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### Native *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus acidophilus* Strains from Traditional Sri Lankan Buffalo Curd Reveal Significant Strain-Specific Antagonistic Potential Against *Listeria monocytogenes* NCTC 11994

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#### ABSTRACT

The excessive use of antibiotics to control foodborne pathogens has contributed to the emergence of antibiotic-resistant *Listeria monocytogenes*, posing a significant global public health concern. In response to this challenge, the present study aimed to evaluate the potential of native *Lactobacillus* species as natural antibacterial agents against *L. monocytogenes*. Cell-free supernatants (CFS) of nine *Lactobacillus* strains comprising *L. plantarum*, *L. fermentum*, and *L. acidophilus* isolated from traditionally fermented buffalo curd in Sri Lanka were tested for antagonistic activity against *L. monocytogenes* NCTC 11994 using the agar well diffusion method on Brain Heart Infusion (BHI) agar. Among the tested strains, *L. plantarum* T3b showed no inhibition, whereas the remaining eight strains demonstrated varying degrees of antagonistic activity, with inhibition zones ranging between 10.77 mm and 14.10 mm, indicating a strain-specific antibacterial effect. These findings highlight the potential of traditional Sri Lankan buffalo curd as a source of native *Lactobacillus* strains with a functional probiotic characteristic and natural bio-preservative properties. The study contributes to the growing body of evidence supporting the use of beneficial microbes as biological alternatives for controlling foodborne pathogens and emphasizes the value of indigenous fermented foods in public health and food safety strategies.

**Keywords:** Agar well diffusion, Antibacterial activity, Buffalo curd, Cell-free supernatant (CFS), *Lactobacillus* and *Listeria monocytogenes*

#### 1.0 INTRODUCTION

Foodborne diseases present considerable health concerns worldwide, with millions of people being affected yearly. According to the World Health Organization (WHO), cases of foodborne diseases caused by pathogens are as high as 600 million and fatalities are around 420,000 each year<sup>[1-4]</sup>. Among foodborne bacteria, *Listeria monocytogenes* is of particular concern due to its lethal consequences, known as listeriosis<sup>[5,6]</sup>. *L. monocytogenes* poses a

significant threat in food processing and storage due to its ability to survive and proliferate in diverse environments, including refrigerated temperatures<sup>[6,7]</sup>. Listeriosis primarily affects the gastrointestinal tract, and it may cause prolonged, asymptomatic bacteremia, which may disseminate to the brain or placenta with the development of meningitis or encephalitis. Listeriosis has a relatively high mortality rate, exceeding 20% in affected individuals<sup>[5-7]</sup>.

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The first documented isolation of *L. monocytogenes* in Sri Lankan food was reported in 1995<sup>[8]</sup>. Since then, the prevalence of *L. monocytogenes* in different types of food in Sri Lanka has been reported. More recently, strains of *L. monocytogenes* were identified in raw milk samples from Polonnaruwa district<sup>[9]</sup>. Moreover, *L. monocytogenes* has also been isolated in Sri Lanka in leafy vegetables as well as in fish from Negombo area<sup>[10,11]</sup>.

Despite these findings, reports of human listeriosis in Sri Lanka remain limited. The first documented human case in Sri Lanka was reported in 1967, followed by two cases of neonatal listeriosis in 1992<sup>[12,13]</sup>. Since then, there has been a notable absence of human cases in the scientific literature. However, in 2021, a sporadic outbreak of listeriosis in goats was recorded in the Jaffna region, indicating the pathogen's continued presence in the environment<sup>[14]</sup>.

*L. monocytogenes* has gained more attention particularly in the international market. According to local and international food safety regulations, including SLS 516:part 15/section 1:2017 and ISO 11290-1:2017, certification confirming the absence of *L. monocytogenes* is required in certain food items<sup>[15,16]</sup>. Therefore, *L. monocytogenes* is one of the primary pathogens routinely tested in industrial food items in Sri Lanka.

