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Probiotic potential of autochthonous *Lactobacillus* species from buffalo calves in controlling multidrug resistant *Escherichia coli*

Srivani Moturi[1]*, Lakshmi Kavitha Kommalapati[2], Muralidhar Metta[3], Venkata Seshaiah Chappidi[4], Srilakshmi Jatavathu[5]

[1] Department of Veterinary Microbiology, N.T.R College of Veterinary Science, Sri Venkateswara Veterinary University, Vijayawada, India; [2] Department of Veterinary Microbiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, India; [3] Department of Animal Genetics and Breeding, College of Veterinary Science, Sri Venkateswara Veterinary University, Vizianagaram, India; [4] Livestock Farm Complex, N.T.R College of Veterinary Science, Sri Venkateswara Veterinary University, Vijayawada, India; [5] Junior Research Fellow, Department of Veterinary Microbiology, N.T.R College of Veterinary Science, Sri Venkateswara Veterinary University, Vijayawada, India.

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Abstract

The aim of this study was to investigate the probiotic potential of autochthonous *Lactobacillus* species isolated from buffalo calves against multidrug-resistant *Escherichia coli*. A total of 252 rectal swabs were collected from healthy neonatal buffalo calves under 30 days old from six districts of Andhra Pradesh, India in a completely randomized design from August 2019 to August 2021, of which 190 *Lactobacillus* strains were isolated based on cultural, morphological, biochemical and molecular tests. Out of 190 isolates, 57 showed high levels of auto-aggregation (> 80.00%) and hydrophobicity (> 60.00%) and 51 of the 57 isolates had a zone of inhibition greater than 15.00 mm in diameter against multidrug-resistant *E. coli* in an Agar well diffusion assay. Among the 51 isolates, 36 were found to be acid and bile tolerant and showed varying levels of sensitivity to antibiotics such as erythromycin, clindamycin, tetracycline, chloramphenicol, and ampicillin. Among the 36 isolates, *Limosilactobacillus reuteri 178*, *L. reuteri 209*, *L. fermentum 182*, *L. fermentum 211*, and *Lactiplantibacillus plantarum 34* were non-hemolytic, and none of the isolates were able to hydrolyse gelatine. Therefore, these five autochthonous *Lactobacillus* species may be used in probiotic or synbiotic formulations against multidrug resistant *E. coli* in buffalo calves.

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Introduction

The natural microbiota of the digestive tract impacts on the biochemistry, immunology, physiology, and non-specific host resistance to infectious diseases.[1] In neonatal calves, stress, dietary changes, and environmental factors contribute to the colonization of opportunistic pathogens in the gut, leading to digestive problems and diarrhea. Antibiotics are frequently used to treat calf's diarrhea in, resulting in the development of antibiotic-resistant bacteria, a serious concern for both human and veterinary medicine. In our previous research, we found that *Escherichia coli* was responsible for 85.04% of e buffalo calf diarrhea cases, with 69.81% of those isolates being multidrug resistant.[2]

The commensal *E. coli*, which lives in the intestines of calves, has the ability to acquire resistance genes from other microorganisms or the environment. Thus, it may act as a potential reservoir for the horizontal trans mission of these genes to various bacterial species found in the food chain.[3-5] To control multidrug-resistant *E. coli* in calves, new strategies must be developed in response to the increasing antibiotic resistance. One novel approach to combat antibiotic -resistant *E. coli* is the use of probiotics, which are defined as live microorganisms that, when administered in adequate amounts, provide a health benefit to the host.[6]

Numerous probiotic strains, such as *Bifidobacterium* sp., *Lactobacillus* sp., *Saccharomyces boulardii*, and *Streptococcus thermophilus*, are being used to improve animal health, feed efficiency, weight gain, and immunocompetence.[7] One of the ideal qualities of an organism to be used as a probiotic species is to adhere and colonize the intestinal lining and must be able to withstand the harsh conditions of bile salt and stomach acid.[8] The normal gut microbiota is more resilient and stable in the face of population changes. Therefore, choosing species specific probiotic organisms from the gut microbiota may

***Correspondence:**

Srivani Moturi. MVSc, PhD
Department of Veterinary Microbiology, N.T.R College of Veterinary Science, Sri Venkateswara Veterinary University, Vijayawada, India
E-mail: srivanimoturi@yahoo.com

