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SCREENING OF LACTIC ACID BACTERIA FOR THEIR USE AS BUFFALO PROBIOTIC

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ABSTRACT

The extensive use of antibiotics in animal feed and development of antimicrobial resistance has led to the development of probiotics to improve the rate of livestock productivity. The present study was designed to isolate breed specific lactic acid bacterial (LAB) strains as animal probiotics. Sixty-eight gram-positive and catalase-negative strains were isolated, from the gastrointestinal tract and milk of *Nilli Ravi* buffaloes. For the selection of the buffalo probiotic, the viable capability of the isolated strains in a medium with different rumen conditions and antimicrobial activity were determined. Six strains (NMCC- PI, NMCC- PT, NMCC- P3n, NMCC- P7, NMCC- P8 and NMCC- F) showed a positive antibacterial as well as maximum cellular viability. These strains were identified as *Enterococcus lactis* (NMCC- PI) *Pediococcus pentosaceus* (NMCC- PT, MK014295), *Enterococcus faecalis* (NMCC-P3n) *Lactobacillus fermentum* (NMCC-P7), *Pediococcus acidilactici* (NMCC-P8) and *Enterococcus ratti* (NMCC-F) through 16 S rRNA gene sequencing. All strains fulfilled the basic criteria of animal probiotic properties however *P. acidilactici* (NMCC-P8) showed best result in terms of survivability at low pH (2.0), better antimicrobial activity and maximum enzymatic potential. Present results demonstrated that *P. acidilactici* may serve as a promising candidate probiotic for use in the buffalo dairy industry. This study was the unique to select LAB, with the potential to be used as probiotic from *Nilli Ravi* buffalo ruminal gut based on the ruminal-gut conditions.

Key words: *Lactobacillus*, Acid tolerance, Lactic acid bacteria, Breed specific probiotic, Phylogenetic analysis.

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INTRODUCTION

Dairy products industry is one of the fastest growing industries of the world. Previous studies have shown that 99% of milk production is coming from ruminants. The lactating animals are highly dependent on their gut microbial communities to fulfil their nutritional needs for maintaining body homeostasis, milk production and growth. The diverse and multifunctional animal microbial flora present in gastrointestinal tract may play a significant function in high or low milk production. Numerous techniques have been employed to enhance animals' productivity by manipulating GIT microbial communities of dairy animals. The use of antibiotics to improve dairy animal's productivity is associated with an increasing emergence of antibiotic resistance in microbiota (Zeineldin *et al.,* 2008). This emergence led to ban on the use of antibiotics as a feed additive in many countries (Lee and Salminen, 2009). Livestock sector is seeking alternative to antibiotics which could safely fulfil the demands of the local markets. The use of friendly bacterial feed supplements (probiotics) is one of the recommended methods to improve the balance of GIT microbes (GU and Roberts 2019). Probiotics are live microbes that benefit the host by maintaining the GIT microbial balance (Fuller, 1989). Further, till date they have been discovered non-pathogenic microbes that occur naturally in the tract of ruminants. Various microorganisms have been used as probiotics (Di-Gioia *et al.,* 2018). Probiotics bacteria give positive impact in young animals, whereas probiotic yeasts are found to be effective in lactating animals (Markowiak and Śliżewska, 2018). The most common ruminant probiotic products available in market are yeast probiotics i.e. *Saccharomyces cerevisiae* and bacterial probiotics i.e. lactic acid bacteria (LAB), *Bifidobacterium*, and some *Bacillus* (Kvan *et al.,* 2018; Grochowska *et al.,* 2012). Among LAB, *Lactobacillus acidophilus, L. plantarum*, *L. casei*, *L. Lactis* and *Enterococcus faecium* are found as one of the best candidates for probiotics. It has been reported that probiotic feed supplements are highly responsive to geographical location of the host, age, diet and breed Taylor *et al*., (2007). Probiotic effectiveness is highly dependent on the host and probiotic strain for maximum colonization Collado *et al*., (2009). The host origin of commercially available probiotic strains is not specific. Therefore, LAB strains fed to buffalo should be isolated from the same animal source or niche for

obtaining maximum benefits in terms of meat and milk production (Shakira *et al.,* 2018). The present study was designed with the objective to isolate and molecular identification of the autochthonous LAB strains present in buffalo gut for their potential use in buffalo feed as buffalo probiotic.

