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Original Article

Tracing probiotic producing bacterial species from gut of buffalo (*Bubalus bubalis*), South-East-Asia

Rastreando espécies bacterianas produtoras de probióticos de intestino de búfalo (*Bubalus bubalis*), sudeste da Ásia

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Abstract

Due to extensive application of antibiotics as growth promoters in animal feed, antimicrobial resistance has been increased. To overcome this challenge, rumen microbiologists search for new probiotics to improve the rate of livestock production. The present study was aimed to isolate and evaluate breed-specific lactic acid bacteria (LAB) as potential animal probiotics. The current study was conducted during 10 months from July 2020 to April 2021, in which a total of n=12 strains were isolated from different samples including milk, rumen, and feces of *Nilli Ravi* Buffaloes. These isolates were evaluated for their antimicrobial potential against common animal pathogens (*Bacillus spp*., *E. coli*, *Staphylococcus aureus*, *Salmonella spp*., *Listeria spp.*). All the isolates were identified using 16S rRNA gene sequencing and the phylogenetic analyses inferred that these strains showed close relations to the species of various genera; *Enterococcus lactis*, *Pediococcus pentosaceus*, *Bacillus subtilis Weissella cibaria*, *Weissella soli*, *Bacillus tequilensis*, *Weissella bombi*, *Bacillus licheniformis*, *Lactococcus lactis, Bacillus megaterium*, *Lactobacillus ruminis,* and *Lactococcus lactis.* NMCC-Ru2 has exhibited the enormous potential of antimicrobial activity, 28 mm, for Salmonella typhimurium;23 mm for Listeria monocytogenes 21 mm for E.coil. Highest resistance was seen in NMCC-Ru2 agasint test antbiotic, like 25.5 mm for Tetracycline. Overall results revesl that the probiotic profile of isolates was achieved using standard criteria, particularly with animal probiotic properties

Keywords: antibiotic resistance, probiotics, 16S rRNA gene sequencing, lactic acid bacteria (LAB).

Resumo

Devido à extensa aplicação de antibióticos como promotores de crescimento na alimentação animal, a resistência aos antimicrobianos aumentou. Para superar esse desafio, os microbiologistas do rúmen buscam novos probióticos para melhorar a produtividade do gado. O presente estudo teve como objetivo isolar e avaliar bactérias lácticas específicas de raças (BAL) como potenciais probióticos animais. 12 cepas foram isoladas de diferentes amostras, incluindo leite, rúmen e fezes de búfalos Nilli Ravi. Esses isolados foram avaliados quanto ao seu potencial antimicrobiano contra patógenos animais comuns (*Bacillus spp*., *E. coli*, *Staphylococcus aureus*, *Salmonella spp*., *Listeria spp*.). Todos os isolados foram identificados por meio do sequenciamento do gene 16S rRNA e as análises filogenéticas inferiram que essas cepas apresentaram estreita relação com as espécies de vários gêneros; *Enterococcus lactis*, *Pediococcus pentosaceus*, *Bacillus subtilis*, *Weissella cibaria*, *Weissella soli*, *Bacillus tequilensis*, *Weissella bombi*, *Bacillus licheniformis*, *Lactococcus lactis*, *Bacillus megaterium*, *Lactobacillus ruminis* e *Lactococcus lactis*. O perfil probiótico dos isolados foi obtido usando critérios padrão, particularmente com propriedades probióticas animais.

Palavras-chave: resistência a antibióticos, probióticos, sequenciamento do gene 16S rRNA, bactéria láctica (LAB).

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1. Introduction

In Pakistan, 23.2% of the GDP (gross domestic product) is earned by agriculture, hence, considered as a principal sector of the economy. Livestock (a foremost sub-sector of agriculture) plays a vital role in improving the quality of food and enhancing the export earnings that make our economy strong. But still per animal production, income and social condition of animal breeder are unsatisfactory. Due to all these reasons, some public health issues such as malnutrition, etc arise; (Casewell et al., 2003). So, animal growth promoters are used to avoid these challenges as well as to enhance animal production. The extensive use of these antimicrobial agents is associated with the emerging resistance among bacterial population which ultimately develops health crises globally. To meet the demands of local markets, Livestock sector is looking for an alternate to these antibiotics that could be used safely. In lactating animals, milk production, weight gain, and nutrient digestibility depend on microbial population present in their gastrointestinal tract (GIT) (Jami and Mizrahi, 2012). It is recommended to improve microbial balance in GIT by the application of microbial growth promoters (probiotics) (Dowarah et al., 2017).