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have advantages as autochthonous probiotic species are easy to colonize the intestine, making it easier to maintain the gut microbiota and get rid of enteric infections.[9]

According to previous studies, *Lactobacillus* isolates from fecal samples of dairy cows have been shown to possess both antibacterial properties and the survival qualities required to be potent probiotics.[10] Similar results were found in swine, where probiotics made from autochthonous *Lactobacillus* isolates were more effective.[11] Due to the rise in multidrug resistant *E. coli* in buffalo calves, the present research aimed to isolate potential autochthonous *Lactobacillus* species to be used as probiotics against multidrug resistant *E. coli.*

Materials and Methods

Sample collection. From August 2019 to August 2021, a total of 252 rectal swabs were collected from healthy buffalo calves less than 30 days old. These calves were from six districts of Andhra Pradesh. The calves had not received any antibiotics since birth. The swabs were immediately placed into De Man–Rogosa–Sharpe agar (MRS) broth, (HiMedia Laboratories, Kennett Square, USA) containing L-cysteine HCl (0.05%) and Bromocresol green (0.004%) with a low pH of 5.00. They were then incubated at 37.00 ˚C for 24 to 48 hr in a 5.00% CO2 incubator.

Isolation and biochemical characterization of *Lactobacillus* species. A loopful of enriched broth culture was streaked on MRS agar with L-cysteine HCl (0.05%) and Bromocresol green (0.004%) plates and incubated at 37.00 ˚C for 24 to 48 hr in a 5.00% CO_2 incubator. Colonies of dark or light green colour, with a diameter of 2.00 - 3.00 mm and a transparent halo surrounding them were examined for Gram's reaction. Gram-positive rods were then streaked on MRS agar plates and incubated for 48 hr at 37.00 ˚C in a 5.00% CO_2 incubator. These colonies were further examined for spore staining, motility and biochemical tests such as catalase, indole and nitrate reduction tests.[12] Catalase negative isolates were then inoculated into MRS broth for molecular characterization.

Confirmation of *Lactobacillus* sp. by genus specific polymerase chain reaction (PCR). Whole cell DNA of *Lactobacillus* isolates was extracted using the boiling and snap chilling method[13] and were subjected to genus-specific PCR using primers Lac 1F (5'-AGCAGTAGGGAATC TTCCA-3') and Lab-0677R, (5'-CACCGCTACACATGGAG-3') targeting the 16S rRNA gene.[14] The reaction mixture consisted of 1.50 µL of DNA template from each isolate, 2.50 µL of 10.00 x Taq buffer (Thermo Fisher Scientific, Waltham, USA), 0.50 µL of 10.00 mM dNTP mix (Thermo Fisher Scientific) 1.50 µL of 25.00 mM $MgCl_2$ (New England BioLabs, Ipswich, Massachusetts, USA) 1.00 µL of 10.00 pmol µL^{-1} forward primer, 1.00 µL of 10.00 pmol µL^{-1} reverse primer, 1.00 µL of 1.00 U µL^{-1} Taq DNA polymerase (Thermo Fisher Scientific) and 16.00 µL of nuclease free water to make a total volume of 25.00 µL. The standardized thermal cycling conditions included an initial denaturation at 95.00 ˚C for 3 min followed by 35 cycles of denaturation at 95.00 ˚C for 30 sec, annealing at 60.00 ˚C for 1 min, elongation at 72.00 ˚C for 1 min and a final elongation at 72.00 ˚C for 7 min, with an expected amplicon size of 341 bp. DNA from *Lacticaseibacillus rhamnosus.* Microbial Type Culture Collection 1,408 was used as a positive control. The amplified PCR products were then analysed using 1.50% agarose gel electrophoresis and the bands were visualized under an ultraviolet transilluminator (Bio-Rad, Hercules, USA).