MATERIALS AND METHODS

Sampling from *Nilli Ravi* buffalo: Fresh fecal samples (10 g) from 18 lactating dairy buffalo (*Nilli Ravi*) were taken from deep rectum using the sterile gloves from Livestock Research Station, National Agricultural Centre (NARC), Islamabad, and district Buner, Khyber Pukhtunkhwa Pakistan. The samples were taken early in the morning in the sterile containers (Deltalab, Spain) and immediately transported to the laboratory for microbiological study.

Microbiological analysis: For microbial profiling, 5 g fecal samples from each group was homogenized in 40 ml saline-peptone water in a falcon tube using vortex mixture for 2 min. The serial dilutions were then prepared in saline (NaCl 0.9 w/v). Microbial suspension were plated on MRS media (Oxoid, UK) and incubated for 24 to 48 h at 37 °C under anaerobic condition (BD BBL™ GasPak™) for total lactic acid bacterial species (*Lactobacillus, Enterococcus, Lactococcus,* and *Bifidobacteria*)

Isolation of buffalo origin bacterial strains: Methods of bacterial isolation were adopted to obtain the LAB strains on DeMan, Rogosa and Sharpe agar, (MRS) (Oxoid, UK) media. Buffalo samples after serial dilution in PBS buffer were spread on MRS media plates. The plates were aerobically incubated at 37 °C for 48 hours (Di-Gioia *et al.,* 2016). Morphological characterisation of bacterial strains was done by using Bergey's manual (1962).

Selection of buffalo origin presumptive probiotic strains: 20 catalase negative and gram positive bacterial strains were inoculated in MRS medium containing 0.01 lactic acid. The concentration was prepared according to lactic acid concentrations in the buffalo rumen (Van Nieuwenhove *et al*., 2007). The bacterial isolates were incubated for 170 h at 28°C, the bacterial colony size was noted every 24 h during that incubation period (Table 8). The strains that showed maximum cellular viable results were selected for antimicrobial test. For this the bacterial isolates were inoculated at 37°C in shaking incubator on MRS media. Five pathogenic bacterial strains *L. monocytogenes, S. aureus, E.coli, B.cereus* and *P.aeroginosa* were selected to test antimicrobial effect by well diffusion agar assay method (Naeem *et al.,* 2018).

Identification of buffalo origin probiotic strains by 16S rRNA gene sequence

Extraction of Genomic DNA: Genomic DNA extraction from the pure bacterial isolates was done by using the methods given by Shakira *et al*., (2018). It involved suspension of purified bacterial colonies of single strain in 20µL of Tris EDTA buffer in micro-PCR strips. The bacterial strain colonies were mixed gently and heating the PCR machine at 95 °C (10 min). The mixture was centrifugate at 6000 rpm (2-3 min). The supernatant obtained by discarding the pellet was used as a DNA template for the amplification of 16S rRNA gene.

PCR-based buffalo origin bacterial strains identification: Takara Pre-Mix Ex-Taq was used to amplify the 16S rRNA gene of the presumptive LAB strains by using the method given by Shakira *et al.,* (2016). The sequencing for the 16S gene of the amplified PCR products was conducted using commercial sequencing service of Macrogen Inc Korea.

Determination of animal probiotic characterization

Acid tolerance: To determine low pH the tolerance of LAB strains, a suspension on MRS broth was prepared. The absorption was adjusted at 600 nm wavelength. The suspension was centrifuged and suspended in phosphate solubilizing bacteria (PSB). Different pH suspension were made and incubated for 3 hours and later on, aliquot inoculated in MRS broth. Strains were serially diluted and inoculated on MRS agar for measure cfu/ml (Hassan zadazar *et al.,* 2012).

Antibiotic resistance: Disc diffusion method was used for determination of the antibiotic resistance (Olatunde *et al.,* 2018). LAB strains were anaerobically grown at 30ºC in MRS broth for 24 h. Antibiotic discs were placed on overlaid prepared plates and incubated at 30ºC for 24 h anaerobically.