Probiotic is an array of microorganisms showing health beneficial effects directly by reducing the prevalence of diseases and by increasing resistance towards intestinal pathogens. Probiotics secret various compounds such as hydrogen peroxide, bacteriocins, and organic acids to inhibit the growth of pathogenic strains in host (Garcia-Gutierrez et al., 2019). Several microorganisms have been used as probiotics (Grochowska et al., 2019). The utmost common ruminant probiotic products available in market are bacterial probiotics (lactic acid bacteria (LAB), *Bacillus*, *Bifidobacterium*, *Propionibacterium,* and yeast probiotics (*Saccharomyces cerevisiae*) have been applied as feed supplements in adult ruminants (Ghazanfar, 2016; Uyeno et al., 2015). Among LAB, *Lactobacillus, L. acidophilus, L. plantarum*, *L. casei*, are used (Emmanuel et al., 2007; Peterson et al., 2007; Qadis et al., 2014; Stanford and Bottini, 2014). Research showed that, the animal health and production can be improveby using the probiotic as feed supplements. In ruminants' probiotics promote health by reducing acidosis, improving food digestion, eliminating pathogenic bacteria, and enhancing weight gain (Elghandour et al., 2020). For maximum colonization probiotics must be isolated and fed to same host. The GIT of buffalo contains diverse beneficial bacteria that should be isolated and fed to gain maximum efficiency. Therefore, the current research was objectively designed to isolate and evaluate probiotic strains from buffalo gut for their possible application in wellbeing of animal health.

2. Materials and Methods

2.1. Specimens collection and identification

The current study was conducted during 10 months from July 2020 to April 2021 in which a total of n=30 specimens, including feces, milk and rumen, were aseptically collected in sterile tubes from different buffalo's houses of Islamabad. All the collected specimens were taken to the laboratory via insulated boxes and stored at 4°C to process within 24 hours. 1ml of each specimen was serially diluted in 9ml of phosphate buffer saline solution (BPS) and allowed to homogenise for 1 minute. The dilution from 10^{-1} to 10^{-5} was inoculated on De Man Rogosa and Sharpe agar (MRS, Oxoid) and was allowed to incubate for 24-48 hours at 37°C (De Man et al., 1960). After morphological identification, the colonies were then purified via sub-culturing and were further identified via biochemical tests including catalase, oxidase, etc. as prescribed by (Wali et al., 2021).

2.2. Molecular identification of selected isolates

Bacterial DNA was extracted from pure isolates as followed and supported by the previous study (Naeem et al., 2018). The colony comprising of a single bacterial strain was suspended and mixed in micro-PCR strips containing 20µl tris EDTA buffer and was run in thermocycler machine at 95°C for 10 minutes. The resulting mixture was then centrifuged at 6000 rpm for 3 minutes and the obtained supernatant was used as DNA template whereas the pellet was discarded. The 16S rRNA gene present on DNA template was amplified in Polymerase Chain Reaction (PCR). The PCR conditions were set as, primary denaturation at 94°C for 2 minutes proceeded by 30 cycles of denaturation at 94°C for 1 min, the annealing at 50°C for 1 min, the extension at 72°C for 1.5 mins and the last extension at 72°C for 5 mins. The final PCR product was run along with 1kb ladder on agarose gel (0.8%) and was examined via gel documentation system. The desired bands were then sent for sequencing through commercial sequencing service Macrogen Inc. (Seoul Korea).

2.3. Growth determination at different temperature

The growth of isolated strains was observed at different temperatures as adapted by the previous study (Kavitha and Devasena, 2013). Briefly, 2ml of fresh culture was inoculated to each test tube containing 110ml MRS broth medium and were allowed to incubate at different temperatures including 6°C, 22°C, 30°C, 37°C, and 44°C. The ideal growth temperature was examined after 24-48 hours.

2.4. Phylogenetic analysis

Following gene sequencing of 16S rRNA, the complete sequenced data of bacterial strains were aligned using Clusta lX software. BioEdit software was applied for assembling the sequences of the 16S rRNA gene. The classification of bacterial isolates at species level was done using nBLAST search using GenBank internet service. For permitting public access to these probiotic strains. The 16S rRNA gene sequences were submitted to GenBank database (www.ncbi.nlm.nih.gov/projects/genome/clone/). To construct the phylogenetic tree, the Ez-BioCloud service was applied to retrieve the sequences of closely related bacterial strains. For molecular and phylogenetic evolutionary analysis of the bacterial strains, MEGA7 Molecular evolutionary Genetic Analysis software was utilized.

2.5. Probiotic characteristics of LAB isolates

The presumptive probiotic bacterial isolates were screened for tolerance to bile salt and acidic environment using MRS broth. The broth was concentrated in different tubes with different concentrations of bile salt such as 0.8, 1.3, 1.8, and 2.0% by adjusting pH 2, 3, and 7. Each tube containing MRS broth was inoculated with fresh culture of Lab isolates and was allowed to incubate at 37°C for 72 hours. After incubation, the resultant growth was inoculated on MRS agar and was allowed to incubate for 24 hours at 37°C. The next day, the grown colonies were counted (Lee et al., 2012).

