

PHENOTYPIC CHARACTERISTICS OF THE SPECIES OF *LISTERIA* OBTAINED FROM DAIRY ENVIRONMENTAL SAMPLES

ABSTRACT

In present study, *Listeria* count in soil, fodder, dung and feed samples collected from KVAFSU dairy farm ranged from 2.30 to 3.14 log₁₀cfu/g, in which fodder showed more followed by feed. Udder swab, pail rinse, pail milk and can milk had *Listeria* count of 0.30 log count while 0.60 log count observed in chilled milk whereas water, swabs of hand, udder and equipment, pail and can rinses, air of 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 per cent of unidentified *Listeria* spp. All the 23 *Listeria* isolates, except *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.

Highlights:

- 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.

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 their 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 per cent) and hospitalization (91 per cent) 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 plates were incubated at 37 °C for 48 h. Grey coloured colonies with black hallow zone were counted (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 *Listeria* Ottaviani Agosti (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 *Staphylococcus* spp. and *Bacillus* spp. 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 their isolation and phenotypic identification 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 swab, handler 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 with prior permission. Dairy environmental samples collected were subjected for enumeration of *Listeria* species as per the procedure of Harrigan (1998). Dairy environmental samples (solid or liquid) of 11 g (solid) or ml (liquid) was weighed (for solid triturate using pestle and mortar) and transferred to the sterile 99 ml flask containing physiological saline to make 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 nutrient 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

Carbohydrate fermentation test, CAMP, coagulase, growth at different temperatures and lecithinase or phospholipase activity tests (Harrigan, 1998) were the specific test to carried out speciate *Listeria* isolates.

| 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 | + | + | + | + | + | + |
| D mannitol | - | - | - | - | - | + |
| D xylose | - | - | + | + | + | - |
| Pathogenic characteristics | | | | | | |
| CAMP with <i>S. aureus</i> | + | - | - | + | - | - |
| Coagulase | | | | | | |
| β Haemolysis | + | - | + | + | - | - |
| Lecithinase activity | + | - | + | D | - | - |

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

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 ($P=.05$) 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)

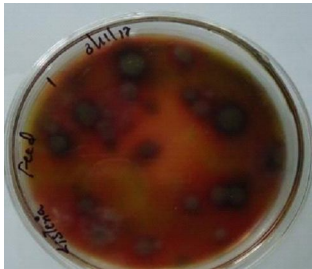
= Mean Sum of squares of the error; r = number of replications; $t_{0.05}$ = table t from value at 0.05 level of significance.

RESULTS AND DISCUSSION

3.1 Enumeration of listeria from dairy environmental samples:

All the solid and liquid environmental samples including air of dairy farm were collected aseptically and subjected for enumeration of total bacteria and listeria. The total bacterial count helped to determine the per cent 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} cfu/g while bacterial counts from 4.35 to 6.31 \log_{10} 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 \log_{10} cfu/g while feed showed lowest count of 2.30 \log_{10} cfu/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. Total bacterial count helped to know the per cent occurrence of listeria count among the samples plated. Fodder, feed, dung and soil possessed 59, 53, 48 and 47 per cents 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 | TBC | Listeria |
|------------------|--|---------------------------------------|---------------------------|
| | | log ₁₀ cfu/g (Per cent) | |
| 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 per cent occurrence
- Different superscripts in the column indicate significant difference at $P=.05$ 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 of the farm when subjected for enumeration of listeria and total bacteria, exhibited 0.00 to 0.60 $\log_{10}\text{cfu/ml}$ and 0.30 to 5.16 $\log_{10}\text{cfu/ml}$, 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 | TBC | Listeria |
|--------|---|--|---------------------------|
| | | log ₁₀ cfu/ml (Per cent) | |
| 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 per cent 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 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 which 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 i.e., 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 listeria 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. The *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, haemolysin production, CAMP test, acid from lactose, xylose, D-mannitol and growth at 10 % sodium chloride. Based on the results obtained, after comparing with identification key (3.11) and further identified as non-halophilic (means showing no growth at 10 per cent 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 per cent 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 listeria Isolates | Names of biochemical tests | | | | | | | Identity of <i>Listeria</i> spp. | |
|---|----------------------------|----------------------------|-------------|----------------------|--------|-------------|--------------------|----------------------------------|--------------------------|
| | Coagu lase | CAMP with <i>S. aureus</i> | Haem olysis | Acid production from | | | Growth at 10% NaCl | | Lecith inase |
| | | | | Lactose | Xylose | D-man nitol | | | |
| L1, L2, L9, L10, L15, L16, L17, L20 (8) | + | + | + | + | - | - | - | + | <i>L. monocyto genes</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> sp. |
| 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 *Listeria ivanovii*, *Listeria monocytogenesi*, *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria innocua* and *Listeria*

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 *Listeria monocytogenes* (13), *Listeria innocua* (4), *Listeria ivanovii* (2), *Listeria seeligeri*(1) and three as unidentified *Listeria* species. Among the species of *Listeria*, *Listeria monocytogenes* (56.50 %) predominated followed by *Listeria innocua* (17.39 %), *Listeria ivanovii* (8.70 %); *Listeria seeligeri* (4.35) while 13.06 per cent were unidentified *Listeria* sp.(Fig. 2).

Fig. 2: Per cent 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 *Listeria 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

All the 23 listeria isolates were streaked on nutrient agar slant in duplicate and one set incubated at 5°C in refrigerator and other set in deep freezer at -18°C for 10 days. After incubation, it was interesting to note that except *L.monocytogenes* L16 (Table 6) , all the isolates grew well at 5°C. At deep freeze temperature, 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 5 °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) stated that *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 AEW. Low temperature stress enhanced resistance of *L. monocytogenes*.

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 (Table 7). 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 8). *Listeria monocytogenes* L10 was one such listeria isolate that showed highest DMC of 8.95 log₁₀/ml as well as haemolysis diameter of 10 mm with significant difference when compared with other isolate. Based on these two features, the isolate phenotyped as *Listeria monocytogenes* L10 was selected for further studies.



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 *Listeria innocua* (17.39 %), *Listeria ivanovii* (8.70 %); *Listeria seeligeri* (4.35). *Listeria monocytogenes*, *Listeria ivanovii* and *Listeria 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.

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