Hemolytic activity: The bacterial strains inoculated on MRS broth and then streaked lined on blood agar dispensed with sterile 5% defibrinated blood incubated at 37°C for 48 hours. Results were noted for a deep hemolysis zone around the bacterial colonies (Halder *et al*., 2017).

Enzymatic Activity: The proteolytic activity of LAB was determined by autoclaving, skim milk agar (SMA) plates. 10 g skim milk powder was added to autoclaved SMA plates. The 24 hours LAB isolate were streaked on the prepared plates and aerobically indicated at 37 ºC for 48 h. For determination of amylolytic activity amylase media plates were prepared. The freshly grown inoculum was streaked on the plates and 37 ºC (Latorre *et al*., 2016).

B-galactosidase activity: The β-galactosidase activity of LAB was determined by method given by Chen *et al*., (2008).

Bile salt tolerance and cholesterol assimilation: Bile salt tolerance and cell surface hydrophobicity of the LAB were determined by the method given by Del Re *et al*., (2000).

Statistical Analysis: All experimental tests were performed in triplicates. Results are presented as mean with standard deviation. The statistical analysis was done by using the statistics (version 8.1) software. The significant difference between the means was assessed by using Tukey's test. ($P \leq 0.05$).

RESULTS AND DISCUSSION

Study samples from buffalo feces (BF) showed total *Lactobacillus* 5.2 □.0.72 log CFU/g in early while 4.2 □0.14 log CFU/g in middle and 4.1 □0.14 log CFU/g in late lactating buffalo. As far as the *Lactococcus* species are concerned, 5.1□0.72 CFU/g in early 4.5 □0.14 and 4.1□0.14 log CFU/g both in middle and late lactating respectively. In addition, total *Enterococcus* numbers in fecal samples were counted at levels between 5.1□0.24 in early 5.3□0.80 in middle and 5.0□0.31 in late lactation stage. As far as the *Bafidiobactrium* are concerned, higher numbers (6.5□0.31 CFU/g) were detected initially at early lactation (Figure 1). Nonetheless, Shakira *et al.* (2018) previously described the similar results of *Lactobacillus* counts (6.5 CFU/g) for cattle manure. In present study, thirty (30) different bacterial colonies were isolated from buffalo fecal samples. After examination of these isolates through sample microscopy and electron microscopy which shown in fig 4 and 5 a total of twenty isolates, found as gram positive, catalase and oxidase negative (Table 1 and 8) were used for antimicrobial testing. *L. monocytogenes, S. aureus, E.coli, B. cereus* and *P. Aeroginosa* (ATCC) strains were used as indicator pathogens as shown in table 2 (Fijan, 2016). Six strains (NMCC-PI, NMCC-PT, NMCC-P3n, NMCC-P7, NMCC-P8 and NMCC-F) showed positive antibacterial activity (Table 3). These strains inhibited the growth of pathogenic bacteria particularly food borne pathogens including *B. cereus, E. coli* and *L. monocytogenes*. All six strains showed antagonistic effects towards tested pathogenic strains, however, the activity of antagonism was different among tested strains. NMCC-PI, NMCC-P7 and NMCC-P8 strains possessed strong activity against *L. monocytogenes*. No significant activity of NMCC-PI was observed against *P. aeruginosa* and *E.coli.