The antimicrobial activity of the isolated strains was assessed by agar well diffusion method. Five pathogens *E. coli*, *salmonella*, *listeria*, *Staphylococcus aureus,* and *Bacillus cereus* were used in this method. The presumptive probiotic strains were inoculated in MRS broth (Oxide, UK) and incubated at 37ºC for 24 hours. After incubation, (cell density 108 cfu/mL the MRS broth culture was shifted into Eppendorf tubes. It was then centrifuged at 8000 rpm for 20 minutes and supernatant was collected, to obtain cell-free supernatant (CFS) it was then passed through a 0.22 mm syringe filter and was placed at 4ºC. Pathogens culture after dilution in PBS was properly spread on surface of MHA media and punctured well into media using 1000µl sterile pipette tip. 10µl soft agar was poured into the bottom of wells, then each well was inoculated with 20-35µl probiotic strains cell-free supernatant that was passed through a sterile filter syringe. The plates were incubated at 37°C for 24-48hrs in upright direction. The antimicrobial activity of isolated strains was interpreted by measuring its zone diameter (mm) using scale.

The susceptibility pattern of Lab isolates was examined via Kirby-Bauer disc diffusion method using Mueller Hinton Agar (MHA) (Bauer et al., 1966). Before inoculation, the isolated strains were adjusted up to 0.5 MacFarland index in phosphate buffer solution (BPS). The inoculum was then inoculated on MHA medium using sterilized cotton swabs and was allowed to dry. Commercially available antibiotics (Oxoid) were exposed and were allowed to overnight incubation for 37°C. After incubation, the zone of inhibition of each antibiotic against each bacterial strain was examined, measured and checked with disc diffusion chart for Lab, to obtain results as sensitive, intermediate and resistant (CLSI, 2016).

The proteolytic activity of each isolate was determined using skim milk agar. Fresh culture of Lab isolates was inoculated on skim milk agar and was allowed to incubate 37°C for 48 hours. After incubation, the plates were examined for translucent halos around the colonies (Pailin et al., 2001). For lipase activity, the presumptive probiotic strains were inoculated on amalgam of tween 80 medium, TSA medium and phenol red as elucidated by a previous study (Sierra, 1957). The petri dishes were incubated at 37°C for 48 hours. The culture plates were examined carefully for changing color from red to yellow-orange. The amylolytic activity of Lab isolate was screened using starch agar medium. The isolated strains were inoculated on starch agar medium in a straight line and were allowed for incubation at 37°C for overnight incubation. After incubation, the culture plates were flushed with 1%-gram iodine to observe the bright zones near the cultured lines (Bernfeld, 1955).

Probiotic with haemolytic capability is assumed as disadvantage. LAB cultures were grown overnight, inoculated on petri dishes having nutrient agar (NA) media and 4% based agar (Hi media). The plates were incubated at 37°C for 48 hours. After incubation, the presence or absence of hydrolysis zone near the cultured colonies were observed (Naeem et al., 2018). The results for haemolytic activity were reported as α-haemolysis (slight hydrolysis involving appearance of green zones around the cultured isolates), β-haemolysis (formation of clean zones of hydrolysis around the colonies) and γ-haemolysis (without any change in the media).

3. Results and Discussion

3.1. Isolation and identification of potential probiotics

While being proposed as a probiotic, the comprehensive resemblance concerning the safety profile and functional property of bacterial strains is a critical stage particularly when the source of isolation is an animal (Naeem et al., 2018). Animal gut, and its milk are a rich source for the isolation of the unique probiotic strains.*Nilli Ravi* Buffalo is the black gold of Pakistan. The ruminal gut/milk of the aimal may contain many useful probiotic strains.

In the present study, 30 samples from different sources of water buffalo i.e., milk, rumen, and fecal were taken, serially diluted in PBS buffer, and then cultured aseptically on De Man, Rogosa, and Sharpe agar for selective growth of *Lactobacilli*. The subsequent sub-culturing of bacterial colonies produced pure cultures which were used for further research. Initially, the bacterial strains were identified using phenotypic methods. The gram reaction of the isolated bacterial strains was determined by observing it under a phase contrast microscope (Phase Contrast 2, Nikon, Japan) after performing gram staining, following the procedure recommended by the manufacturers. Lactic acid bacteria were observed as gram-positive, catalase-negative. 12 bacterial isolates were identified based on their morphology and biochemical properties namely, NMCC-Ru1, NMCC-M1, NMCC-Ru2, NMCC-f5, NMCC-Ru3, NMCC-f12, NMCC-f13, NMCC-f14, NMCC-f15, NMCC-f16, NMCC-f17, and NMCC-M16, all exhibited either coccus or rod shape and were further viewed under Scanning Electron Microscope (Mira 3, Tescan SEM). The ideal growth temperature for NMCC-Ru1, NMCC-M1, NMCC-Ru2, NMCC-f5, NMCC-Ru3 and NMCC-M16 was observed to be 37ºC, whereas for NMCC-f12, NMCC-f13, NMCC-f14, NMCC-f15, NMCC-f16 and NMCC-f17 was observed to be 30ºC (Table 1 and Figure 1).

Probiotic starins identification is a basic step for the prepration of the unique probiotic. We used the 16 s rRNA gene sequences for the indentification of the microbial starins. Based on 16S rRNA, six major genera were molecularly identified including, species of *Weissella*, *Lactococcus*, *Enterococcus*, *Bacillus*, *Lactobacillus,* and *Pediococcus*. The bacterial species isolated from faecal

Table 1. Growth of bacterial strains at different temperature.