Auto-aggregation assay. This test was conducted following the method described Janković *et al.*[15] with slight modifications. *Lactobacillus* cultures grown overnight in MRS broth were harvested by centrifugation at 3,000 rpm for 5 min, then washed and suspended in Phosphate-buffered saline (PBS)to achieve a final optical density of 1.00×10^9 colony-forming unit (CFU) mL^{-1} at 600 nm. The optical density values were measured with Multiskan GO (Thermo Fisher Scientific) at 5 and 24 hr. Auto-aggregation was calculated as follows:

$$Auto\text{-}aggregation\ (\%) = [1 - (A_t/A_0)] \times 100$$

where, A_0 represents the absorbance at 0 hr and A_t is the absorbance at 5 and 24 hr.

Cell surface hydrophobicity. *Lactobacillus* cultures grown overnight were centrifuged at 7,500 rpm for 5 min at 4.00 ˚C, washed twice with PBS buffer and then suspended in the same buffer. The initial absorbance (A_0) at 600 nm was measured. The 2.00 mL of bacterial suspension was then transferred into a round bottom test tube and 0.40 mL of hydrocarbon (N-Hexadecane and xylene; Sigma-Aldrich, Burlington, USA) was added. The tubes were vortexed for 2 min and then left undisturbed for 1 hr to allow the phase separation. The aqueous phase was separated and the absorbance (A_1) was measured at 600 nm. Hydrophobicity was calculated as the percentage decrease ($H\%$) in the absorbance of the bacterial suspension using the following formula:[16]

$$H\ (\%) = [1 - (A_1/A_0)] \times 100$$

Detection of antibacterial activity. The antibacterial activity against multidrug resistant *E. coli* was determined using agar well diffusion method.[17] The degree of inhibition of the tested pathogen was interpreted as high (> 15.00 mm diameter of the zone of inhibition; +++), medium (10.00 - 15.00 mm diameter of zone of inhibition; ++), low (< 10.00 mm diameter of the zone of inhibition; +) and absent (-).[17]

Acidic pH tolerance test. Overnight grown *Lactobacillus* cultures were inoculated (1.00 % v/v) into MRS broth that had been previously adjusted to pH values 2.50, and 7.00 (control) with 1.00 N HCl or NaOH (Sisco Research Laboratories Pvt. Ltd., Mumbai, India) incubated at 37.00 ˚C for 3 hr in a 5.00% CO_2 incubator. The biomass

(CFU mL^{-1}) of each culture obtained in the assays, which were conducted in triplicate, was enumerated on MRS agar incubated anaerobically at 37.00 ˚C for 24 hr. The reduction in cell count compared with the control tube was assessed as a Log reduction in CFU[18] with slight modifications.

Bile salt tolerance. Overnight grown cultures (1.00 % v/v) were inoculated into MRS broth containing 0.50% (w/v) bovine bile (Sigma-Aldrich) and incubated aerobically at 37.00 ˚C for 2, 4 and 6 hr.[19] The pH of both control and test cultures was adjusted to 6.00 with 1.00 N HCl or NaOH. The turbidity of the cultures was monitored spectroscopically at 2 hr intervals for growth at 600 nm. The control (blank) consisted of MRS broth without bile.

Antibiotic susceptibility test. Antibiotic susceptibility testing was conducted on selected isolates.[20] The bacterial suspension density was adjusted until visible turbidity matched 0.50 McFarland standard (1.50 × 10^{8} CFU mL^{-1}). The inoculum was spread across MRS agar plates. Antibiotic paper discs (HiMedia Laboratories, Mumbai, India) containing ampicillin (10.00 µg), gentamicin (10.00 µg), cefotaxime clavulanic acid (30.00 µg), vancomycin (30.00 µg), tetracycline (30.00 µg), chloramphenicol (30.00 µg), nalidixic acid (30.00 µg), cotrimoxazole (25.00 µg), erythromycin (15.00 µg), penicillin (10.00 U), ciprofloxacin (5.00 µg), clindamycin (2.00 µg), streptomycin (300 µg) and nitrofurantoin (300 µg) were placed on the plates and incubated at 37.00 ˚C in a 5.00% CO_2 incubator for 24 hr. The diameters of inhibition zone were measured and the results were interpreted as resistant, intermediate, or susceptible according to established standards.[21]

Sequencing and phylogenetic analysis. The 16S rRNA PCR amplified products of different *Lactobacillus* isolates were sequenced using Sanger on a 3,500 genetic analyser (Applied Biosystems, Foster City, USA). To identify the species of the isolate, a similarity search was performed using BLAST in the NCBI database. The phylogenetic analysis of sequences was conducted by MEGA Software (version XI; Biodesign Institute, Tempe, USA).[22] A model test was performed to identify the best model that explains the sequence evolution.[23] A neighbour joining (NJ) tree with 1,000 boot strap replications was created using the orthologous 16s rRNA sequence of *B. subtilis* as an outgroup.