* Two isolated strains viz NMCC-P7 and NMCC-P8 had capacity to inhibit a number of pathogenic bacteria. Therefore, these two best strains (NMCC-P7 and NMCC-P8) could further be exploited as potential antimicrobial probiotic candidates against animal pathogens and be considered for their health implications. Antimicrobial activity of LAB towards pathogens may be due to production of the metabolites like, organic acids, hydrogen peroxide, bacteriocins (Lahtinen *et al.,* 2009). Molecular identification of the presumptive probiotic bacterial strains is very crucial step to access and evaluate the safety of the microbes of an animal origin. These isolates were molecular identified as *Lactobacillus fermentum, Pediococcus acidilactici, Pediococcus pentosaceus, Enterococcus faecalis, Enterococcus ratti* and *Enterococcus lacti*s (Table 4). Acid tolerance is a basic criteria for screening probiotics species. All LAB had a wide range of pH tolerance form 1 to 4. However, NMCC- PT, NMCC- P8 and NMCC-P7 showed significantly ($P \leq 0.05$) good acid tolerance (Table 5). Antibiotics acceptability is another important criteria of a probiotics strains beneficial for human and animal consumption (FAO/WHO, 2001). In the present study, none of strains were entirely susceptible to all tested antibiotics. Most of the strains showed resistance to most antibiotics (Table 6; Figure 2). The strain NMCC-P7 was moderately susceptible to Ampicilin. However, NMCC-PT, NMCC-P8 and NMCC-P7 strain showed maximum resistant to all antibiotics except Chloramphenicol and Erythromycin as shown in fig 2. The LAB strains showed resistance to penicillin G and Ampicillin in number of the studies Coppola *et al.,* 2005; Ribeiro *et al.,* 2014 and Papagianni and Anastasiadou 2009. In the present study, most of the strains were resistant to antibiotic Vancomycin, which inhibits cell wall synthesis. This may be due to the presence of D-Ala-D-lactate in their peptidoglycan chains rather than the D-ala-D-ala dipeptide Meziane-Cherif *et al.,* (2013) *L. fermentum* is capable of suppressing primarily gram-negative bacteria but to some extent it also suppresses *Enterococci* and *S. aureus*. *P. acidilactici* in addition to ordinary properties, like resistance to heat, acid and bile tolerance, has the ability to produce antimicrobial peptides with antimicrobial spectrum, which makes it an interesting potent probiotic (Papagianni and Anastasiadou 2009). The *Pediococcus* strains showed the maximum temperature tolerance (Ribeiro *et al.,* 2014). The capability of the bacterial strains to stand varying acidic, bile, pancreatic enzymes conditions and to adhere to intestinal epithelial cells has been considered as promising indicator for the survival of a bacterial strain. One of the main aspects of probiotic strains is the cholesterol assimilation. Microbial strains required lipid derivatives during their growth pattern. These fatty acids played a vital role in the synthesis and protection of eukaryotic cell membranes. In present study Cholesterol assimilation assay showed significant variation among microbial strains with better results in NMCC-P8 (Table 7). Present findings depicted that all six isolates showed good acid tolerance at pH 3; however, capacity of tolerance varied among the strains. All strains especially