Strain ID	Temperature					MRS (Oxoid)	MRS (*Lactobacillus* Specific)
	06°C	22°C	30°C	37°C	44°C		
NMCC-M1	_	_	++	++	_	++	++
NMCC-f5	_	_	_	++	+	_	++
NMCC-f12	_	_	++	+	_	+	++
NMCC-f13	_	_	++	+	_	+	++
NMCC-f14	_	_	++	+	_	+	++
NMCC-f15	_	_	++	+	_	+	++
NMCC-f16	_	_	++	+	_	+	++
NMCC-f17	_	_	++	+	_	+	++
NMCC-M16	_	_	++	+	_	+	++
NMCC-RU1	_	_	_	++	+	_	++
NMCC-RU2	_	_	_	++	_	_	++
NMCC-RU3	_	_	_	++	+	_	++

(+, weak) (++, strong) (–, no growth). MRS = De Man, Rogosa and Sharpe agar

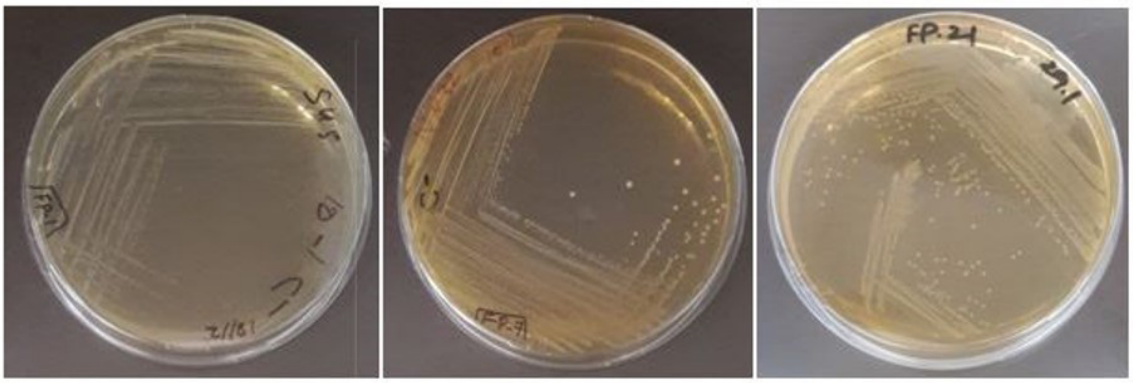

Figure 1. Growth pattern of isolates on MRS (lactobacillus specific) media.

sample were presumed to be; *Bacillus megaterium* (NMCC-f5), *Pediococcus pentosaceus* (NMCC-f12), *Weissella bombi* (NMCC-f13), *Weissella cibaria* (NMCC-f14), *Enterococcus lactis* (NMCC-f15), *Lactobacillus ruminis* (NMCC-f16), *Weissella soli* (NMCC-f17) and *Weissella confuse* (NMCC-M1). Three bacterial species were isolated from rumen including *Bacillus licheniformis* (NMCC-Ru1), *Bacillus tequilensis* (NMCC-Ru2) and *Bacillus subtilis* (NMCC-Ru3), whereas, *Lactococcus lactis* (NMCC-M16) was isolated from milk sample (Table 2 and Figure 2). Althoughlarge variety of bacterial strains that have been introduced as a probiotic in the market; however, the heavily claimed beneficial potential of such bacterial strains has not been evaluated in most cases. The autochthonous bacterial strains may prove more compatible with the animal gut microflora; therefore, investigation of the probiotic potential of new indigenous strains is indispensable (Naeem et al., 2018), i.e. *Lactobacillus ruminis* (MK463977), *Pediococcus pentosaceus* (MK463958), *Weissella soli (*MK463978). *Weissella bombi (MK463957).*

3.2. Acid tolerance

It is important to understand that once in transit through the gastrointestinal tract, resistance and survivability of probiotic bacteria extremely depend on bacterial strain. Furthermore, it is well established that each strain has its unique acid tolerance property. This exclusive property varies immensely between strains and species of lactic acid bacteria (Gu et al., 2008). While selection of a good probiotic is an important criterion that it must be resistant to low pH. As it is difficult for microorganisms to sustain in unfavorable conditions (pH 2–3) of stomach during transport, where it takes 2-4 hours for the processing of food (Montoro et al., 2016). All the tested bacterial strains in this study exhibited stability at pH 2 for 2-3 hours deprived of showing any substantial decline in viability. Our results supported by the previous two studies as they had observed survivability of probiotic bacteria at pH 3 for 2.5 hours (Lee et al., 2012; Patel et al., 2014). This huge acid tolerance capacity of bacterial isolates

Table 2. 16S rRNA based gene analysis of putative probiotic strains.