Hemolytic activity. The hemolytic activity of isolates was determined using Columbia agar containing 5.00% (w/v) sheep blood (HiMedia Laboratories, Mumbai, India). The plates were then incubated at 37.00 ˚C for 48 hr. The hemolytic activity of the isolated strains was evaluated and classified based on lysis of red blood cells in the medium surrounding the colonies. Green zones around colonies indicated α-hemolysis, clear zones indicated β-hemolysis, and no zones indicated γ-hemolysis on Columbia blood agar plates. Only strains with γ- hemolysis are considered safe.[24]

Gelatine liquefaction test. A gelatine medium (Sigma-Aldrich) containing 12.00% gelatine was inoculated with *Lactobacillus* strains at a concentration of 1.00 × 10^{9} CFU mL^{-1} and incubated for 48 hr at 37.00 ˚C. Gelatine liquefaction of strains was assessed by storing the medium in a refrigerator for 24 hr and checking whether the gelatine was hydrolysed or not.[25]

Results

A total of 190 *Lactobacillus* spp. was isolated from 252 samples based on colony morphology, Gram staining (+), spore formation (non-spore formers), motility (non-motile) and different biochemical tests like catalase (-), oxidase (-), indole (-), and nitrate reduction (-) tests. These isolates were further confirmed to be *Lactobacillus* sp. using the genus-specific 16S rRNA PCR (Fig. 1). This study observed a high (100%) isolation rate of *Lactobacillus* sp. from fecal samples of 1 - 2-week age group calves, followed by 97.95% in less than one week age group. In the 2 - 3 weeks and 3 - 4 weeks age groups, 51.78 and 55.81% isolation rates were observed, respectively. The suitability of these isolates for use as probiotic species was further investigated. All of the isolates were tested for auto aggregation and the hydrophobicity of the cell surface using microbial adhesion to hydrocarbons. Among 190 isolates, 57 showed both a high degree of auto-aggregation (> 80.00%) and hydrophobicity (> 60.00%) and were initially selected. The details of the isolates are presented in Table 1. Among the tested hydrocarbons, the maximum adhesion score was observed for N-hexadecane (56.65%) compared to xylene (54.25%).

Fig. 1. Molecular detection of *Lactobacillus* sp. by genus-specific polymerase chain reaction targeting 16s rRNA gene (341bp). Lane 1: MTCC1408, Lanes 2-5: *Lactobacillus* isolates, Lane 6: Negative control, and Lane M: Marker.

Table 1. Auto-aggregation and hydrophobicity (%) of *Lactobacillus* isolates.

