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Screening of cattle gut associated *Bacillus* strains for their potential use as animal probiotic

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ABSTRACT

Use of antibiotic in animal feed pose a significant risk to human health. Over the last decade uses of probiotics increasingly viewed as an alternative to antibiotic. *Bacillus* are currently being used as feed supplements in animal diet, due to their enhanced tolerance rate and survivability under harsh gut environment. Animal gut is a rich source for *Bacillus* species isolation. There is still a scarcity of information on the *Bacillus* species of livestock species. The present study was carried out to identify *Bacillus* species from animal gut to evaluate their probiotic potential. Three (NCCP-2004a, NCCP-2029 and NCCP-2029) strains were selected as putative probiotics strains based on their super acid and bile survival rates. These isolates were identified as *Bacillus subtilis* subsp. inaquosorum (NCCP-2029, NCCP-2025). *Bacillus tequilensis* (NCCP-2004a) by 16S rRNA gene sequencing. NCCP-2004a elicited best results regarding antimicrobial potential and its ability to survive on acid and bile conditions. The results of present study demonstrated that the *B. tequilensis* showed a promising alternative probiotic candidate to the use of antibiotics in the dairy feeding system.

Key words: Animal probiotics, *Bacillus tequilensis*, Intestinal microbiota, *Sahiwal* cattle.

INTRODUCTION

Dairy items describe a significant industry all over the world. It is estimated that 99% of this milk production come from the ruminants (Dill-McFarland *et al.*, 2017; Chitra *et al.*, 2018; Sahin *et al.*, 2017). The diverse complex of microflora in animal gut may play a critical role for their low or high production performance.

Probiotic are living microbes that provide beneficial effects on host by maintaining its gastrointestinal tract microbial flora equilibrium (Zoumpopoulou *et al.*, 2018). The most frequently used strains of probiotic microbes are lactic acid bacteria, *Bifidobacteria* and yeast. The selection criteria of the probiotics are very important factors which affect the probiotic quality, safety and validity of commercial probiotic products. A felicitous probiotic bacteria must fulfill some essential standards, like its ability to adhere on gut epithelial cell wall, to overcome possible obstruction inside gut, like low pH and high concentration of bile acids, compete with pathogen (Anandharaj *et al.*, 2015). The probiotic strains must tolerate the manufacturing, transportation, storage and application steps. Previously literature indicated that, probiotic strains like, lactic acid bacteria, *Bifidobacteria* and yeast, has been facing some difficulties in term of storage transportation point of views (Frizzo *et al.*, 2018). Researches show that for long storage term storage, we need some techniques like, microencapsulation and lyophilization which leads to additional cost (Bora *et al.*, 2018).

To overcome this drawback, now a days scientists use spore forming bacteria, especially from genus *Bacillus* as feed supplements in animal and human diets, because of long shelf life and their significant resistance to unfavorable conditions (Petruk *et al.*, 2018). *Bacillus* can be considered as a metabolically active member of host microbial population (Grutsch *et al.*, 2018). In addition, survival ratio of some gut associated *Bacillus* may be linked to their capability to synthesize biofilms, which is a protecting material against the GIT unfavorable conditions. Nevertheless, current researches revealed that some *Bacillus* spp. also provide the vital probiotic properties and have the capability to produce some important antimicrobial substance, like; bacterocin, which has a comprehensive inhibitor on many pathogen (Mingmongkolchai *et al.*, 2018). *Bacillus* species widely used as animal probiotics in South East Asia markets (Cutting, 2011). These reports motivated us to identify the animal origin *Bacillus* species from lactating dairy cattle of *Sahiwal* breed for their incorporated into feed as probiotic.

