key for the identification of *Listeria* species (Ludwig *et al.*, 2009)

Characteristics	<i>L. monocytogenes</i>	<i>L. innocua</i>	<i>L. ivanovii</i>	<i>L. seelgeri</i>	<i>L. welshimeri</i>	<i>L. gravi</i>
Preliminary Tests						
Gram staining	+	+	+	+	+	+
Motility	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+
Oxidase test	-	-	-	-	-	-
Methyl red test	+	+	+	+	+	+
Nitrate reduction test	-	-	-	-	-	-
Specific Tests						
Growth in 10% NaCl	D (variable)	+	d	D	+	+

Carbohydrate Fermentation						
Lactose	+	+	+	+	+	+
L-Rhamnose	+	d	-	-	d	+
D-Mannitol	-	-	-	-	-	+
D-Xylose	-	-	+	+	+	-
Pathogenic characteristics						
CAMP with <i>S. aureus</i>	+	-	-	+	-	-
Coagulase						
β Haemolysis	+	-	+	+	-	-
Lecithinase activity	+	-	+	d	-	-

2.3.3 Psychrotrophic and Psychrophilic nature of the *Listeria* isolates:

All the isolates were streaked on to nutrient agar slants in duplicate and one set incubated at 7°C for 10 days to observe the psychrotrophic growth and in deep freeze at -18°C for 20 days to study the psychrophilic nature of the isolates (Harrigan, 1998). After the incubation period, slant tubes were kept at room temperature and noted the growth at refrigeration and deep freeze temperatures.

2.4 Statistical analysis

All the values obtained regarding counts of total bacteria and *Listeria* in the result of the present study were average of three trials. The data was analysed using R software (R-4.3.1 for Windows) for statistical computing. ANOVA tables were prepared to analyse the data and the critical difference was calculated ($P=0.05$) and used to identify the significant differences that are indicated in the result tables through superscripts. The formula for the

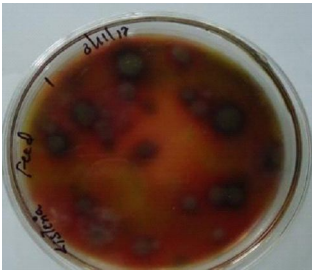
critical difference (CD) = $\sqrt{2} \times \text{MSS (E)} \times t_{\alpha}$ @ 0.05 level of significance where, MSS (E) = Mean Sum of squares of the error; r = number of replications; $t_{0.05}$ = table t from value at 0.05 level of significance.

RESULTS AND DISCUSSION

3.1 Enumeration of *Listeria* from dairy environmental samples:

All the solid and liquid environmental samples including air from dairy farm were collected aseptically and subjected for enumeration of total bacteria and *Listeria*. The total bacterial count helped to determine the percent of *Listeria* count in the samples.

3.1.1 *Listeria* in solid dairy environmental samples:



Soil, fodder, dung and feed considered under solid samples

of dairy farm of the university when plated for enumeration on selective media for *Listeria* counts that ranged from 2.30 to 3.14 $\log_{10}\text{cfu/g}$ while bacterial counts from 4.35 to 6.31 $\log_{10}\text{cfu/g}$ (Fig.1, Table 1). Among soil, fodder, dung and feed samples of dairy farm of

the university, fodder had more *Listeria* count of 3.14 log₁₀cfu/g while feed showed lowest count of 2.30 log₁₀cfu/g. Soil and dung samples showed nearly 3 log viable counts of *Listeria*. If total bacterial count was considered, dung had highest count followed by soil, fodder and feed but occurrence of *Listeria* count varied among samples with respect to the trend observed. There was no significant difference with respect to *Listeria* counts among the solid environmental samples. Total bacterial count helped to know the percent occurrence of *Listeria* count among the samples plated. Fodder, feed, dung and soil possessed 59, 53, 48 and 47 percents of *Listeria* count out of total bacterial count.