Isolate No.	Auto-aggregation	Hydrophobicity		Isolate No.	Auto-aggregation	Hydrophobicity	
		Xylene	N-Hexadecane			Xylene	N-Hexadecane
L10	90.18 ± 0.01	88.52 ± 0.08	89.14 ± 0.05	**L103**	86.71 ± 0.02	64.37 ± 0.03	75.45 ± 0.09
L12	92.25 ± 0.08	75.80 ± 0.03	80.35 ± 0.01	**L108**	85.01 ± 0.04	65.73 ± 0.05	75.65 ± 0.03
L15	90.95 ± 0.01	60.30 ± 0.02	61.49 ± 0.03	**L109**	84.18 ± 0.02	73.37 ± 0.04	76.82 ± 0.04
L21	93.14 ± 0.05	85.63 ± 0.03	75.89 ± 0.05	**L125**	80.91 ± 0.03	71.13 ± 0.05	87.69 ± 0.05
L33	80.34 ± 0.04	95.49 ± 0.04	62.22 ± 0.05	**L126**	82.24 ± 0.06	75.70 ± 0.06	78.12 ± 0.07
L34	90.14 ± 0.01	73.34 ± 0.02	87.65 ± 0.03	**L134**	81.70 ± 0.03	73.72 ± 0.04	77.13 ± 0.06
L35	90.73 ± 0.03	94.22 ± 0.03	84.59 ± 0.05	**L135**	85.74 ± 0.04	78.69 ± 0.03	69.90 ± 0.02
L37	95.27 ± 0.04	86.62 ± 0.04	89.43 ± 0.02	**L136**	87.32 ± 0.05	85.31 ± 0.05	88.65 ± 0.03
L38	84.14 ± 0.06	84.56 ± 0.04	82.34 ± 0.04	**L143**	80.64 ± 0.03	78.21 ± 0.03	84.26 ± 0.04
L39	94.89 ± 0.04	78.28 ± 0.02	86.77 ± 0.05	**L144**	81.65 ± 0.06	85.34 ± 0.04	88.78 ± 0.05
L42	87.16 ± 0.05	89.76 ± 0.01	92.56 ± 0.09	**L145**	86.20 ± 0.04	63.25 ± 0.09	86.01 ± 0.07
L43	93.93 ± 0.02	94.46 ± 0.02	95.03 ± 0.02	**L162**	83.28 ± 0.03	62.82 ± 0.06	63.83 ± 0.04
L44	80.47 ± 0.04	66.09 ± 0.03	93.77 ± 0.05	**L163**	84.38 ± 0.04	60.31 ± 0.03	72.67 ± 0.05
L45	89.08 ± 0.01	66.42 ± 0.03	64.53 ± 0.05	**L169**	82.05 ± 0.02	60.70 ± 0.07	61.97 ± 0.06
L51	80.36 ± 0.05	93.11 ± 0.04	94.91 ± 0.03	**L171**	85.85 ± 0.03	67.34 ± 0.03	69.87 ± 0.04
L57	93.59 ± 0.03	74.81 ± 0.05	88.91 ± 0.03	**L173**	89.27 ± 0.04	66.05 ± 0.05	70.86 ± 0.08
L58	87.92 ± 0.02	75.43 ± 0.01	94.35 ± 0.04	**L177**	84.97 ± 0.05	59.61 ± 0.02	70.78 ± 0.03
L59	95.27 ± 0.04	63.12 ± 0.01	62.04 ± 0.04	**L178**	89.76 ± 0.02	60.12 ± 0.04	68.04 ± 0.04
L60	94.20 ± 0.01	78.37 ± 0.03	65.10 ± 0.03	**L182**	87.26 ± 0.03	61.40 ± 0.03	69.06 ± 0.06
L63	92.73 ± 0.03	85.67 ± 0.09	89.71 ± 0.04	**L187**	92.35 ± 0.02	95.27 ± 0.05	95.66 ± 0.05
L66	93.45 ± 0.06	74.25 ± 0.02	68.39 ± 0.04	**L191**	85.85 ± 0.01	77.51 ± 0.04	75.08 ± 0.07
L68	90.22 ± 0.05	60.63 ± 0.03	73.01 ± 0.05	**L196**	89.84 ± 0.02	69.03 ± 0.05	62.21 ± 0.06
L70	84.25 ± 0.01	72.39 ± 0.04	68.71 ± 0.03	**L202**	80.13 ± 0.03	62.41 ± 0.07	68.52 ± 0.05
L71	80.85 ± 0.03	75.74 ± 0.05	73.58 ± 0.01	**L203**	92.53 ± 0.04	95.68 ± 0.03	95.95 ± 0.07
L73	85.03 ± 0.04	68.00 ± 0.04	72.75 ± 0.03	**L204**	80.69 ± 0.02	95.50 ± 0.04	95.79 ± 0.05
L80	85.49 ± 0.02	68.44 ± 0.03	60.35 ± 0.04	**L207**	88.06 ± 0.03	72.40 ± 0.05	65.28 ± 0.07
L94	85.01 ± 0.05	62.85 ± 0.04	64.92 ± 0.03	**L209**	92.02 ± 0.04	88.25 ± 0.02	74.36 ± 0.06
L99	87.91 ± 0.07	69.15 ± 0.09	85.09 ± 0.06	**L211**	89.27 ± 0.03	60.56 ± 0.04	62.67 ± 0.05
L100	90.35 ± 0.00	60.80 ± 0.01	60.93 ± 0.05				

The results of the agar well diffusion assay of 57 *Lactobacillus* isolates against multidrug- resistant *E. coli* is presented in Table 2. Of these isolates, 51 exhibited a zone diameter of inhibition (ZDI) greater than >15mm. The isolates L12, L21, L33, L35, L39 and L.143 showed a ZDI less than 15.0 mm.