Strain ID	Identified Species	Source (Nilli Ravi Buffalo)
NMCC-M1	*Weissella confusa*	Faecal
NMCC-f5	*Bacillus megaterium*	Faecal
NMCC-f12	*Pediococcus pentosaceus*	Faecal
NMCC-f13	*Weissella bombi*	Faecal
NMCC-f14	*Weissella cibaria*	Faecal
NMCC-f15	*Enterococcus lactis*	Faecal
NMCC-f16	*Lactobacillus ruminis*	Faecal
NMCC-f17	*Weissella soli*	Faecal
NMCC-M16	*Lactococcus lactis*	Milk
NMCC-Ru1	*Bacillus licheniformis*	Rumen
NMCC-Ru2	*Bacillus tequilensis*	Rumen
NMCC-Ru3	*Bacillus subtilis*	Rumen

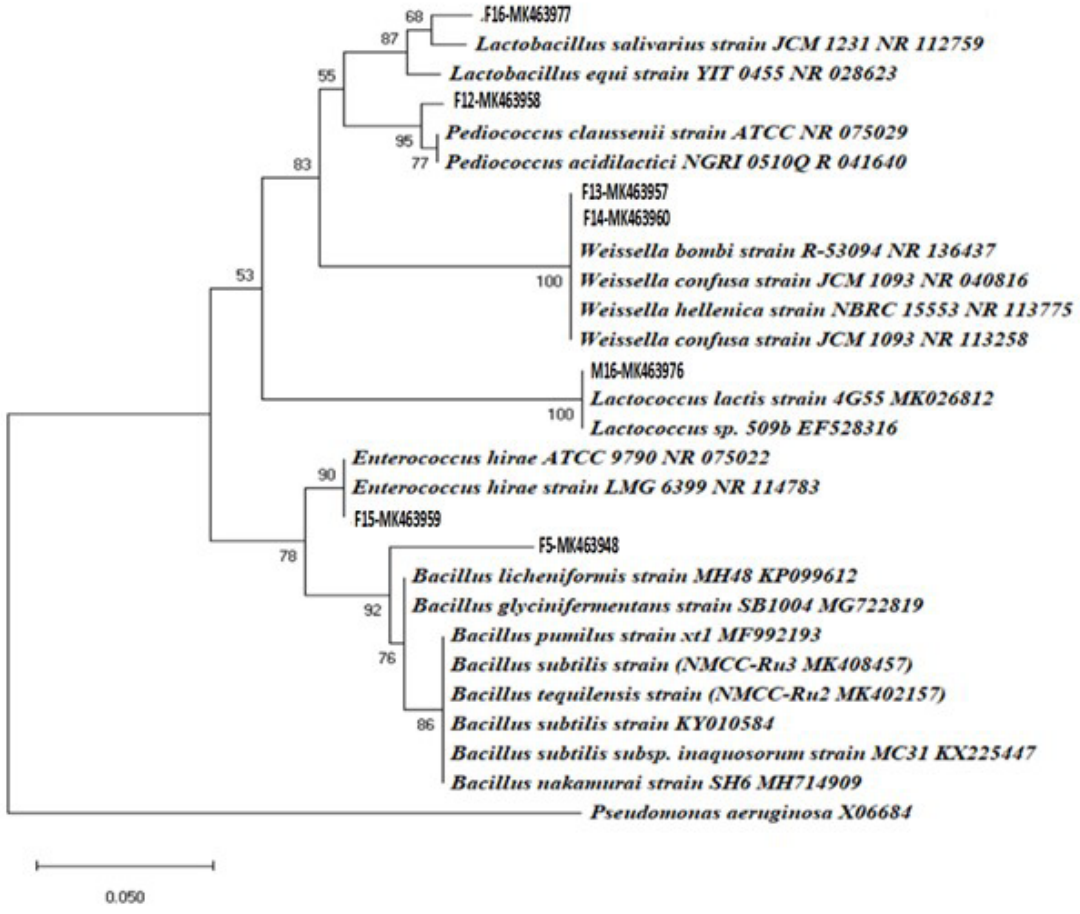

Figure 2. Phylogenetic tree of the bacterial isolates exhibiting the inter-relationship of most closely related type species inferred from 16S rRNA analysis

might be relying upon the source and H+-ATPase activity (Matsumoto et al., 2004).

