*International Multidisciplinary Research Journal - ISSN 2424-7073*
*Agriculture & Climate Change – (July 2019): Pages 65-70*
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# *In vitro* antibacterial activity of *Lactobacillus* strains in spontaneously fermented curd from Kanthale, Sri Lanka

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**Abstract**

*In the need of wild Lactobacillus strains to contest for the survival in a hostile environment, they produce higher amounts of antimicrobials such as bacteriocins. The Food & Agricultural Organization (FAO)/World Health Organization (WHO) has stipulated, that the In vitro production of antimicrobial substances as a major criterion for probiotic evaluation. In this study, 36 Lactobacillus strains were isolated from spontaneously fermented, traditional, buffalo curd samples and morphologically and biochemically identified up to species level. In vitro antibacterial effect of cell-free supernatants of isolated Lactobacilli was determined against; Salmonella typhi (NCTC 10787), Staphylococcus aureus (ATCC 25923), Bacillus cereus (ATCC 10876), Enterococcus faecalis (ATCC19433), Pseudomonas aeruginosa (ATCC 27853), Listeria monocytogenes (NCTC 11994) and Escherichia coli (ATCC 25922), using agar well diffusion method. Antibacterial effect was clearly observed against all indicator organisms and the antibacterial property of the isolates against them was strain specific rather than species specific. The highest percentage of antibacterial activity was against Listeria monocytogenus (72.22%). This survey reveals that the Lactobacillus flora in spontaneously fermented curd withholds a natural ability to act against pathogenic and spoilage bacteria. Further, Lactobacillus strains encompassing the antibacterial activity could be developed as an innovative approach for controlling food-borne bacterial disease-causing agents and spoilage bacteria in food.*

*Keywords: Lactobacillus, Antibacterial activity, Spontaneous fermentation, Curd*.

## Introduction

Genus *Lactobacillus* is a highly heterogeneous genus; encompassing bacteria with a wide range of biochemical and physiological properties (Felis & Dellaglio, 2007). Lactobacilli are Generally Recognized as Safe (GRAS) and naturally present or added intentionally to fermented dairy products for technological reasons or to generate health benefits for the consumer (Coeuret, 2003). Traditional Sri Lankan buffalo curd is a spontaneously fermented dairy product where traditional method of backslopping or the inoculation of the boiled milk with a small quantity of a previously performed successful fermentation called as "Muhun' was done instead of adding commercial starter cultures. Spontaneous agency of Genus *Lactobacillus* in to milk plays a dominant role in the process of converting lactose in buffalo milk into lactic acid.

Spontaneously fermented foods are reported to consist a higher diversity of microorganisms than fermented food prepared by addition of commercial starter cultures. Also, it is observed that pure cultures isolated from complex ecosystems of traditionally fermented foods encompass a diversity of metabolic activities that considerably deviate from the comparable strains used as industrial starter cultures. These characteristics include competitive growth behavior in mixed cultures, adaptation to raw materials, antimicrobial properties, and flavour, aroma, and quality attributes. In the need of wild strains to survive and persist in their hostile natural environment by withstanding the competition of other microorganisms; they often produce higher amounts of antimicrobials such as bacteriocins (Ayad, 2002).Therefore, a recent trend has emerged in the isolation of wild-type strains from spontaneously fermented foods to be used as starter cultures, which will act as bio-preservatives and probiotics.

Antimicrobial effects of Lactobacilli are incurred by producing some substances such as organic acids, carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Dunne, 2001). One of the most important parameters by which potentially new probiotic strains must be characterized is the production of antimicrobial substances under *in vitro* conditions (FAO/WHO, 2002). Probiotics including *Lactobacillus*, *Streptococcus spp*. and *Bifidobacterium* are

known to inhibit the growth of a wide range of intestinal pathogens in human. Many efforts have been made to search potential probiotic *Lactobacillus* strains isolated from dairy products, recently due to their healthful properties. *Lactobacillus* originally isolated from milk and milk products are considered to be potent for improving the microbiological safety of these foods because they are well adapted to the conditions in milk and should therefore be more competitive than bacteria from other sources. Prolix failures in antibiotic treatment due to antibiotic resistance of disease-causing microorganisms has led to the rise of studies, on the development of antimicrobial from natural products and the recent approaches are increasingly directed towards the antibacterial potential of *Lactobacillus*.