P. Acidilactici strains showed positive cellulolytic, proteolytic and amylolytic activity (Table 6; Figure 3). *P. acidilactici* strains isolated from the buffalo GIT showed best probiotic results. Dairy industry has been facing a lot of problems worldwide, one of which is the use of unidentified microbial based feed products (Shakira *et al.,* 2016; Lahtinen *et al.,* 2009). Identification of the microbial based feed though latest molecular methods can generate unique information for the preparation of the animal probiotic. Present study was reported for the isolation and molecular identification of the animal based microbial flora. In the present study, 6 lactic acid bacterial strains were isolated from *Nilli Ravi* buffalo fecal samples. All strains showed good probiotic potential but overall results showed that the *P. acidilactici* can be used as probiotic for animal use.

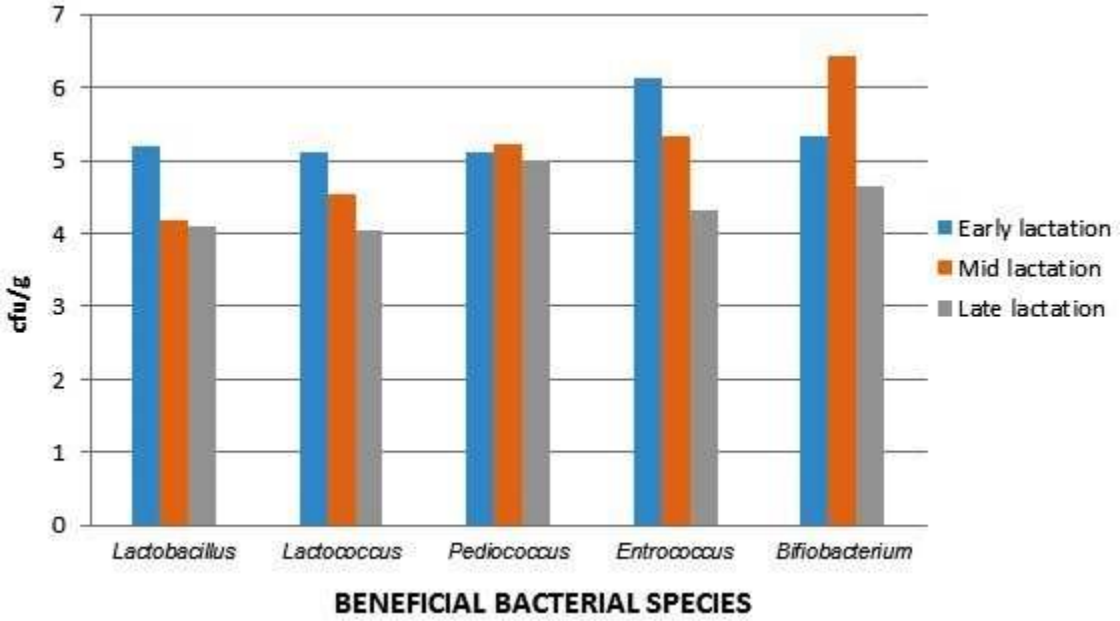

Figure 1. Total *Lactobacillus, Lactococcus, Pediococcus, Enterococcus* and *Bifidobacterium bacteria* (CFU/g) from buffalo feces at different lactation stage: Values are represented as mean of three replicates. Different subscripts lowercase letters showed significant different at the level of p≤0.05.

Table 1. Morphological and biochemical characterization of LAB strains as candidate for buffalo probiotics.

Characterization	*Enterococcus lactis*	*Pediococcus pentosaceus*	*Enterococcus faecalis*	*Lactobacillus fermentum*	*Pediococcus acidilactici*	*Enterococcus ratti*
Shape	Cocci	Cocci	Cocci	Rod shape	Cocci, occurs in tetrads	Cocci
Gram Staining	+	+	+	+	+	+
Catalase	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Gas from Glucose	+	-	-	+	-	-
Fermentation	Homo	Homo	Homo	Hetero	Homo	homo
Hemolysis	GAMMA	GAMMA	GAMMA	GAMMA	GAMMA	GAMMA
Growth at temperature (^{O}C)						
04	-	-	-	-	-	-
10	+	-	+	-	-	+
37	++	++	++	+	++	++
45	+	+	+	++	++	+
50	-	+	-	-	+	-

Table 2. The antibacterial activity of LAB strains as candidate for buffalo probiotics against five pathogens and their zones diameter (mm).

NMCC Strains	Test Pathogen				
	E. coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Listeria monocytogenes*	*Bacillus Cereus*
NMCC-PI	-	-	+	+++	+
NMCC- PT	-	+	+	+++	-
NMCC-P3n	-	+	-	++	+++
NMCC-P7	++	+	++	+	+
NMCC-P8	++	+	+++	+++	++
NMCC-F	-	+	++	++	++

ATCC: American type culture collection, Virgina, USA. E. coli: (ATCC8739); *Pseudomonas aeruginosa* (ATCC9027), *Staphylococcus aureus;* (ATCC6538); *Listeria monocytogenes* (ATCC13932), *Bacillus Cereus (ATCC-11778)*

Table 3. 16S rRNA based gene analysis of LAB strains as candidate for buffalo probiotics.

NMCC Strain	Bacterial strain name (genus)	Length of 16S r RNA (ntds)	Accession number	Bacterial Taxonomy	Similarities
NMCC-PI	*Enterococcus*	1028	MK007471	***Enterococcus lactis***	98.66
NMCC- PT	*Pediococcus*	1119	MK014295	***Pediococcus pentosaceus***	97.74
NMCC-P3n	*Enterococcus*	1196	MK007347	***Enterococcus faecalis***	95.32
NMCC-P7	*Lactobacillus*	1242	MK007323	***Lactobacillus fermentum***	94.45
NMCC-P8	*Pediococcus*	1176	MK007346	***Pediococcus acidilactici***	96.58
NMCC-F	*Enterococcus*	1190	MK014298	***Enterococcus ratti***	97.67

Table 4. Different pH (2, 4, 5, and 6) effects on survival of LAB strains as candidate for buffalo probiotics.