3.3. Antimicrobial susceptibility assay

The bacterial strains have displayed various spectrum of antagonistic properties towards foodborne bacteria, NMCC-Ru2 has shown the strongest antibacterial action towards *Salmonella typhimurium* followed by NMCC-f12, NMCC-M1 and NMCC-Ru1 (Table 3 and Figure 3). The high antimicrobial mechanism against *Listeria monocytogenes* was displayed by NMCC-f12, NMCC-Ru2, NMCC-f16, NMCC-f13 and temperate activity by NMCC-M16. NMCC-Ru7 has elicited the strongest competitive exclusion mechanism against *Staphylococcus aureus*, while NMCC-M1 displayed moderate activity. In case of *Escherichia coli,* Ru7 exhibited potent antibacterial activity followed by NMCC-f5, NMCC-f13, NMCC-f14 and NMCC-f15. Overall, NMCC-Ru2 displayed maximum competitive inhibition against the tested pathogenic strains. This conclusion corresponded with the previous research; *Bacillus tequilensis* has been revealed to exhibit high antilisterial activity and purification of the antilisterial peptide has revealed it to be subtilisin A (Parveen Rani et al., 2016). One possible mechanism for showing excellent antibacterial activity from presumptive probiotic bacteria might be the secretion of some useful antimicrobial compounds such as niacin, bacteriocin, and lactic acid (Fisher and Phillips, 2009). The usage of bacteriocins in food items acts against pathogens and prolongs their shelf life, therefore the production of bacteriocins is considered an invaluable probiotic feature (Yang et al., 2014). In addition, bacteriocins have a key role as auspicious alternatives to fight against emergent resistance shown by microorganisms (Cotter et al., 2013; Hammami et al., 2013). Overall, NMCC-Ru2 has exhibited the enormous potential of antimicrobial activity against the tested pathogens.

3.4. Antibiotic resistance profile

Bacterial strains were tested using the procedure provided by Clinical and Laboratory Standards Institute

Table 3. The antimicrobial susceptibility spectrum of bacterial isolates against pathogens and their inhibitory zones diameter (mm).

Strain ID	*Salmonella typhimurium*	*Listeria monocytogenes*	*Escherichia coli*	*Staphylococcus aureus*
NMCC-M1	21mm	_	_	18mm
NMCC-f5	_	_	20mm	_
NMCC-f12	25mm	24mm	_	_
NMCC-f13	_	20mm	19mm	_
NMCC-f14	_	_	19mm	_
NMCC-f15	_	_	15mm	_
NMCC-f16	_	23mm	_	_
NMCC-f17	_	_	_	_
NMCC-M16	_	17mm	_	_
NMCC-Ru1	16mm	_	_	_
NMCC-Ru2	28mm	23mm	21mm	35mm
NMCC-Ru3	_	_	_	_

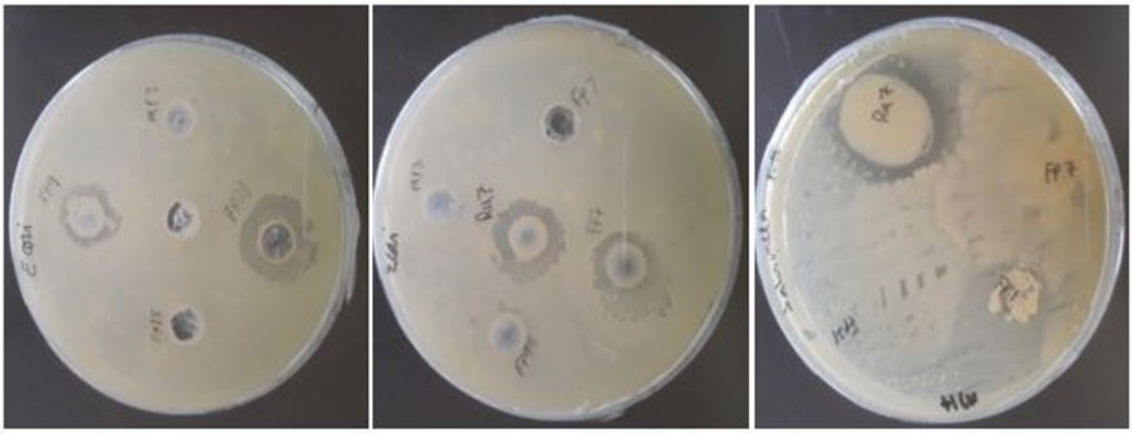

Figure 3. Antimicrobial activity of isolated strains against pathogens on MRS media plates.

(CLSI, 2016) for their resistance towards commonly used antibiotics. The antibiotics used in this research included Tetracycline, Ampicillin, Kanamycin, Chloramphenicol, Streptomycin and Gentamycin. The sensitivity of bacterial isolates against antibiotics can be seen in Table 4. Various patterns of resistance had shown by the isolated bacterial strains against the tested antibiotics. The indiscriminate use of antibiotics as growth promoters in human and veterinary medicine leads to the production of drug-resistant strains among microorganisms (Robredo et al., 2000). Strong susceptibility had shown by NMCC-M16 towards all the selected antibiotics, while for ampicillin it showed intermediate resistance previous finding is correspondent with the susceptibility spectrum of *Lactococcus lactis* (Khemariya et al., 2013; Liasi et al., 2009; Ozdogan et al., 2012). In contrast NMCC-f13, NMCC-f17 and NMCC-M1 are sensitive to Chloramphenicol, Gentamycin, Kanamycin and Tetracycline, but mainly resistant to ampicillin and streptomycin. These figures were in accordance with previously cited literature (Lee et al., 2012) excluding the statistic that they described *Weissella* strains are sensitive towards ampicillin; whereas NMCC-f14 displayed resistance against ampicillin, gentamycin, kanamycin and streptomycin and sensitivity to tetracycline and chloramphenicol. Various pattern of resistance towards ampicillin and sensitivity against tetracycline, chloramphenicol and gentamycin was shown by NMCC-Ru1, NMCC-Ru2, NMCC-Ru3 and NMCC-f5. Yet NMCC-Ru1 and NMCC-f5 showed resistance towards streptomycin and kanamycin; NMCC-Ru2 and NMCC-Ru3 have displayed sensitivity for these two drugs. Likewise, NMCC-f16 was sensitive against all the antibiotics used in this study but it has shown intermediate resistance for ampicillin. The antibiotic resistance spectrum exhibited by Lactobacillus and Bacillus species was also reported by different researchers (Anandharaj et al., 2015; Nguyen et al., 2015; Parveen Rani et al., 2016). NMCC-f15 has shown to be more sensitive against applied antibiotics excluding ampicillin (Braïek et al., 2018). NMCC-f12 demonstrated sensitivity to chloramphenicol, gentamycin, tetracycline and resistance to ampicillin, kanamycin, and streptomycin (Cao et al., 2016; Cavicchioli et al., 2019; Fernandes et al., 2018), whether antibiotic resistance among putative probiotic strains is a desirable attribute or not is an extremely controversial subject. Some researchers have the opinion that probiotic bacteria having antibiotic resistance is mainly dangerous as they can pass these resistance determinants to the host gut microflora (Angmo et al., 2016). Whereas, others claim that it is useful as the antibiotics along with probiotics may enhance the healthy microflora that was worn during antibiotic treatments.