At present, consumers highly prefer minimally processed foods, prepared without chemical preservatives because it is reported that food additives like preservative can be linked to hyperactivity, asthma and cancer. As a result, lactic acid bacteria including *Lactobacillus* are used as an integral part of hurdle technology, to effectively control spoilage bacteria and other pathogens and can inhibit the activities of a wide spectrum of organisms, including inherently resistant, gram negative and positive bacteria (Vuyst and Leroy ,2007).

The present research was carried out in the major perspective to isolate and identify autochthonous *Lactobacillus spp*. from spontaneously fermented traditional Sri Lankan buffalo curd samples and to detect the *in vitro* antibacterial effect of them towards some human pathogenic bacteria and food spoilage bacteria as a new innovative approach for controlling food-borne bacterial disease causing agents and food spoilage bacteria.

## Methodology

### Isolation and Identification of *Lactobacillus* Species

Spontaneously fermented traditional buffalo curd samples in clay pots were collected from Kanthale, Sri Lanka. Curd preparation procedures were observed to make sure that, no industrially prepared starter cultures were added. Samples were enriched in sterile modified Man Rogosa Sharpe (MRS) broth by incubating at 37°C for 24 hours undisturbed. *Lactobacillus* strains were isolated by dilution plate technique and agar overlay method in modified MRS agar medium and incubating plates at 37˚C for 24-36 hours under anaerobic conditions. Morphologically different colonies were subjected in to biochemical tests. $CO_2$ production from glucose in the Gibson's semi-solid medium was performed to determine homofermentative and heterofermentative *Lactobacillus* cultures. Arginine hydrolysis test was done using Arginine MRS broth. Carbohydrate fermentation tests were performed using, MRS fermentation broth containing 2% filter sterilized carbohydrate (Arabinose, Cellobiose, Esculin, Galactose, Maltose, Mannose, Melibiose, Raffinose, Ribose, Sucrose, Trehalose, Xylose, Salicin, Sorbitol, Mannitol, Rhamnose, Lactose, Fructose, Amygdalin) Growth at 45˚C and growth at pH 9.0 were also tested. Species were classified based on the 80%-90% of similarity with the standard reference test results given Bergy's Manual of Systematic Bacteriology (1986, 2009).

### Preparation of the Cell Free Supernatant (CFS)

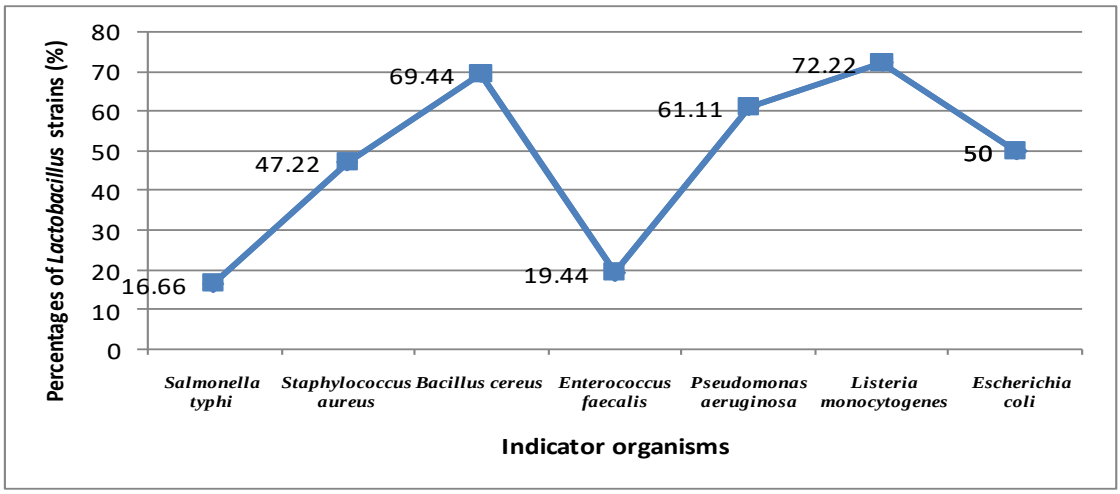
The MRS broth inoculated with *Lactobacillus* cultures were incubated for 24 hours at 37˚C under anaerobic conditions. The cultures were centrifuged at 10,000 rpm for 10 minutes at 4˚C.The cell free supernatants were collected and were sterilized by filtering using 0.45 µm membrane filters. The supernatant was stored at 8˚C.