NMCC Strain	pH (2.0)	pH (4.0)	pH (5.0)	pH (6.0)
		log CFU/ml		
NMCC-PI	6.47±0.04^{a}	3.71±0.02^{a}	5.32±0.04^{a}	5.65±0.06^{a}
NMCC- PT	6.89±0.03^{a}	3.54±0.06^{ab}	5.45±0.06^{c}	6.55±0.07^{a}
NMCC-P3n	6.34±0.06^{a}	3.12±0.08^{b}	5.35±0.08^{a}	6.34±0.04^{a}
NMCC-P7	7.86±0.08^{a}	6.36±0.06^{b}	5.29 ±0.03^{c}	6.24±0.088^{ab}
NMCC-P5n	7.01±0.11^{a}	6.55±0.04^{b}	6.23±0.07	6.54±0.09
NMCC-F	6.34±0.09^{a}	3.12±0.05^{a}	5.35±0.01^{a}	6.33±0.02^{a}

Values are represented as mean ± SD of three replicates. The significant difference between the means was assessed by using Tukey's test ($p \leq 0.05$).

Table 5. Antibiotic resistance profiles of LAB strains as candidate for buffalo probiotics against commonly used antibacterial compounds.

NMCC Strain	Stre^{a} (10ug)	Cipr^{b} (20ug)	Van^{c} (30ug)	Metr^{d} (10ug)	Ampi^{e} (5ug)	Chlora^{f} (30ug)	Kana^{g} (30ug)	Eryt^{h} (15ug)	Pen^{i} (10ug)	Tet^{j} (30ug)
NMCC-PI	S	S	R	R	R	S	S	R	R	R
NMCC- PT	R	R	R	R	R	I	R	S	R	S
NMCC-P3n	S	I	S	R	R	S	S	R	S	R
NMCC-P7	R	R	R	R	I	S	R	S	R	R
NMCC-P8	R	R	R	R	S	I	R	S	R	R
NMCC-F	S	S	S	R	S	R	S	R	R	R

R: 0 mm; SR: 0-4mm; S: 8-12mm; ES>12m [a] **Streptomycin, [b] Ciprofloxacin, [c] Vancomycin, [d] Metronidazole, [e]Ampicillin, [f]Chloramphenicol[i], Kanamycin [h], Erythromycin [i], Penicillin [j], Tetracycline [k]**, Zone of inhibition (R) Resistant, (I) Intermediate resistant (S) Susceptible

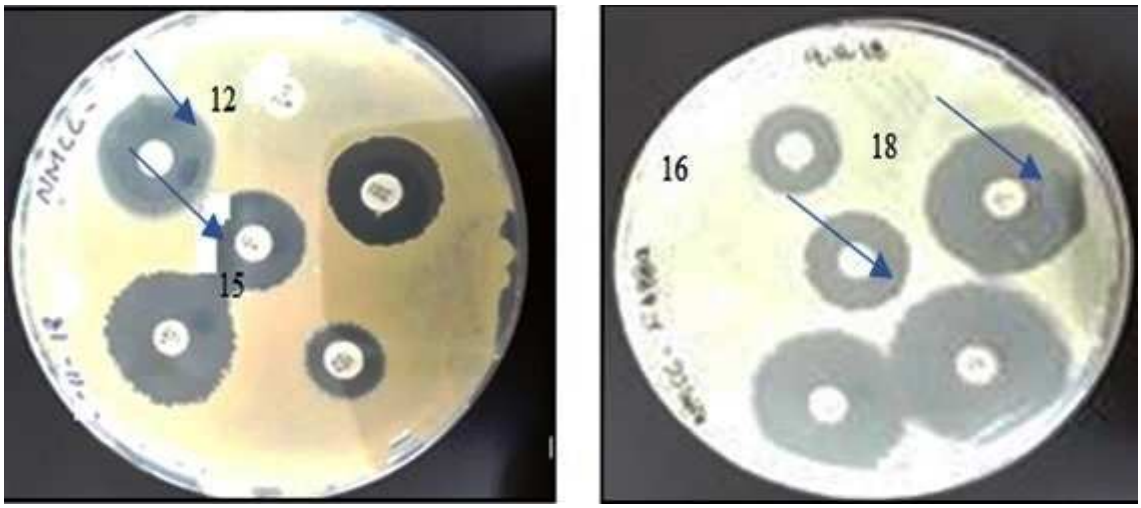

Figure 2. Antibiotic activity of putative probiotic (Zone in mm)

Table 6. Enzymatic potential of LAB strains as candidate for buffalo probiotics.