3.5. Lipolytic activity screening

Lipases hold much attention since they involve in a wide range of reactions; moreover, lipases play a significant role in many industrial processes including food industry in enhancing flavour, texture and aroma of products. Hence, lactic acid bacterial strains having lipolytic potential are of vital importance (Lopes et al., 2002). The selected bacterial strains had shown variable responses to in vitro screening of lipase production. NMCC-M1, NMCC-f15, NMCC-f16 and NMCC-M16 had shown strong *in vitro* lipolytic activity which has been documented before. Whereas NMCC-f17, NMCC-f14, NMCC-f13, and NMCC-f12 also bear lipase activity as seen in other studies. While all of the putative probiotic strains had shown considerable lipolytic potential NMCC-RU1, NMCC-RU2, NMCC-RU3, and NMCC-f5 are an exception and this also concurs with previous studies (Table 5 and Figure 4).

3.6. Screening for proteolytic potential

Proteolytic arsenal by LAB is not only crucial for their growth in milk but it also has a key role in the development of taste and aroma in fermented foods (Siezen, 1999).

Table 4. Antibiotic resistance profiles of isolated strains against commonly used antibacterial compounds.

Strain ID	Amp[a]	Chl[b]	Gen[c]	Kan[d]	Str[e]	Tet[f]
NMCC-M1	12±0.00 (R)	30±0.70 (S)	22±0.00 (S)	21.5±1.41(S)	17±0.70 (S)	22.5±0.70 (S)
NMCC-f5	27±1.41 (I)	23±0.70 (S)	15.5±2.12(S)	2±0.00 (R)	3±0.70 (R)	26±0.00 (S)
NMCC-f12	26.5±2.12 (I)	23±2.82 (S)	29±2.12 (S)	9±0.70 (R)	2±0.00 (R)	20±1.41 (S)
NMCC-f13	15±1.41 (R)	31±0.70 (S)	22±0.00 (S)	20.5±0.70(S)	13.5±2.82 (I)	21±0.00 (S)
NMCC-f14	21±0.00 (R)	27±1.41 (S)	11±0.00 (R)	15±0.70 (I)	10.5±0.70 (R)	22±1.41 (S)
NMCC-f15	19±0.00 (R)	28±0.00 (S)	20.5±2.12 (S)	23.5±0.70 (S)	20±2.82 (S)	25±0.707 (S)
NMCC-f16	21.5±0.70 (I)	26.5±3.53 (S)	23±0.70 (S)	22±0.00 (S)	17.5±0.70 (S)	29±0.00 (S)
NMCC-f17	19±0.00 (R)	28±0.70 (S)	20±0.70 (S)	23.5±0.00 (S)	20±0.00 (S)	24.5±0.70 (S)
NMCC-M16	30±2.82 (S)	29.5±0.70 (S)	28±0.00 (S)	21.5±0.70 (S)	12±0.00 (I)	31±1.41 (S)
NMCC-Ru1	22.5±3.53 (I)	26.5±0.70 (S)	16±0.70 (S)	4±0.00 (R)	9.5±0.70 (R)	20±0.00 (S)
NMCC-Ru2	18.5±0.70 (R)	25.5±0.70 (S)	21.5±0.70 (S)	23±0.00 (S)	17±0.00 (S)	25±5.65 (S)
NMCC-Ru3	23.5±1.41 (I)	28±0.00 (S)	23.±0.70 (S)	23.5±0.70 (S)	15±1.41 (S)	20.5±0.00 (S)

Zone of inhibition: Resistant, Intermediate resistant, Susceptible; a) Ampicillin, b) Chloramphenicol, c) Gentamycin, d) Kanamycin, e) Streptomycin, f) Tetracycline. P-values for antibiotics are as follows; Ampicillin (0.000), Chloramphenicol (0.0017), Gentamycin (0.002), Kanamycin (0.000), Streptomycin (0.000) and Tetracycline (0.003).