MATERIALS AND METHODS

Three healthy *Sahiwal* lactating dairy cattle were raised at the Livestock Research Station, National Agriculture

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Research Center, Islamabad, Pakistan. The nutrient requirements of the dairy cows were fulfilled by offering the diet composed of 4 kg concentrates feed, 50 kg fodder. Fresh fecal samples from all animals were collected by hand from deep in the rectum by using gloves. Sterile polythene bags were used for sample collection and samples were delivered to laboratory for further preservation and processing. For the isolation of bacterial strains, 1gm of fecal sample was mixed in phosphate buffer saline. The samples were further processed and examined on De Man, Rogosa and Sharpe agar, media. This media composed of $C_6H_{12}O_6$ (18.5 g L^{-1}), agar technical (15g/L) meat peptone (10g/L), beef extract (8 g/L), yeast extract (4g/L), $C_2H_3NaO_2$ (3 g/L), K_2HPO_4 (2g/L), $C_6H_{17}N_3O_7$ (2g/L), Tween 80 (1g/L), $MgSO_4.7H_2O$ (0.2g/L) and $MnSO_4.4H_2O$ (0.05 g/L). The samples were spread on MRS plates and incubated aerobically at 37 °C for 24 h. The acid and bile salt tolerance test of 10 isolates have been performed and results indicated that only 3 strains (NCCP-2004a, NCCP-2029 and NCCP-2029) showed bile and acid tolerance. Colony morphology of strains were observed by using phase contrast microscope (Phase contrast 2, Nikon, Japan). For complete morpho logical characterization of the isolates, we used scanning electron microscope (MIRA3, Tescan SEM). For extraction of template DNA from the pure bacterial colony, single colony of each strain was picked and mixed properly with 20µL of Tris-EDTA buffer in PCR strips. The mixture was heated (95 °C) for 10 min in the PCR machine. After centrifugation the supernant was removed, which served as template DNA. Amplification of the 16S rRNA gene was done by the using PCR machine. We used 25 µL of the TAKARA Pre-mix Ex-Taq; 2 µL of Universal forward primers 9F (5`′-GAGTTGATCCTGGCTCAG-3`′) and 2 µL of Universal reverse primers 1510R (5`′-GGCTA CCTTGT TACGA -3`′); 20 µL PCR water, and 1 µL template DNA (total volume 50 µ L) were used for the PCR amplification of DNA. The amplified PCR products were sequenced by using Macrogen sequencing, Korea (http://dna.macrogen .com). The strains were identifed at species level by using the EzBioCloud server (https://www.ezbiocloud.net/ identify). All probiotic bacterial strains sequences were submitted to NCBI for getting the accession numbers. The tree was constructed from unambiguously arranged nucleotides sequence using an algorism by using MEGA 7 (Saitou and Nei, 1987). The acid tolerance was determine by methods given by Parveen *et al*, (2016). Briefly, *Bacillus* strains were growth in MRS broth (pH; 1.0, 2.0, 3.0 and 7.0) and culture plates were incubated at 37 °C for 20 h aerobically. The bacterial cells count (log CFU/mL) was done by growing the isolates (MRS agar) at 37 °C for 20 h aerobically. The experiments were performed in triplets and means were calculated. The bile salt resistance of the *Bacillus* strains was calculated by using MRS broth having different concentration of bile salts (Oxgall, Merck). Freshly grown *Bacillus* culture were centrifugate and re-suspended in the MRS broth (0.3 or 0.5% bile salts) and then kept in an incubator at 37 °C. After 3 and 5 h, I ml of samples was withdraw and plated on MRS agar and incubated at 37 °C aerobically for 24 hours. The experiments were performed in triplets and means were calculated. Antimicrobial activities of the *Bacillus* strains were determine by using the pathogenic strains such as, *Pseudomonas aeruginosa* (ATCC9027), E.coli (ATCC8739) and *Staphylococcus aureus* (ATCC6538) based on Shakira *et al*. (2018). Briefly, 100µl of pathogenic strain was suspended in 2.5ml of (0.75% TSA) soft agar. In order to prepare the lawn of pathogenic strains, soft agar suspension was poured into petri plates having the TSA media. The prepared plates were incubated aerobically at 37 °C for 3 h. Sterile disks were set on the lawn of indicator strains and poured the *Bacillus* strains supernatant (10µl) on filter paper disks and incubator at 37 °C for 48 h. Results of antimicrobial activity were observed in terms of its zone diameter (nm). A clear zone formation around the disks, determine the antimicrobial activity of the testing strains (Fig 1). Antibiotic susceptibility profiles of the *Bacillus* isolates were generated by using diûerent cell wall synthesis and protein synthesis inhibitor and other antibiotic disks given by European Food Safety Authority (EFSA) panel in 2012 (Amoxicillin, Cefuroxime, Chloramphenicol, Erythromycin, Kanamycin, Gentamicin, Metronidazole, Penicillin, Vancomycin, Streptomycin, Clindamycin and Tetracycline (Bioanalyse, Turkey) were used. *Bacillus* culture (100 µl) was spread over the MRS plates (4 mm) and prepared disks were seeded on the agar surface and incubated aerobically at 37 °C for 24 h.