Fig. 1: Colonies of *Listeria* on PALCAM agar

Table 1: Enumeration of total bacteria and *Listeria* from solid dairy environmental

samples

Sl. No.	Name of the solid dairy environmental Sample	Counts of	
		Total bacteria	<i>Listeria</i>

		log₁₀ cfu/g (Percent)	
1	Soil	6.08 ^{ab} (100)	2.87 ^a (47)
2	Fodder	5.30 ^{ab} (100)	3.14 ^a (59)
3	Dung	6.31 ^a (100)	3.04 ^a (48)
4	Feed	4.35 ^b (100)	2.30 ^a (53)
CD(P=.05)		1.36	1.14

Note:

- All the values are the average of three trials
- Values in the parentheses indicated percent occurrence
- Different superscripts in the column and same superscripts indicates significant and non-significant differences, respectively at $P=0.05$ level.

3.1.2 *Listeria* in liquid dairy environmental samples:

The liquid based dairy farm samples like water, swab of hand, udder and equipment, pail and can rinses, milk from pail, can, chilled milk, pasteurized milk as well as air from the farm when subjected for enumeration of *Listeria* and total bacteria, exhibited 0.00 to 0.60 log₁₀cfu/ml and 0.30 to 5.16 log₁₀cfu/ml, respectively with no significant difference among the samples (Table 2). Chilled milk had more *Listeria* count (0.60 log) compared to other samples, whereas bacterial counts were more in pail milk followed by can milk compared to other dairy environmental samples.

Table 2: Enumeration of total bacteria and *Listeria* from liquid dairy environmental samples

Sl. No	Name of the liquid dairy environmental Sample	Counts of	
		Total bacteria	<i>Listeria</i>
		log ₁₀ cfu/ml (Percent)	
1	Water	2.52 ^{ab}	0.00 ^a
2	Air (log ₁₀ cfu/min)	0.30 ^b	0.00 ^a
3	Animal's udder swab	3.64 ^a (100)	0.30 ^a (8)
4	Milker's hand swab	2.78 ^{ab}	0.00 ^a

5	Pail rinse	3.07 ^{ab} (100)	0.30 ^a (10)
6	Can rinse	4.68 ^a	0.00 ^a
7	Swab of Float Balance Tank	2.60 ^{ab}	0.00 ^a
8	Aseptic milk	2.08 ^{ab}	0.00 ^a
9	Pail milk	5.16 ^a (100)	0.30 ^a (6)
10	Can milk	5.12 ^a (100)	0.30 ^a (6)
11	Chilled milk (5 °C)	4.30 ^a (100)	0.60 ^a (14)
12	Pasteurized milk	4.45 ^a	0.00 ^a
CD(P=.05)		1.89	0.35

Many of the samples like farm water supply, milker's hand swab, swab of float balance tank, aseptic milk, pasteurized milk including air on milk parlour did not show the presence of *Listeria* when plated using selective medium ie PALCAM agar. Swab of

udder, pail rinse, can rinse, milk from pail, and can showed only 0.30 log₁₀cfu/ml, while chilled milk sample had 0.60 log₁₀cfu/ml. Total bacterial counts were more in pail milk followed by can milk compared to other samples. Out of total bacterial counts, *Listeria* percent accounted for 14, 10, 8, 6 and 6 in chilled milk, pail rinse, udder swab, pail milk and can milk, respectively.

Most of the research studies of *Listeria* were on isolation but not on enumeration. Secondly, the samples analysed were milk and milk products and very few studies conducted on the dairy farm environmental samples. A local study by Matto *et al.* (2018) confirmed the presence of 70, 52 and 33 % of *Listeria innocua* in soil, dung and in feed stuff while 5.2 and 2.6 % of *L. monocytogenes* in dung and feed stuff, respectively, of pasture-based dairy farms in Paysandú, Uruguay. Another study by El-Hag *et al.* (2020), among the analysed dairy environmental samples collected in Khartoum State, Sudan, high prevalence of *Listeria* spp. was observed in water (24.2%) followed by teats (20.0%), feed (16.7%) and utensils (14.2%) compared to milk (7.5%) and air (5.8%).

Deepti *et al.* (2015) observed prevalence of *Listeria* spp. in 10 out of 100 samples of milk and milk products. (icecream, milk shake and fruit salad) obtained from Navsari, Gujrat. As per Sreeja *et al.* (2016), 2 raw milk and 3 branded pasteurized milk revealed the presence of *Listeria* spp. out of 12 samples collected from Tiruchengode, Tamil Nadu. Of the 380 milk samples tested, 21 (5.5%) were positive for *L. monocytogenes* by using two way enrichment method and isolated on selective medium ALOA (Agar *Listeria* Ottavani & Agosti). It was shown that contaminated teat cups might contribute to the transmission of *L. monocytogenes* in the herd (Skowron *et al.*, 2019).