For probiotic bacteria to survive in the gut, they must be resistant to stomach acidity and have a high tolerance for bile salts. Among the 51 isolates tested, 36 were found to be both acid and bile tolerant. These selected isolates showed a less than a 2-log reduction in cell viability (CFU mL^{-1}) after being incubated at a pH of 2.50 for 3 hr (acid tolerant) and were also tolerant to 0.50% oxgall (bile tolerant). Since the isolates were able to survive in the simulated gastric acid and intestinal environments, we have selected these *Lactobacillus* sp. for further assessment of their antimicrobial activity.

The antibiotic sensitivity test of *Lactobacillus* sp. revealed varying levels of sensitivity to tested antibiotics ranging from 94.44% for ampicillin to 2.78% for vancomycin and streptomycin. All isolates showed 94.44% resistance to vancomycin.

Nucleotide sequencing was performed on 36 *Lactobacillus* isolates which were resistant to bile and acid as well as had > 15.00 mm ZDI on agar well diffusion assay. Among these 22 isolates showed nucleotide sequence similarity of 99.00% as *Limosilactobacillus fermentum*, six isolates as *Limosilactobacillus reuteri*, four isolates as *Ligilactobacillus agilis*, two isolates as *Lactiplantibacillus plantarum* and one isolate each as *Ligilactobacillus salivarius and Limosilactobacillus mucosae.* The hemolysis test revealed that of these 36 isolates five (*L. reuteri 178*, *L. reuteri 209*, *L. fermentum 182*, *L. fermentum 211*, and *L. plantarum 34*) did not exhibit hemolysis (γ-hemolysis) and 31 isolates showed a clear zone of hemolysis (β-hemolysis) around the colonies. Gelatin hydrolysis was not detected in any of the 36 isolates.

The sequences were submitted to the NCBI database with the following accession numbers: *L. fermentum* (OQ283736- OQ283765), *L. plantarum* sub sp*. plantarum* (OQ283773- OQ283774); *L. reuteri* (OQ283780 - OQ283785), *L. mucosae* (OQ283804), *L. agilis* (OQ283800 - OQ283803) and *L. salivarius* (Q283979). The model test revealed that the K2P + G model best explained the sequence evolution. The NJ tree revealed two major clusters (Fig. 2). The first cluster consisted of *L. fermentum*, *L. reuteri*, *L. mucosae* and *L. plantarum* species; with three sub-clusters: *L. fermentum* subgroup*, L. plantarum* subgroup and *L. reuteri* subgroup which included *L. mucosae*. The second cluster included *L. agilis* and *L. salivarius*. All the *Lactobacillus* isolates were grouped distantly from *Bacillus subtilis*.

Table 2. Agar well diffusion test (inhibitory zone) of *Lactobacillus* isolates against multidrug resistant *Escherichia coli*.