### Agar Well Diffusion Method

Indicator organisms, *Salmonella typhi (NCTC 10787)*, *Staphylococcus aureus (ATCC 25923)*, *Bacillus cereus (ATCC 10876)*, *Enterococcus faecalis (ATCC 19433)*, *Pseudomonas aeruginosa (ATCC 27853)*, *Listeria monocytogenes (NCTC 11994)* and *Escherichia coli (ATCC 25922)* were inoculated in to Tryptone Soy Broth (TSB) medium and incubated at 30˚C for overnight. Concentrations of the cultures were adjusted to 106 CFU/ml by adjusting the optical density of the culture at 600 nm. Lawns of indicator organisms were made by spreading the cell suspension over the surface of Brain Heart Infusion (BHI) agar plates with sterile cotton swabs. The plates were allowed to dry and uniform wells with a diameter of 8 mm were cut in the agar pates using sterile cork borers. Each well was filled with 70 µl of filter sterilized cell free supernatant of different *Lactobacillus* strains. All assays were carried out in triplicates. Plates were incubated at 37˚C for 24 hours. The diameter of the inhibition zone around the wells was measured using the millimeter scale of the ruler, excluding the diameter of wells.

**Results and Discussion**

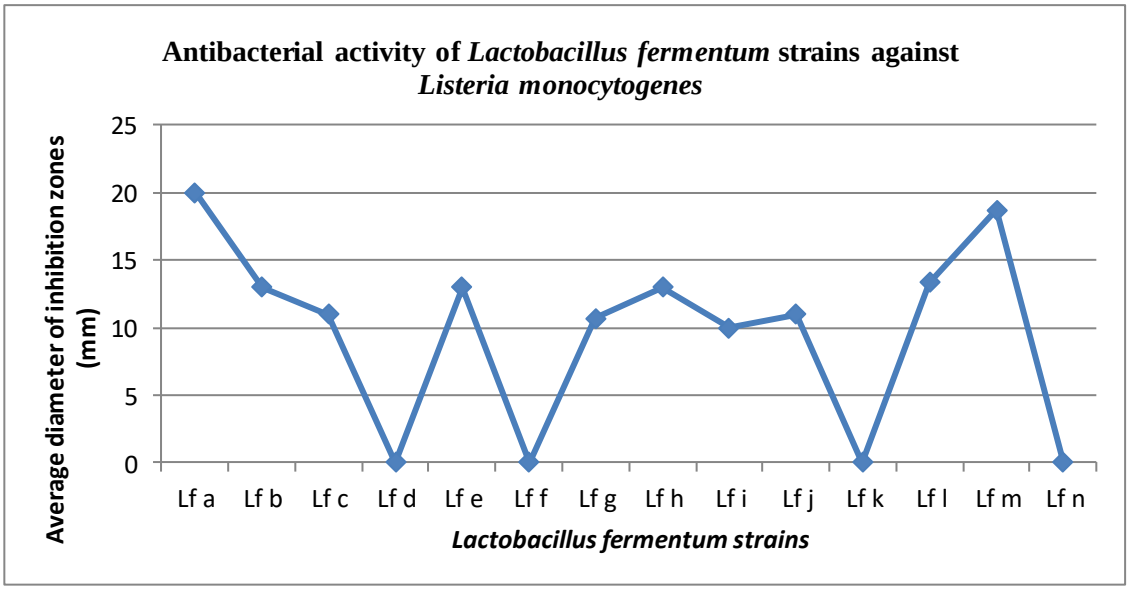
In the current study, a total of 36 pure *Lactobacillus* cultures were subjected in to biochemical tests and 16 species of *Lactobacillus* were identified among them. Along with relative percentages of abundance, they are *Lactobacillus acidophilus* (6%), *Lactobacillus amylolyticus* (3%), *Lactobacillus buchneri* (3%), *Lactobacillus casei* (3%), *Lactobacillus corinyformis subsp. coryniformis* (3%), *Lactobacillus delbrueckii subsp bulgaricus* (3%), *Lactobacillus delbrueckii subsp indicus* (6%), *Lactobacillus delbrueckii subsp lactis* (6%), *Lactobacillus ferintoshensis* (3%), *Lactobacillus fermentum* (39%), *Lactobacillus frumenti* (3%), *Lactobacillus helveticus* (3%), *Lactobacillus hilgardii* (3%), *Lactobacillus paracasei subsp paracasei* (3%), *Lactobacillus plantarum subsp. plantarum* (14%), *Lactobacillus rhamnosus* (3%). The most abundant species among the cultures was identified as *Lactobacillus fermentum* with a relative abundance of 39%.