3.1.3 Isolation of *Listeria* species from dairy environmental samples:

A total of 23 numbers of isolates of *Listeria* were obtained from the dairy environmental samples that showed the counts of *Listeria*. As soil had more *Listeria* count, hence more isolates from soil (6) followed by dung(4); feed (4); fodder(3) and remaining udder swab, pail rinse, can rinse, pail milk, can milk and chilled milk samples that had lower counts of *Listeria* ie., one each were obtained (Table 3).

Table 3: Number of *Listeria* isolates obtained from dairy environmental samples

Name of the Dairy environmental sample	Codes of <i>Listeria</i> isolates	Number of isolates obtained
Soil	L1, L2, L3, L4, L5, L6	6
Fodder	L7, L8, L9	3
Dung	L10, L11, L12, L13	4
Feed	L14, L15, L16, L17	4
Animal swab	L18	1
Handler swab	L19	1
Pail rinse	L20	1
Can rinse	L21	1
Pail milk	L22	1
Chilled milk	L23	1
Total		23

3.2 Phenotypic characterization of *Listeria* isolates

All the 23 *Listeria* isolates obtained from various dairy environmental samples were subjected for preliminary tests like staining, motility, catalase, oxidase, methyl red and nitrate reduction tests. *Listeria* isolates were Gram positive rods, motile, catalase, oxidase, methyl red positive and reduced nitrate. Further specific tests were conducted to speciate the *Listeria* isolates with coagulase, CAMP test, haemolysin production, acid from lactose, L-Rhamnose, D-Mannitol, D-Xylose, growth at 10 % sodium chloride and lecithinase activity. Based on the results obtained, after comparing with identification key (2.3.2.1) and further identified as non-halophilic (means showing no growth at 10 percent sodium chloride) 8 numbers of *Listeria monocytogenes* (L1, L2, L9, L10, L15, 16, L17, L20); 4 numbers of *Listeria innocua* (L3, L4, L5, L19); 2 numbers of *Listeria* sp.(L6, L7); 1 number of *Listeria ivanovii* L18 and 1 number of *Listeria seeligeri* (L23). The halophilic group (means showing growth at 10 percent sodium chloride) had 5 numbers of *Listeria monocytogenes* (L8, L11, L13, L14, L21); 1 number of *Listeria* sp. (L12) and one number of *Listeria ivanovii* L22 (Table 4).

"All the collected samples (milk (n= 30), curd, [buttermilk](#), [paneer](#), and [ghee](#) (n= 22), street food (panipuries, sauces, and juices) (n=33), as well as salad (fruit and vegetables) (n= 7).) from different street vendors and dairy booths in and around the city of Belagavi, North Karnataka, India, were screened for the presence of *Listeria* spp on PALCAM agar. A total of 11 putative *Listeria* spp colonies were picked and taken through a number of biochemical analyses for further characterization. Out of the 11 isolates, 7 originated from milk, 2 from buttermilk and 2 from street food; none was

detected from fruits or vegetable salads. Gram staining assay revealed that all 11 colonies contained Gram-positive rod bacterial cells. Besides, MR-VP, [oxidase](#) and [catalase](#) assays were all positive tests, which are typical of the species *Listeria*" (Sharma *et al.*, 2024).

Table 4: Phenotypic characterization of *Listeria* isolates obtained from dairy environmental samples

Codes of <i>Listeria</i> Isolates	Names of biochemical tests								Identity of <i>Listeria</i> spp.	
	Coagulase	CAMP with <i>S. aureus</i>	Haemolysin	Acid production from				Growth at 10% NaCl		Lecithinase
				Lactose	L-Rhamnose	D-Mannitol	D-Xylose			
L1, L2, L9, L10, L15, L16, L17, L20 (8)	+	+	+	+	+	-	-	-	+	<i>L. monocytogenes</i>
L8, L11, L13, L14, L21 (5)	+	+	+	+	+	-	-	+	+	<i>L. monocytogenes</i>
L3, L4, L5, L19 (4)	+	-	-	+	-	-	-	+	-	<i>L. innocua</i>
L6, L7 (2)	+	-	-	+	-	-	-	-	-	<i>Listeria</i> spp.
L12 (1)	+	+	-	+	-	-	-	+	-	<i>Listeria</i> sp.
L18 (1)	+	-	+	+	-	-	-	-	+	<i>L. ivanovii</i>
L22(1)	+	-	+	+	-	-	-	+	+	<i>L. ivanovii</i>
L23 (1)	+	+	+	+	-	-	+	-	+	<i>L. seeligeri</i>