NMCC Strain	Lipolytic Activity	Proteolytic Activity	Amylolytic Activity
NMCC-PI	+	+	-
NMCC- PT	++	+	+
NMCC-P3n	++	+	-
NMCC-P7	+	++	++
NMCC-P8	++	++	+
NMCC-F	+	-	-

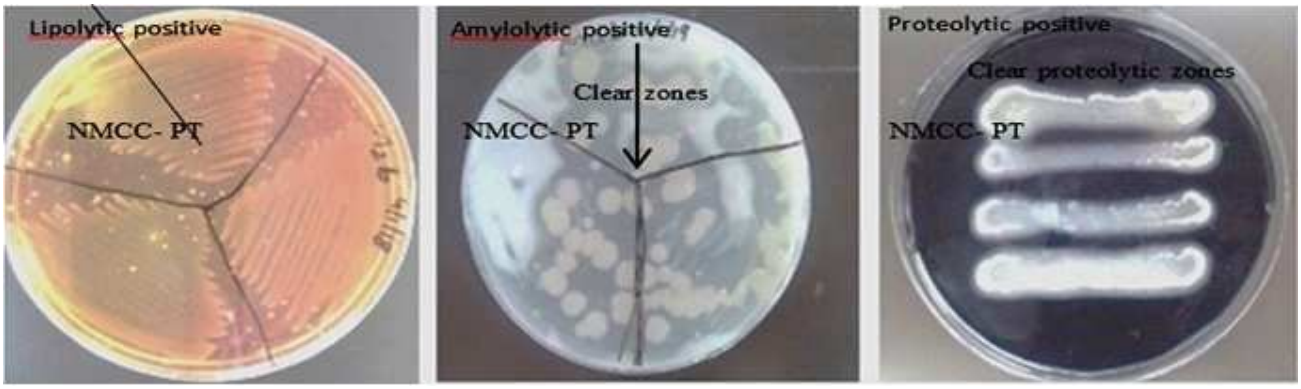

Figure 3: Enzymatic potential displayed by *Pediococcus acidilactici*
(a) Lipolytic activity (b) Amylolytic activity (c) Proteolytic activity

Table 7. In-vitro tests of LAB strains as candidate for buffalo probiotics (n=3).

NMCC Strain	Cholesterol assimilation (%)	β-galactosidase activity
NMCC-PI	**37.34**±1.76^{a}	**410.12**±25^{a}
NMCC- PT	**46.98**±1.34^{b}	**290**±41b
NMCC-P3n	**37.13**±1.65^{c}	-
NMCC-P7	**41.34**±1.54^{d}	347 ± 47^{c}
NMCC-P8	**44.35**±1.45^{e}	**276**±29^{d}
NMCC-F	**36.55**±0.39^{a}	**421**±54

Values are represented as mean plus minus SD of three replicates. Different subscripts lowercase letters showed significant different at the level of p≤0.05, as measured by one way ANOVA.