***Graph 01***: Percentages of the number of *Lactobacillus* strains that encompass antibacterial activity against indicator organisms.

Regarding antibacterial activity, results gave clear evidence to prove the antagonism of genus *Lactobacillus* against all indicator organisms and that the antibacterial activity of the isolates against them was strain specific rather than species specific. It was observed that the highest percentage of antagonistic activity of 36 isolates was against *Listeria monocytogenes* (72.22%) and the lowest was against *Salmonella typhi* (16.66 %).

*Listeria monocytogenes*, a gram-positive, ubiquitous, intracellular pathogen, has been implicated as the causative organism in several outbreaks of forborne disease for past decades. Listeriosis is found mainly among pregnant women, their fetuses, and immunocompromised persons. A total of 26 isolates which were identified as different species including *Lactobacillus acidophilus*, *Lactobacillus amylolyticus*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus delbrueckii subps bulgaricus*, *Lactobacillus delbrueckii subps indicus*, *Lactobacillus delbrueckii subps*, *Lactobacillus ferintoshensis*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus plantarum subsp. plantarum*, *Lactobacillus rhamnosus* expressed antibacterial activity towards *Listeria monocytogenus*. The highest average diameter of inhibition zone showed against *Listeria monocytogenes* was 24.66±0.57 mm by one of the isolates which was identified as *Lactobacillus plantarum subsp. plantarum*. Antibacterial activity of *Lactobacillus fermentum* against *Listeria monocytogenes* clearly expressed strain specific results.




***Graph 02***: Strain specificity of the antibacterial activity of *Lactobacillus fermentum* against *Listeria monocytogenes*. The values are arithmetical means of inhibition zones (mm).

*Bacillus cereus* is a spore-forming food-borne pathogen often associated with food products such as meat, vegetables, soup, rice, and milk and other dairy products. A total of 25 isolates of *Lactobacillus* demonstrated antagonistic effect against *Bacillus cereus*. Among them were isolates identified as *Lactobacillus acidophilus*, *Lactobacillus amylolyticus*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus corinyformis subsp. coryniformis*, *Lactobacillus delbrueckii subps bulgaricus*, *Lactobacillus delbrueckii subps indicus*, *Lactobacillus delbrueckii subps lactis*, *Lactobacillus fermentum*, *Lactobacillus hilgardii*, *Lactobacillus plantarum subsp. plantarum*, *Lactobacillus rhamnosus*. The highest average diameter of inhibition zone showed against *Bacillus cereus* was 16.66±0.57 mm by the isolate which was identified as *Lactobacillus plantarum subsp. plantarum*. Antagonistic activity of *Lactobacillus fermentum* and *Lactobacillus plantarum subsp. plantarum* against *Bacillus cereus* was strain specific.