Note: All the 23 isolates were Gram positive rods, motile, catalase positive, oxidase, methyl red positive and reduced nitrate

3.2.1 Distribution of phenotyped *Listeria* isolates in dairy environmental samples

Soil isolates of *Listeria* (6) identified as *Listeria monocytogenes* (2); *L.innocua* and one remained as unidentified species of *Listeria*. Fodder *Listeria* isolates of three

numbers were identified as *L.monocytogenes* (2) and one remained as unidentified species of *Listeria*. Dung isolates of 4 numbers were characterized as *L.monocytogenes* while among 4 feed isolates, 3 were *L.monocytogenes* and one remained as unidentified species of *Listeria*. Each isolate obtained from animal's udder swab, pail rinse, can rinse, pail milk, can milk and chilled milk samples were identified as *L. ivanovii*, *L.monocytogenesi*, *L. monocytogenes*, *L. ivanovii*, *L. innocua* and *L. seeligeri*, respectively (Table 5).

Table 5: Distribution of phenotyped *Listeria* species isolated from dairy environmental samples

Sources	<i>Listeria</i> species				
	<i>L.monocytogenes</i>	<i>L.innocua</i>	<i>L.ivanovii</i>	<i>L. seeligeri</i>	<i>Listeria</i> sp.
Soil	2 (8.7)	3 (13.0)	0	0	1 (4.3)
Fodder	2 (8.7)	0	0	0	1 (4.3)
Dung	3 (13.0)	0	0	0	1 (4.3)
Feed	4 (17.4)	0	0	0	0
Animal swab	0	0	1 (4.3)	0	0
Handler swab	0	1 (4.3)	0	0	0
Pail rinse	1 (4.3)	0	0	0	0
Can rinse	1 (4.3)	0	0	0	0
Pail milk	0	0	1 (4.3)	0	0

Chilled milk	0	0	0	1 (4.3)	0
Total	13 (56.5)	04 (17.4)	02 (8.6)	01 (4.3)	03 (12.9)

3.2.2 Occurrence of *Listeria* spp. in dairy environmental samples

Among 23 isolates of *Listeria* obtained from dairy environmental samples, the species identified were *L. monocytogenes* (13), *L. innocua* (4), *L. ivanovii* (2), *L. seeligeri* (1) and three as unidentified *Listeria* species. Among the species of *Listeria*, *L. monocytogenes* (56.50 %) predominated followed by *L. innocua* (17.39 %), *L. ivanovii* (8.70 %); *L. seeligeri* (4.35) while 13.06 percent were unidentified *Listeria* sp.(Fig. 2).

Fig. 2: Percent occurrence of *Listeria* spp. in dairy environmental samples

El Hag *et al.*, (2020) found the presence of *Listeria* spp. in milk and were identified as *L. monocytogenes* (2.5%), while 1.7% for each of *L. seeligeri*, *L. welshimeri* and *L. ivanovii* in 720 total dairy farm samples collected from 120 dairy farms in Khartoum state, Sudan. The most frequently isolated species was *L. welshimeri* (33.3%). Ahimed *et al.* (2022), detected *Listeria monocytogenes* in silage feed, cow barn, milk from cow teat, milking parlour floor, pooled milk at supply and milk measuring equipment. Apart from *Listeria monocytogenes* other detected *Listeria* species were *L. innocua* 10 (5%), *L. gray* 6 (3%), *L. ivanovii* 5 (2.5%) and *L. seeligeri* and *L. welsheri* at 4 (2%) . The silage sample was found positive for all *Listeria* species except for *L. ivanovii*. Milk from cow's teat was positive for all isolates except for *L. ivanovii* and *L. seeligeri*.