Isolate No.	Diameter (mm)	Isolate No.	Diameter (mm)
L10	16.00 ± 0.04	**L103**	16.00 ± 0.04
L12	14.00 ± 0.01	**L108**	16.00 ± 0.03
L15	19.00 ± 0.07	**L109**	19.00 ± 0.03
L21	13.00 ± 0.03	**L125**	20.00 ± 0.01
L33	14.00 ± 0.09	**L126**	18.00 ± 0.04
L34	18.00 ± 0.06	**L134**	18.00 ± 0.05
L35	14.00 ± 0.03	**L135**	18.00 ± 0.09
L37	18.00 ± 0.01	**L136**	18.00 ± 0.08
L38	14.00 ± 0.04	**L143**	14.00 ± 0.01
L39	13.00 ± 0.05	**L144**	17.00 ± 0.00
L42	22.00 ± 0.01	**L145**	19.00 ± 0.03
L43	15.00 ± 0.03	**L162**	18.00 ± 0.03
L44	19.00 ± 0.05	**L163**	18.00 ± 0.00
L45	20.00 ± 0.06	**L169**	17.00 ± 0.01
L51	19.00 ± 0.04	**L171**	15.00 ± 0.02
L57	20.00 ± 0.07	**L173**	19.00 ± 0.02
L58	16.00 ± 0.09	**L177**	18.00 ± 0.04
L59	17.00 ± 0.08	**L178**	17.00 ± 0.06
L60	15.00 ± 0.02	**L182**	19.00 ± 0.05
L63	23.00 ± 0.04	**L187**	16.00 ± 0.03
L66	18.00 ± 0.03	**L191**	20.00 ± 0.06
L68	20.00 ± 0.02	**L196**	20.00 ± 0.09
L70	18.00 ± 0.03	**L202**	16.00 ± 0.05
L71	18.00 ± 0.04	**L203**	15.00 ± 0.05
L73	16.00 ± 0.02	**L204**	20.00 ± 0.07
L80	17.00 ± 0.02	**L207**	20.00 ± 0.05
L94	16.00 ± 0.03	**L209**	20.00 ± 0.02
L99	19.00 ± 0.02	**L211**	19.00 ± 0.01
L100	17.00 ± 0.01		

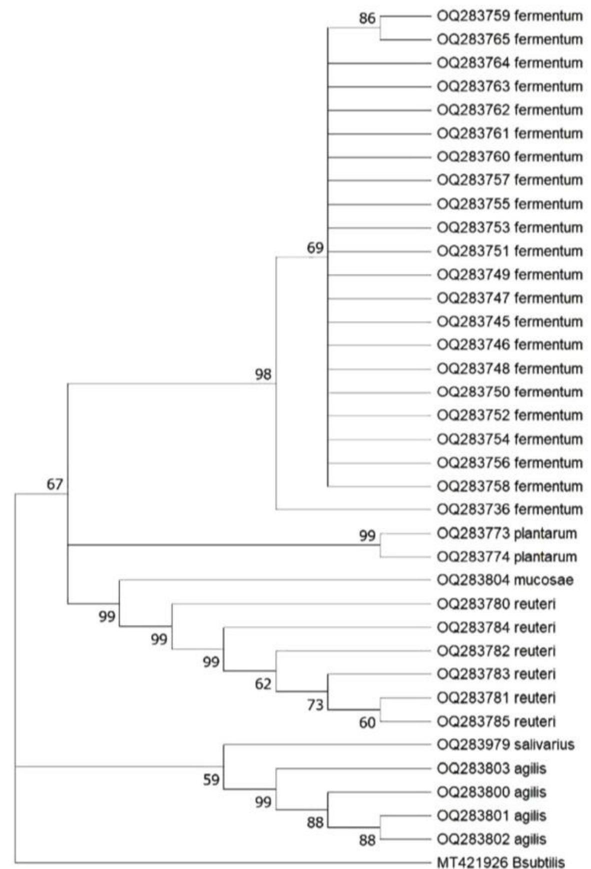

Fig. 2. Phylogenetic analysis of *Lactobacillus* species.

Discussion

A total of 190 *Lactobacillus* sp. was isolated and confirmed as *Lactobacillus* based on cultural, morphological, biochemical and molecular testes. Consistent with present findings, several authors earlier isolated and identified *Lactobacillus* as Gram-positive, catalase-negative, non-sporulated rods from fecal samples of different animals.[26-28] The isolates were further confirmed to be *Lactobacillus* using genus-specific PCR. In neonatal calves, switching their diet from colostrum to whole milk increased the number of milk-using bacteria such as *Lactobacillus, Parabacteroides*, and *Bacteroides* in the calf gut[29] up to 2 weeks of age. After two to three weeks, calves are gradually introduced to concentrate and hay in addition to milk, which promotes the growth of amylolytic and fibrinolytic bacteria like *Succino-vibrionaceae, Fibrobacteraceae*, and *Prevotellaceae* in the developing rumen.[30] This may explain the decrease in *Lactobacilli* isolation rates in older calves.