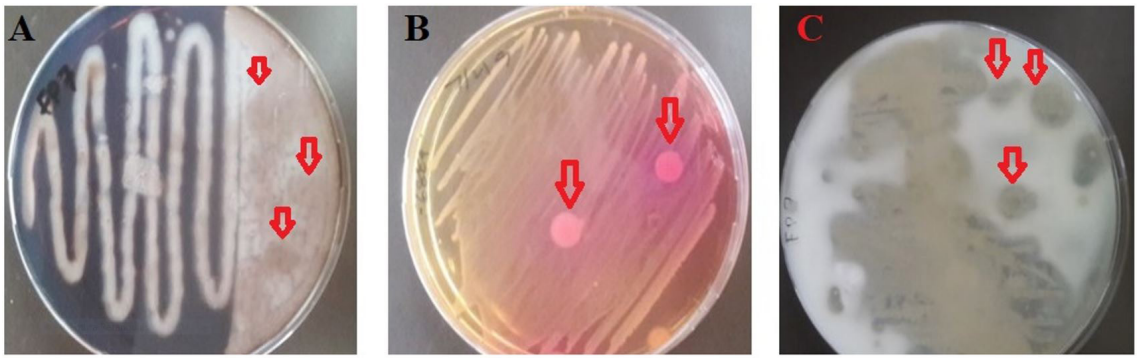

Figure 4. Enzymatic Potential of *P. pentosaceus* SPARC2: (A) Amylolytic (B) Lipolytic (C) Proteolytic.

Table 5. Spectrum of enzymatic potential displayed by LAB isolates.

Strain ID	Amylolytic	Lipolytic	Proteolytic
NMCC-M1	++	++	++
NMCC-f5	++	_	++
NMCC-f12	++	+	++
NMCC-f13	++	+	++
NMCC-f14	++	+	++
NMCC-f15	+	++	+
NMCC-f16	++	++	+
NMCC-f17	++	+	+
NMCC-M16	++	++	++
NMCC-RU1	++	_	++
NMCC-RU2	++	_	++
NMCC-RU3	++	_	++

(+, weak) (++ strong positive) (– no activity).

Since the milk products do not contain a sufficient amount of low molecular peptides and amino acids which are necessary for LAB growth .To hydrolyse milk proteins into free amino acids s an active system of proteases is required (de Souza and Dias, 2017; Leboš Pavunc et al., 2012). Impressively, all the LAB strains tested in this study have exhibited strong proteolytic properties with NMCC-RU1, NMCC-RU2, NMCC-R3, NMCC-f5, NMCC-f12, NMCC-f13, NMCC-f14, NMCC-M16, and NMCC-M1 displaying the highest potential subsequently followed by NMCC-f17, NMCC-f15, and NMCC-f16 (Table 5 and Figure 4).

3.7. Screening for amylolytic potential

Amylolytic Lactic acid bacteria (ALAB) have exceptional significance because they produce alpha amylases and lactic acid by modifying the structural properties of starch and are therefore used for a broad spectrum of industrial applications (Sundarram and Murthy, 2014). With the prevention of chemical oxidants like potassium bromate (used as a bread improver), screening and selection of suitable amylase-producing strains is required (Amapu et al., 2016). All the selected LAB strains had shown maximum amylolytic activity with NMCC-M1, NMCC-f5, NMCC-f12, NMCC-f13, NMCC-f14, NMCC-f16, NMCC-f17, NMCC-M16, NMCC-RU1, NMCC-RU2 and NMCC-RU3 whereas NMCC-f15 had also been able for hydrolysis of starch substrate with considerable potential. The amylolytic potential spectrum has been given in Table 5 and Figure 4.

3.8. Screening of haemolysin production

The most crucial characteristic of a probiotic strain is the absence of virulence traits. Production of hemolysin has to be screened progressively for the selection of a probiotic strain. The tested LAB strains exhibited negative results for the production of both α-and β-hemolysis.

4. Conclusion

The frequent use of antibiotics as animal growth promoter is causing huge anxiety, to overcome this problem, microbiologists are trying to introduce new alternatives agents having least adverse reactions on host health and production. According to present research, live microbial feed supplements (probiotics) could be used as an best perfromaing alternative of antibiotics if they are isolated and fed to the same host. This is the first report on isolation and evaluation of probiotics from Nili Ravi buffalo in Pakistan. Our work exposed that LAB isolated from buffalo gut were tolerated to acid and bile condition, showed excellent probiotic potentials and antagonistic activity towards tested animal pathogens. This research revealed that the isolated probiotics may have a role in balancing the stability between pathogenic bacteria and normal microflora of animals. This study has suggested that probiotics in animal feed can reduce certain enteric infections like diarrhoea, food indigestion, gastroenteritis, and rumen acidosis.

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