Likewise, milk measuring equipment was positive for all species, except for *L. innocua* and *L. seeligeri*. Kananub *et al.* (2024) revealed that, one out of 99 farms exhibited *Listeria* contamination, confirmed to be *L. marthii*. *Listeria* spp. were not detected in the processed milk samples collected from from milk-collecting centers and small-scale retailers of central part of Thailand.

Matto *et al.* (2018) collected samples from bovine faeces, feedstuffs, bulk tank milk, drinking water and soil from the entry and exit pens of the milking parlour for detection of *Listeria* spp., particularly *L. monocytogenes*, in cattle and environment of pasture based dairy farms in Paysandú, Uruguay using *Listeria* UVM II enrichment broth. *Listeria* spp. were isolated from eight of ten dairy farms, whereas *L. monocytogenes* in three of them. *L. monocytogenes* or *L. innocua* excreted from clinically healthy milking cows was detected via faeces. In feedstuffs, only one *L. monocytogenes* isolate from a pasture was obtained.. No isolation of *Listeria* spp. was retrieved from the bulk tank milk or drinking water from any of the farms. *Listeria innocua* was detected in 13 feedstuffs and seven samples of soil from the entry and exit pens of the milking parlour. This is a first local study that confirms the presence of *Listeria* spp. including *L. monocytogenes* in healthy cattle and environment of pasture-based dairy farms. These results suggest the potential role that healthy cattle and their sub-products would play as a source of these agents for humans and/or others animals.

The overall isolation rate of *Listeria* spp. was 16% from three hundred and fifty samples including; normal raw milk (n=200), mastitis milk (n=50), feces of dairy cattle (n=50) of dairy cows in Sharkia and Dakahlia Governorates, Egypt. The prevalence rates of *Listeria monocytogenes* were 8%, 4% and 2% in feces of dairy cows, normal milk and

mastitis milk, respectively. The prevalence of *L. ivanovii* and *L. welshimeri* in milk of dairy cows were 6% and 4%, respectively. *Listeria* spp. isolated from normal milk, feces and mastitis milk, potentially can cause human illness (EL-Sayed *et.al.*, 2019). According to Idland *et al.* (2022), silage (32%) showed higher prevalence of *Listeria monocytogenes* followed by dung (30%), milk filter (13%) and teat swab (5%), but teat milk and bulk milk samples did not show the presence of the pathogen in Norwegian dairy cattle farms

3.3 Psychrotrophic and psychrophilic nature of *Listeria* isolates obtained from dairy environmental samples

It was interesting to note that except *L.monocytogenes* L16 (Table 6) , all the isolates grew well at 7°C. At deep freeze temperature (-18°C), only 9 out of 23 *Listeria* isolates showed growth that included *Listeria monocytogenes* (L8, L9, L10, L13, L21 – 5 nos.), *Listeria innocua* (L5 – 1 no.), *Listeria* sp. (1 no.), *Listeria ivanovii* (L22 – 1 no.) and *Listeria seeligeri* (L23 – 1 no).

Table 6: Species of *Listeria* isolated from dairy environmental samples that grow at refrigeration

and deep freeze temperature

Species of <i>Listeria</i> with codes that grew at 7 °C (refrigeration temperature)	Species of <i>Listeria</i> with codes that grew at -18 °C (Deep freeze temperature)
<i>Listeria monocytogenes</i> [L1, L2, L8, L9, L10, L11, L13, L14, L15, L17, L20, L21] (12)	<i>Listeria monocytogenes</i> [L8, L9, L10, L13, L21] (5)
<i>Listeria innocua</i> [L3, L4, L5, L19] (4)	<i>Listeria innocua</i> [L5] (1)

<i>Listeria</i> sp. [L6, L7, L12] (3)	<i>Listeria</i> sp. [L12] (1)
<i>Listeria ivanovii</i> [L18, L22] (2)	<i>Listeria ivanovii</i> [L22] (1)
<i>Listeria seeligeri</i> [L23] (1)	<i>Listeria seeligeri</i> [L23] (1)
22 nos.	9 nos.

Saha *et al.* (2015) "*L.monocytogenes* is a psychrotroph with resistance to high temperatures.