One of the key characteristics of a potent probiotic organism is the adherence to cell surfaces and competitive exclusion of pathogenic bacteria. An increase in hydrophobicity of the cell surface causes enhanced adhesion, and vice versa.[31] Among the two tested hydrocarbons, N-hexadecane (56.65%) showed the maximum score of adhesion compared to xylene (54.25%). Similar results showing higher adhesion for hexadecane compared to xylene have been reported.[32] This study observed that the *Lactobacillus* isolates from calves fed only milk had better auto aggregation than those from calves fed both milk and roughage. Furthermore, isolates from organized dairy farms demonstrated a high ability for auto aggregation compared to isolates from calves kept by individual farmers. The adhesion process is influenced by a number of factors, including surface exopolysaccharides, S-layer protein, and lipoteichoic acid.[33]

Out of 57 isolates, 51 exhibited more than 15.00 mm ZDI which is categorised as highly active.[34] The organic acids produced by *Lactobacillus* strains reduced the pH of the culture media[35] potentially inhibiting the growth of *E. coli.* These organic acids may also act as permeabilizers of outer membrane of Gram-negative bacteria[36] enhancing the activity of other antimicrobial metabolites against *E. coli*. Earlier *in vitro* studies have also demonstrated the antimicrobial activity of *Lactobacillus* strains against *E. coli*[37] and *Staphylococcus aureus.*[38]

A potent probiotic bacteria must overcome the physiological barriers of the host, including the hostile acidic environments[6] and bile toxicity present in the

stomach and duodenum, respectively, in order for the chosen strain to survive in sufficient numbers to express their health-promoting functions in the gut to the best of their ability. The gastric pH of neonatal calves can range from 2.50 to 3.00.[39] Therefore, we tested the strains with more than 15.00 mm ZDI for tolerance to acid (pH 2.50) and bile (0.50%). However, considerable variation was observed in acid and bile tolerance among the *Lactobacillus* isolates. Similar findings were also observed in *Lactobacillus* isolates from buffalo calves.[26]

The antimicrobial susceptibility test revealed that all isolates except *L. fermentum 66,* showed 94.44% resistance to vancomycin. The resistance to vancomycin was previously documented as intrinsic or natural.[40] According to European Food Safety Authority 2012 guidelines, *Lactobacillus* sp*.* to be used as a feed additive must be susceptible to ampicillin, streptomycin, gentamicin, erythromycin, clindamycin, tetracycline and chloramphenicol.[41] The observed antimicrobial resistance of *Lactobacillus* sp*.* against vancomycin, streptomycin and gentamicin in this study may be chromosomally encoded and is an intrinsic feature of *Lactobacillus,* which is non-transferable.[42,43]

The nucleotide sequencing results of this study revealed that *L. fermentum* is highly prevalent in buffalo calves in this geographic area. The probiotic qualities of *L. fermentum* were discovered in an animal model experiment, including adhesion properties, lifespan extension, immune system strengthening, and other health-promoting capabilities.[44] Further, it has been found to have synbiotic benefits by increasing antioxidant and immune function in aged mice.[45] This is consistent with previous findings demonstrating the probiotic qualities of *L. reuteri*[46] and *L. plantarum.*[47]

Prior to being used as probiotic species *in vivo*, they need to pass tests for other biosafety needs such as hemolysis and gelatine hydrolysis in addition to antibiotic resistance. Hemolysis on blood agar medium occurs when a test bacterium destroys red blood cells by producing g hemolysin, [48] resulting in a red transparent liquid. Since hemolysis is associated with pathogenicity, the test probiotic species should be non-hemolytic. In the present study we observed that among 36 *Lactobacillus* isolates *L. reuteri 178, L. reuteri 209, L. fermentum 182, L. fermentum 211* and *L. plantarum 34* were non-hemolytic (γ-hemolysis) and none of the 36 isolates showed gelatine hydrolysis. Therefore, these isolates were selected as probiotic species.

In conclusion, based on the results of this study, autochthonous probiotics may be used as feed additives to control multidrug resistant *E. coli* in buffalo calves. Further research is required to determine the therapeutic potential of these probiotics in treating cases of multidrug resistant *E. coli* associated diarrhea in buffalo calves.

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Conflict of interest

The authors declare that they have no conflict of interest.

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