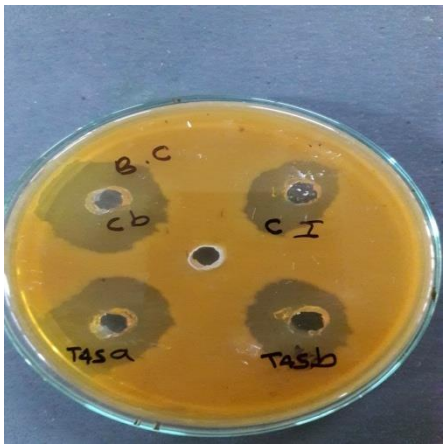



***Figure 01***

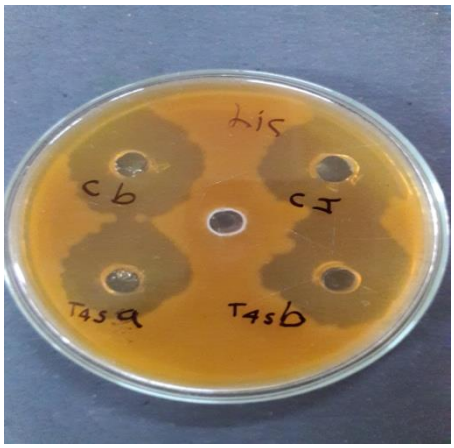



***Figure 02***

***Figure 1***- Inhibition effect of cell free supernatants of *Lactobacillus* cultures (Cb - *Lactobacillus fermentum*, CI, T45a - *Lactobacillus plantarum subsp. plantarum*, T45b - *Lactobacillus delbrueckii subsp indicus*) against *Bacillus cereus* by the agar well diffusion assay.

***Figure 2***- Inhibition effect of cell free supernatants of *Lactobacillus* cultures (Cb - *Lactobacillus fermentum*, CI, T45a - *Lactobacillus plantarum subsp. plantarum*, T45b - *Lactobacillus delbrueckii subsp indicus*) against *Listeria monocytogenes* by the agar well diffusion assay.

*Pseudomonas aeruginosa* was reported as a food spoilage organism that spoils foods at low temperatures as a result of its lipolytic and proteolytic activity (Oliveira, 2015) and was reported to be sensitive to *L. casei* and *L. plantarum* (Çon and Gokalp, 2000). In the present research antibacterial activity against *Pseudomonas aeruginosa* was shown by a total of 22 cultures including *Lactobacillus acidophilus, Lactobacillus amylolyticus, Lactobacillus buchneri, Lactobacillus casei, Lactobacillus corinyformis subsp.coryniformis, Lactobacillus delbrueckii subps indicus, Lactobacillus fermentum, Lactobacillus hilgardii, Lactobacillus plantarum subsp. plantarum, Lactobacillus rhamnosus, Lactobacillus helveticus* and *Lactobacillus frumenti*. The highest average diameter of inhibition zone showed against *Pseudomonas aeruginosa* was 18.33±1.52 mm by the isolate which was identified as *Lactobacillus rhamnosus*. Antibacterial activity of the isolate identified as *Lactobacillus frumenti* was demonstrated only against *Pseudomonas aeruginosa* with a zone of inhibition of 14.33±0.57 mm.

Among isolates that showed antagonistic activity against *Escherichia coli*, there were 18 isolates including *Lactobacillus acidophilus, Lactobacillus buchneri, Lactobacillus corinyformis subsp. coryniformis, Lactobacillus delbrueckii subsp indicus, Lactobacillus delbrueckii subsp lactis, Lactobacillus fermentum, Lactobacillus hilgardii, Lactobacillus plantarum subsp. plantarum, Lactobacillus rhamnosus* and *Lactobacillus helveticus*. The highest diameter of inhibition zone showed against *E. coli* was 21.66±0.57 mm by isolates which were identified as *Lactobacillus plantarum subsp. plantarum* and *Lactobacillus corinyformis subsp. coryniformis*.