It has wide temperature range for growth. It multiplies at temperatures ranging from 1.5°C to 45°C.

Yu *et al.* (2023) found *L. monocytogenes* cells cultivated at low temperature presented a less damage

and had a higher survival rate when exposed to acid electrolysed water. "

3.4 Pathogenic nature of species of *Listeria* obtained from dairy environmental samples

The phenotyped *Listeria* isolates of 23 numbers when streaked on blood agar, blood agar with *Staphylococcus aureus* (CAMP test Fig. 3), egg yolk agar and further tested for coagulase using bovine serum, 14 numbers of *Listeria* spp. showed haemolysis, zone around *S.aureus* in CAMP test, hazy zone and coagulated blood serum indicating the pathogenic characteristics of the isolates that included *Listeria monocytogenes* (L1, L2, L8, L9, L10, L11, L13, L14, L15, L16, L17, L20, L21 -13 nos.) and *Listeria seeligeri*

{L23 – 1 no.), while *Listeria ivanovii* (L18, L22 -2 nos.) showed all the characteristics except negative for CAMP test. *Listeria innocua* (L3, L4, L5, L19 - 4 nos.) and *Listeria* sp.(L6, L7, L12 - 3 nos.) only revealed haemolysin production on blood agar while other pathogenic characteristics were absent. With regard to haemolysis, 7 *Listeria* isolates did not show any haemolysis, 3 isolates showed 4 mm haemolysis, 9 isolates had 5 mm haemolysis, 3 isolates of 6 mm and 1 isolate of 10 mm diametric haemolysis (Table 7).



Fig.3: CAMP test for species of *Listeria* with *Staphylococcus aureus* as test organism

Table 7: Pathogenic characteristics of species of *Listeria* isolated from dairy environmental samples

Name of <i>Listeria</i> species with codes	Tests conducted to determine the pathogenicity			
	Coagulase	CAMP with <i>S. aureus</i>	Haemolysis	Lecithinase
<i>Listeria monocytogenes</i> [L1, L2, L8, L9, L10, L11, L13, L14, L15, L16, L17, L20, L21] (13)	+	+	+	+
<i>Listeria seeligeri</i> [L23] (1)	+	+	+	+
<i>Listeria ivanovii</i> [L18, L22] (2)	+	-	+	+
<i>Listeria innocua</i> [L3, L4, L5, L19] (4)	+	-	-	-
<i>Listeria</i> sp. [L6, L7, L12] (3)	+	-	-	-

"The interaction between *Staphylococcus aureus* and *L. monocytogenes* was studied through CAMP Test: CAMP" (Christie, Atkins and Munch Peterson). "In this test blood agar plates were used, where in *S. aureus* was streaked on the center of the agar plate as straight line and *L. monocytogenes* was streaked as perpendicular to the central staphylococcal inoculum, starting about 5 mm from the central streak and extending towards the periphery of the agar, incubated at 37°C for 24 hours" (Sreeja *et al.*, 2016). Thomas *et al.* (2022) "opined that β -haemolysis and the CAMP tests are recommended to differentiate pathogenicity among species of *Listeria*. *Listeria* Isolates were cultured from food and environmental swabs of several food processing facilities across all of the provinces in South Africa during the listeriosis outbreak period. Among 50 *Listeria* species, 20 *L. monocytogenes* strains; 27 *L. innocua*, two *L. seeligeri* and one *L. welshimeri*. β -haemolysis

was performed on sheep blood agar while for CAMP test *Staphylococcus aureus* ATCC strain 25923 was used. β -haemolysis and the CAMP test were absent in 18% of the isolates of *L. monocytogenes*; one out of the 27 *L. innocua* isolates was positive for both β -haemolysis and the CAMP test. However, these tests can give delayed positivity (up to three days later) ".

CONCLUSION

The dominant species in the present study in various dairy environmental samples was *Listeria monocytogenes* (56.50 %) followed by *L. innocua* (17.39 %), *L. ivanovii* (8.70 %); *L. seeligeri* (4.35). *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* also exhibited coagulase, haemolysin as well lecithinase activities indicating their pathogenic nature. Hence the presence of *Listeria* species in dairy environment suggest the significant role of hygiene required in the dairy farm. The present study concluded that the diversity in the prevalence of *Listeria* spp. in the dairy farm environment necessitates the implementation of *Listeria* risk assessment in the milk supply chain starting with milk production. Dairy farm hygiene and environment showed significant impact on the prevalence of *Listeria* spp.