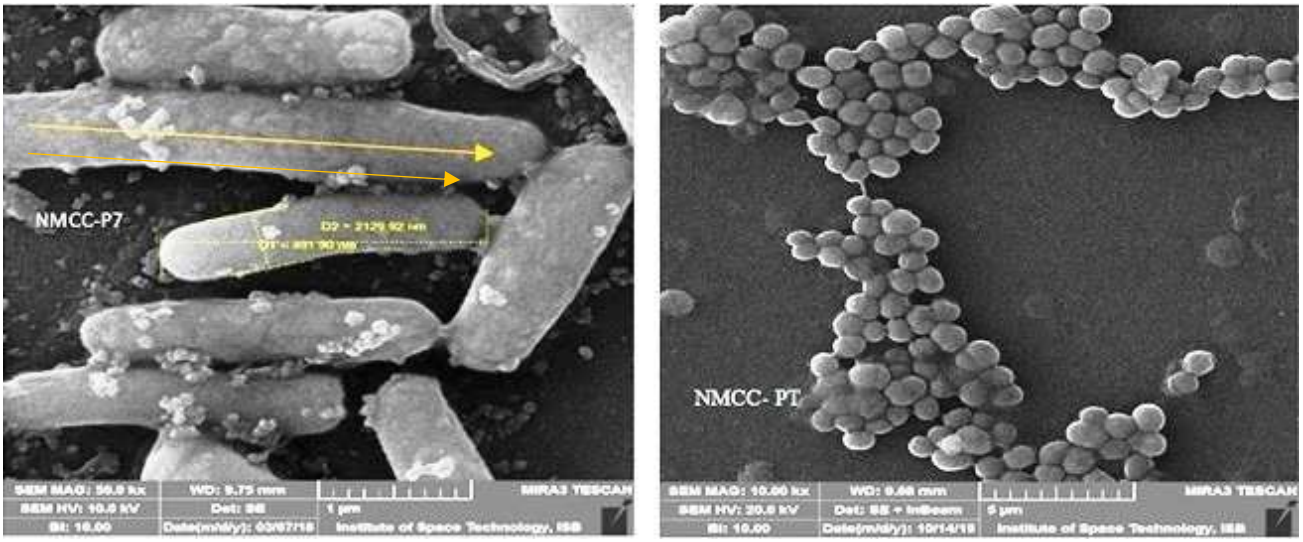

Figure 4. Scanning electron microscopy view of LAB strains as candidate for buffalo probiotics.

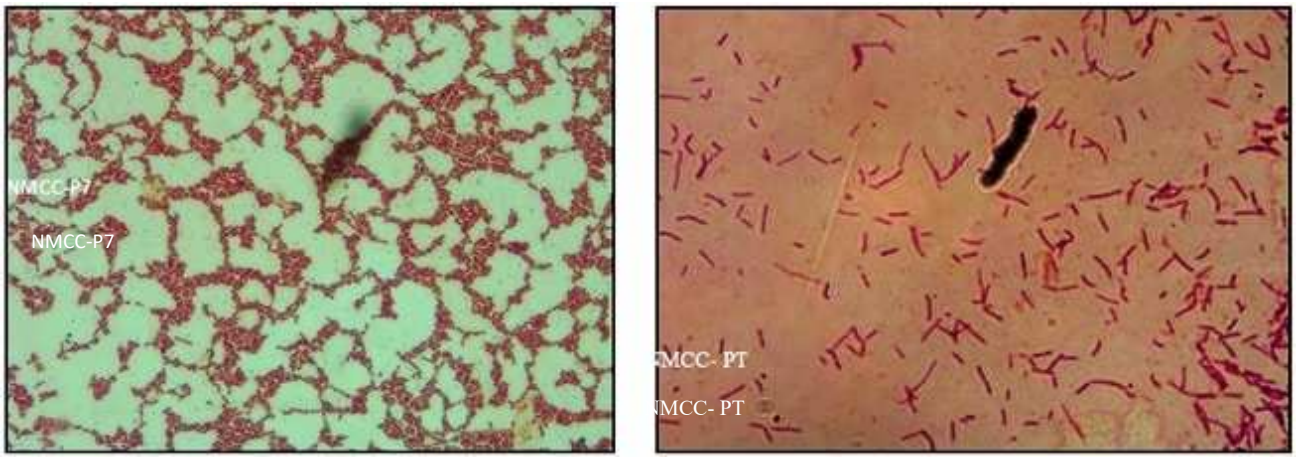

Figure 5. Simple Microscopy of the of LAB strains as candidate for buffalo probiotics.

Table 8. LAB strain shows cell viability, temperature and lactic acid assimilation.

Bcateria[1]	Cell viability in rumen fluid (Log_{10} cfu, mL^{-1})[2]	Temperature (°C)			Lactic acid assimilation
		37	40	41	0.01 %
NMCC-P1	4.34	+	+	+	W
NMCC-PT	5.11	+	+	+	W
NMCC-P3n	6.44	+	+	+	W
NMCCP7	6.87	+	+	+	W
NMCC4	6.12	+	+	+	W
NMCCP8	6.09	+	+	+	W
NMCCP-	6.81	+	+	+/-	W
NMCC-F	6.79	+	+	+	W
NMCC8	6.70	+	+	+	W
NMCC9	6.51	+	+	+	W
NMCC9	6.61	+	+	+/-	W
NMCC10	6.49	+	+	+/-	W

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