Currently, treatments for listeriosis are entirely dependent on antibiotics. The CDC has recommended antibiotics such as penicillin, ampicillin, or amoxicillin as treatments for *Listeria* infections<sup>[17-19]</sup>. However, the emergence of antibiotic-resistant bacteria has become a global issue which poses a significant threat to the public health<sup>[20,21]</sup>. This trend is also seen in *L. monocytogenes*, with antibiotic-resistant strains becoming increasingly common worldwide. In 2024, studies conducted in Egypt, Romania and in Italy, reported multidrug-resistant *L. monocytogenes* strains recovered from different food items<sup>[22-24]</sup>. In a most recent Sri Lankan report from 2022, antibiotic-resistant *L. monocytogenes* has been isolated from raw milk samples. Among the

tested isolates, the highest resistance was observed against ampicillin (60%), while lower resistance was noted for streptomycin (18%) and tetracycline (8%)<sup>[25]</sup>. If the issue of overall antibiotic resistance is not well addressed, it has been evaluated that it will cause 10 million deaths every year by 2050 and will have a devastating effect on the economy<sup>[20,26,27]</sup>.

With the rising problem of antibiotic resistance, it is necessary to develop alternative approaches for controlling foodborne pathogens. Techniques involving probiotics and antibacterial compounds that are produced by beneficial bacteria offer promising prospects<sup>[28,29]</sup>. *Lactobacillus* species, recognized for their Generally Regarded as Safe (GRAS) status, are notable for their capacity to generate many antibacterial compounds, such as organic acids, hydrogen peroxide, bacteriocins etc<sup>[30,31]</sup>. *Lactobacillus* species are naturally present in naturally fermented dairy products. Sri Lankan buffalo curd, known as “*meeekiri*”, is a natural reservoir of many native *Lactobacillus* species, which contribute to its’ unique flavors and potential health benefits. Traditional buffalo curd is fermented buffalo (*Bubalus bubalis*) milk produced by back-slopping by local home-based dairy farmers without the addition of commercial starter cultures<sup>[32-35]</sup>. Many researchers in Asia and in Middle East have reported that naturally fermented cow and buffalo milk, similar to Sri Lankan curd, is a rich source of local *Lactobacillus* species<sup>[36-41]</sup>.

The antibacterial properties of various *Lactobacillus* strains sourced from traditional dairy products similar to curd have been widely studied by researchers against varieties of microorganisms around the world<sup>[42-45]</sup>. However, in Sri Lanka, there is a deficiency in reported studies in this capacity, particularly, against *L. monocytogenes*. Therefore, this study focused on native *Lactobacillus* species isolated from back-slopped fermented Sri Lankan buffalo curd to evaluate their possible antagonistic activity against the foodborne pathogen *L. monocytogenes*.

## 2.0 MATERIALS AND METHODOLOGY

### 2.1 Bacterial cultures used in the study

#### 2.1.1 *Lactobacillus* strains

All *Lactobacillus* strains used in this study were previously isolated from back-slopped fermented traditional buffalo curd samples collected from local dairy farmers in Kanthale, Sri Lanka<sup>[35]</sup>. *Lactobacillus* species recovered with the highest percentage in that study were selected for the evaluations in the current study; *Lactobacillus fermentum* accounted for 39%, followed by *Lactobacillus plantarum* (14%) and *Lactobacillus acidophilus* (3%). Three strains representing each species were used to test the antibacterial activity. The strains K1, T4b, and T4a were biochemically identified as *Lactobacillus acidophilus*<sup>[35]</sup>. Molecular biological identification based on 16S rRNA gene sequencing confirmed the strains L-12, L-15, and L-18 as strains of *Lactobacillus fermentum* and the strains T3b, T45a, and T5a as strains of *Lactobacillus plantarum*<sup>[46]</sup>. The cultures were preserved in modified Man Rogosa Sharpe (MRS) broth in glycerol and stored at -80 °C until further use. To recover the *Lactobacillus* strains, they were inoculated into MRS broth and incubated at 37 °C for 24 - 48 h under anaerobic conditions in an anaerobic jar. Following incubation, subcultured in MRS agar and incubated under the same conditions and used as working cultures.

#### 2.1.2 *Listeria monocytogenes* (NCTC 11994)

*L. monocytogenes* strain was originally obtained from the National Collection of Type Cultures, USA, and maintained in the freeze-dried form in the microbial culture collection in the Department of Microbiology, Faculty of Science, University of Kelaniya, Sri Lanka. The bacterium was recovered in *Listeria* selective agar, followed by subculturing in Trypticase Soy agar (TSA), at 37 °C for 24 - 48 h.