*Staphylococcus aureus* causes forborne illness by growing in temperature abused food and producing a heat stable toxin (Schelin, 2011). Staphylococcal food-borne disease (SFD) is one of the most common food-borne diseases worldwide resulting from the contamination of food by preformed *S. aureus* enterotoxins. Outbreak investigations report that improper food handling practices in the retail industry account for the majority of SFD outbreaks. *S. aureus* in many food products including raw retail meat indicate that consumers are at potential risk of *S. aureus* colonization and subsequent infection (Kadariya, 2014). Antagonistic effect against *Staphylococcus aureus* was observed from 17 isolates including *Lactobacillus amylolyticus, Lactobacillus casei, Lactobacillus buchneri, Lactobacillus corinyformis subsp. coryniformis, Lactobacillus delbrueckii subsp lactis, Lactobacillus fermentum, Lactobacillus plantarum subsp. plantarum, Lactobacillus rhamnosus* and *Lactobacillus helveticus*. Highest average diameter of inhibition zone shown against *Staphylococcus aureus* was 16.33±1.15 mm by an isolate which was identified as *Lactobacillus delbrueckii subps lactis*. Antagonistic activity of *Lactobacillus fermentum* and *Lactobacillus plantarum subsp. plantarum* against *Staphylococcus aureus* was strain specific.

Enterococci have recently emerged as nosocomial pathogens. They are frequent finding in foods as contaminants. They are low grade pathogens but their intrinsic resistance to many antibiotics and their acquisition of resistance to the few antibiotics available for treatment in clinical therapy, such as the glycopeptides, have led to difficulties and a search for new drugs and therapeutic options (Giraffa, 2002). Epidemiological data also indicate that *E. faecalis* is the most common species among the enterococci isolated from human illnesses (Girffa, 2002). Antibacterial activity against *Enterococcus feacalis* was shown by 7 isolates including *Lactobacillus delbrueckii subps indicus, Lactobacillus fermentum, Lactobacillus plantarum subsp. plantarum, Lactobacillus acidophilus* and *Lactobacillus amylolyticus*. The highest diameter of inhibition zone showed against *Enterococcus feacalis* was 23.33±0.57 mm was by *Lactobacillus fermentum*.

*Salmonella typhi* which is solely a human pathogenic serotype causes typhoid fever. Infection typically occurs due to ingestion of food or water contaminated with human waste (de Jong, 2012). Only 6 *Lactobacillus* isolates expressed antagonistic effect against *Salmonella typhi*. Those were three isolates identified as *Lactobacillus fermentum*, isolates identified as *Lactobacillus hilgardi*, and *Lactobacillus corinyformis subsp. coryniformis*. The highest diameter of inhibition zone showed against *Salmonella typhi* was18.33±0.57 mm was by *Lactobacillus hilgardi*.

Traditional spontaneously fermented Buffalo Curd itself is a food which carries an antibacterial effect by serving as a significant delivery vehicle for the growth of probiotic bacteria. It could be widely used as major portion of the total functional foods available and could be used as a good source of probiotic developments.

In conclusion, current study of the antibacterial activity of autochthonous *Lactobacillus* in traditional buffalo curd, manifest isolates to be used as starter cultures for improving the microbiological safety of fermented food products and for increasing their shelf life. They could be

used for construction of specific starter cultures with probiotics and bio-preservative potentials. In this study, essentially it is noted that the antibacterial activity of *Lactobacillus* isolates is strain specific rather than species specific. The strains should be investigated further for other probiotic bioactivities that have human health benefits and further studies are necessary to define the chemical nature, classification and characterization of antimicrobial compounds produced by these strains.

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