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Abou Elez, R.M.M., Elsohaby, I., Al-Mohammadi, A.R., Seliem, M., Tahoun, A.B., Abousaty, A. I., *et al.* 2023. Antibacterial and anti-biofilm activities of probiotic *Lactobacillus plantarum* against *Listeria monocytogenes* isolated from milk, chicken and pregnant women. *Front Microbiol.*, 14:1201201.

<https://doi.org/10.3389/fmicb.2023.1201201>

Ahimed, H.M, Hiko, A., Abdellah, A., Muktar, Y. and Gutema, F. D. 2022. Isolation and multidrug drug resistance profile of *Listeria* species in selected Dairy Farm's Operational stages in Oromia Regional State, Ethiopia. *Sci Afr.*, 16: e01167.

<https://doi.org/10.1016/j.sciaf.2022>

Chen, J.Q., Regan, P., Laksanalamai, P., Healey, S. and Hu, Z. 2017. Prevalence and methodologies for detection, characterization and subtyping of *Listeria monocytogenes* and *L. ivanovii* in foods and environmental sources. *Food Sci Hum wellness*, 6(3):97-120.

Chow, J. T. H., Gall, A.R., Johnson, A.K. and Huynh, T.N. 2021. Characterization of *Listeria monocytogenes* isolates from lactating dairy cows in a Wisconsin farm: Antibiotic resistance, mammalian cell infection and effects on the fecal microbiota. *J Dairy Sci.*, 104

(4):4561- 4574.

de Noordhout, C. M., Devleeschauwer, B., Angulo, F. J., Verbeke, G., Haagsma, J., Kirk, M., *et al.* 2014. The global burden of listeriosis: a systematic review and meta-analysis. *Lancet Infect Dis.*, 14(11):1073-1082.

Deepti, N.N., Savalia, C.V., Kalyani, I.H., Kumar, R. and Kshirsagar, D.P. 2015. Isolation, identification, and characterization of *Listeria* spp. from various animal origin foods. *Vet World*, 8(6):695-701.

El Hag, M. M. A., El Zubeir, I. E. M. and Mustafa, N.M. 2020. Prevalence of *Listeria* species in dairy farms in Khartoum State (Sudan). *Food Control*, 107699. doi:10.1016/j.foodcont.2020.107699

El-Sayed, Y.M., El Naenaey, E. Y., [Abdelwahab](#), A., Abdallah, M.A., Merwad, A.M.A. and Abdou, H. M. A. 2019. Prevalence of *Listeria* species in dairy cows and pregnant women with reference to virulotyping of *Listeria monocytogenes* in Egypt. *Zagazig Vet J.*, 47(3):248-258.

Hagos, H. A., 2017. Review of animal listeriosis, diagnosis, etiology and its public health. *World J. Adv. Healthc Res.*, 1(1): 9-24

Harrigan, W. F. 1998. *Laboratory Methods in Food Microbiology*. Pub: Elsevier -

Academic Press.

HiMedia. 1998. HiMedia Manual for Microbiology Laboratory Practice. HiMedia Laboratories Pvt Limited, Mumbai.

Idland, L., Granquist, E. G., Aspholm, M. and Lindbäck, T. 2022. The occurrence of *Campylobacter* spp., *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* in Norwegian dairy cattle farms; a comparison between free stall and tie stall housing systems. J Appl Microbiol., 132(5):3959 – 3972.

ISO 11290-1:2017 - Microbiology of the food chain horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. Part 1: Detection method, International Organization for Standardization, ISO Central Secretariat, Geneva, Switzerland.

[Kananub, S.](#), [Lertsakkongkul, P.](#), [Aryatawong, P.](#), [Horhirunkhajohn, W.](#), [Pinniam N.](#), [Krajanglikit, P.](#), *et al.* 2024. *Listeria* contamination in milk-processing chain and proficiency in *Listeria monocytogenes* decontamination of small-scale milk retailers. J Food Qual., 6263938. <https://doi.org/10.1155/2024/6263938>

Korasapati, N.R., Kumar, S., Sing, A. and Thippareddi, H. 2016. Behaviour of *Listeria monocytogenes* in Butter milk, an Indian Fermented Milk Beverage. Indian J Dairy Sci., 69(4): 447-454.