### 2.2 Detection of antibacterial activity

#### 2.2.1 Preparation of *Lactobacillus* cell-free supernatant (CSF)

Each *Lactobacillus* strain was grown in 100 ml of MRS broth and incubated at 37 °C, for 24-48 h under anaerobic conditions. The cultures were centrifuged at 10,000 rpm (revolutions per minute) for 10 minutes at 4 °C, and the supernatants were filtered through 0.45 µm sterile membrane syringe filters. The CFS of each strain was stored under refrigerated conditions and used within 12 - 18 h.

#### 2.2.2 Agar well diffusion assay

Antibacterial activity was analyzed using the agar well diffusion method in Brain Heart Infusion (BHI) agar. *L. monocytogenes* was grown in Trypticase Soy Broth (TSB) and incubated overnight at 37 °C. The optical density of the culture was adjusted to 0.1 at 600 nm to achieve an equivalent cell concentration of 10<sup>6</sup> CFU (colony-forming units)/mL. BHI agar plates were seeded with this culture using sterile cotton swabs to obtain a uniform bacterial layer on the top of the agar medium. Uniform wells of 8 mm diameter were bored with a sterile cork borer in the agar plates seeded with *L. monocytogenes*. From the CFS of each *Lactobacillus* culture, 100 µL was added to each well. Sterile MRS broth was used as the control in one well. All experiments were performed in three independent biological replicates. The plates were incubated at 37 °C for 24 h. Following incubation, the diameter of the inhibition zones was measured with a millimetre ruler. Further to increase the accuracy of the diameter readings, three measurements were taken at three different spots in each well.

## 3.0 RESULTS AND DISCUSSION

The current study evaluated the antagonistic activity of CFS of nine *Lactobacillus* strains, three strains from each of *L. plantarum*, *L. fermentum* and *L. acidophilus* sourced from back-slopped fermented buffalo milk (*meeekiri*) against *L. monocytogenes* NCTC 11994.

The findings of this study are consistent with previous research on the strain-specific nature of the antibacterial activity of *Lactobacillus* spp. isolated from naturally fermented dairy products against *L. monocytogenes*. In the current study,

the size of inhibition zones observed for each *Lactobacillus* strain against *L. monocytogenes* are summarized in Table 1.

As shown in Table 1, all tested *Lactobacillus* strains, except *L. plantarum* T3b, demonstrated measurable antagonistic activity against *L. monocytogenes*, with inhibition zone diameters ranging from 10.77 mm to 14.10 mm. Among the *L. fermentum* group, strain L-12 recorded the highest mean inhibition zone 13.77 mm, while L-15 and L-18 showed slightly lower activity 12.55 mm and 12.99 mm, respectively, indicating consistent but moderate variation within the group.

In the *L. plantarum* group, strain T5a showed the strongest inhibition 14.11 mm, whereas T45a displayed slightly weaker activity 11.99 mm, and T3b showed no inhibition at all, highlighting clear strain-specific variation even within the same species.

In the *L. acidophilus* group, strain T4a exhibited the most potent activity 13.35 mm, closely followed by T4b 13.33 mm, while strain K1 showed comparatively lower inhibition 10.77 mm.

Notably, the standard deviation (SD) values indicate minimal variability in most strains, suggesting consistent replicates. The relatively higher SD in T45a (1.67 mm) likely resulted from an outlier replicate with a zero-inhibition zone. These results reinforce that antibacterial activity varies significantly at the strain level, rather than species level.

The findings of this study support the strain-specific antagonistic behavior of native *Lactobacillus* species isolated from traditionally fermented buffalo curd against *L. monocytogenes*. These observations align with previous reports highlighting the selective antimicrobial effects of *Lactobacillus* strains from fermented dairy products.