Lianou, D.T., Skoulakis, A., Michael, C.K., Katsarou, E.I., Chatzopoulos, D.C., Solomakos, N., *et al.* 2022. Isolation of *Listeria ivanovii* from bulk-tank milk of sheep and goat farms—from clinical work to bioinformatics studies: prevalence, association with milk quality, antibiotic susceptibility, predictors, whole genome sequence and phylogenetic relationships. *Biology (Basel)*, 11(6):871 doi: 10.3390/biology11060871.

Ludwig, W., Schleifer, K. and Whitman, W. B. 2009. Family Liateriaceae, *Bergey's manual of systematic bacteriology*, 2nd Edn. vol.3, The Firmicutes, Springer, pp. 244-256.

Mary, M. S. and Dandapani, S. 2017. Pervasiveness of *Listeria monocytogenes* in milk and dairy products, *J. Food Microbial Saf. Hyg.*, 2(3):1-5.

Matto, C., Varela, G., Braga, V., Vico, V., Ruben, E., Giannechini, R. E. and Rivero, R. 2018. Detection of *Listeria* spp. in cattle and environment of pasture-based dairy farms. *Braz J Vet Res.*, 38(9):1736-1741.

NIAID, 2014. National Institute of Allergy and Infectious Diseases. Emerging Infectious Diseases/Pathogens.<https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens>.

R-4.3.1 for Windows. The R Foundation for statistical Computing. <https://cran.r-project.org/bin/windows/base/>

Saha, M., Debnathand, C. and Pramanik, A.K. 2015. *Listeria monocytogenes*: An Emerging Food Borne Pathogen. Int J Curr Microbiol Appl Sci., 4(11): 52-72.

Sharma, R.K., Jalalpure, S. S., Pathak, S., Ganapathy, S., Desvaux, M., Roy, S. and Hegde, S. 2024. Molecular detection of *Listeria monocytogenes* from different dairy and street food sources in North Karnataka, India. J. Infect. Public Health, 17(4):696-703.

[Skowron, K., Wałęcka-Zacharksa, E., Grudlewska, K., Wiktorczyk, N., Kaczmarek, A., Gryń, G., et al.](#) 2019. Characteristics of *Listeria monocytogenes* strains isolated from milk and humans and the possibility of milk-borne strains transmission. Pol. J. Microbiol., 68(3): 353-369.

Sreeja, S., Moorthy, K., Vivek, N. And Upasani, V. N. 2016. Prevalence of *Listeria monocytogenes* in Raw and Pasteurized Milk Samples from Tiruchengode (TN), India. Int. J. Inn. Res. Sci. Eng. Technol., 5(2):1419-1424.

[Thomas, T. S. M., Thomas, J., Roux, K.L., Duze, S. T., Mkhwanazi, F. and Duse, A.](#) 2022. Diagnostic challenges with accurate identification of *Listeria monocytogenes* isolates from food and environmental samples in South Africa. Afr. J. Lab. Med., 11 (1):1482 doi: [10.4102/ajlm.v11i1.1482](https://doi.org/10.4102/ajlm.v11i1.1482).

Usman, U. B., Kwaga, J. K. P., Kabir, J., Lonitola, O. S. O., Radu, S. and Bande, F.

2016. Molecular characterization and phylogenetic analysis of *Listeria monocytogenes* isolated from milk and milk products in Kaduna, Nigeria. Canadian J. Infec. Dis. Med. Microbiol., 1-7. 4313827 doi: 10.1155/2016/4313827.

WHO, 2014. World Health Organization - Study measures global burden of *Listeria*.
<https://www.foodsafetynews.com/2014/10/study-measures-global-burden-of-Listeria/>

Yu, M., Jiang, C., Meng, Y., Wang, F., Qian, J., Fei, F., *et al.* 2023. Effect of low temperature on the resistance of *Listeria monocytogenes* and *Escherichia coli* O157:H7 to acid electrolyzed water. [Food Res. Int.](#), 168: 112776. doi: 10.1016/j.foodres.2023.112776.