**Table 1.** Statistical analysis of the diameter values of inhibition zones of *Lactobacillus* strains against *L. monocytogenes* NCTC 11994

<i>Lactobacillus</i> strain	Test 1 Clear zone diameter (mm)	Test 2 Clear zone diameter (mm)	Test 3 Clear zone diameter (mm)	Mean Clear zone diameter (mm)	Standard deviation (mm)	95% confidence level
<i>L. fermentum</i> L-12	13.66	13	14.66	13.773	0.836	(9.808, 11.738)
<i>L. fermentum</i> L-15	12.66	12.33	12.66	12.550	0.191	(11.585, 13.515)
<i>L. fermentum</i> L-18	13.33	12.33	13.33	12.997	0.577	(12.032, 13.962)
<i>L. plantarum</i> T3b	0	0	0	0.000000	0.000000	(-0.965070, 0.965070)
<i>L. plantarum</i> T45a	13.66	10.33	12	11.997	1.665	(11.032, 12.962)
<i>L. plantarum</i> T5a	13.66	13.33	15.33	14.107	1.072	(13.142, 15.072)
<i>L. acidophilus</i> K1	10.33	11.66	10.33	10.773	0.768	(9.808, 11.738)
<i>L. acidophilus</i> T4b	13.33	13.66	13	13.330	0.330	(12.365, 14.295)
<i>L. acidophilus</i> T4a	13.38	13.25	13.43	13.3533	0.0929	(12.3883, 14.3184)

**Note:** Pooled standard deviation = 0.796 mm

Findings from other countries, reinforce the global applicability of this research. *L. fermentum* strains isolated from fermented goat and cow milk in Tunisia and fermented curd in India have been reported to exert antibacterial activity against *L. monocytogenes*<sup>[44,47]</sup>. In comparison, a recent research conducted in Iran, both *L. plantarum* and *L. fermentum* derived from traditional curd, have exhibited much stronger antibacterial effects against *L. monocytogenes* compared to our results. Here, the specific strains, *L. plantarum* C39 and 58, exhibited inhibition zones of  $20.0 \pm 0.5$  mm and  $20.9 \pm 0.5$  mm, respectively, while *L. fermentum* strains C3 and C18 demonstrated inhibition zones of  $16.2 \pm 0.4$  mm and  $18.8 \pm 0.6$  mm<sup>[48]</sup>. A similar study conducted on *L. fermentum* from fermented buffalo milk in Pakistan reported that, out of four strains, three exhibited only moderate inhibition (0-4 mm) against *L. monocytogenes*<sup>[42]</sup>.

Studies on fermented milk products other than curd, have provided further insights into the antibacterial properties of *L. acidophilus* to inhibit *L. monocytogenes*<sup>[49]</sup>.

A study isolated *L. reuteri*, *L. plantarum*, and *L. acidophilus* from traditional yogurt, with both *L. plantarum* and *L. acidophilus* demonstrating antibacterial effects against *L. monocytogenes*<sup>[43]</sup>. The size of their inhibition zones (8-11 mm) were quite closer to the results obtained in this study.

In contrast, in some studies, none of the tested *Lactobacillus* strains have positive inhibition against *L. monocytogenes* as same as *L. plantarum* T3b in our study. For instance, *L. plantarum* and *L. acidophilus* were isolated from fermented buffalo milk and yogurt in Iran, but none of the strains inhibited *L. monocytogenes*<sup>[50]</sup>. Similarly, none of the 20 *Lactobacillus* strains isolated from curd in India exhibited inhibitory activity against *L. monocytogenes*<sup>[51]</sup>.

#### 4.0 CONCLUSION

This study provides valuable insights into the strain-specific antagonistic nature of the most common native *Lactobacillus* species, *L. fermentum*, *L. plantarum* and *L. acidophilus*

derived from traditional Sri Lankan buffalo curd against *L. monocytogenes* NCTC 11994. Except for *L. plantarum* T3b, the diameter values of inhibition zones of all other *Lactobacillus* strains varied in-between 10.77 mm - and 14.10 mm highlighting a significant level of antibacterial activity against *L. monocytogenes*. These results align with similar previous studies across different regions of the world. The research also revealed traditional Sri Lankan buffalo curd as a rich food source harbouring beneficial native *Lactobacillus* strain, not only for their role in fermentation but also for their functional properties that could promote consumer health.

Overall, the findings contribute to the growing body of global research, supporting the potential use of *Lactobacillus* strains as natural antibacterial agents to inhibit *L. monocytogenes*, advancing the field of food safety and sustainable food production practices.

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