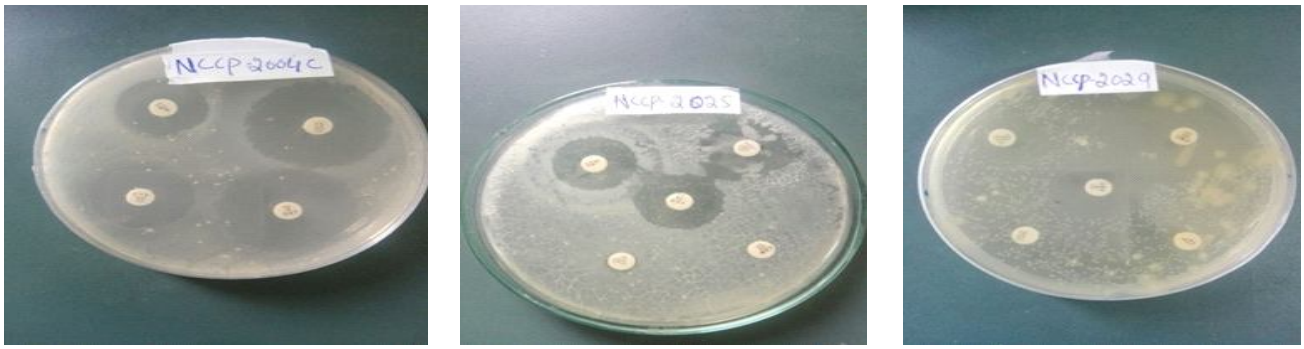

Fig 1: Antimicrobial sensitivity of bacterial isolates as candidate for animal probiotics

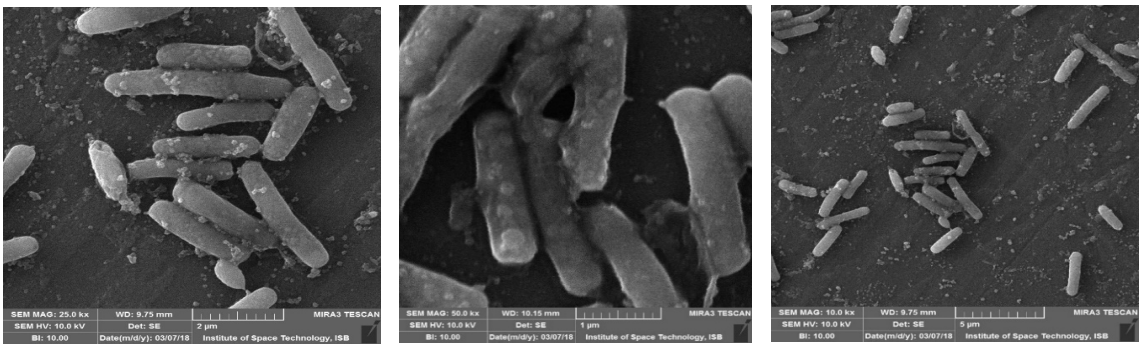

Fig 2: Scanning electron microscopy view of bacterial isolates as candidate for animal probiotics

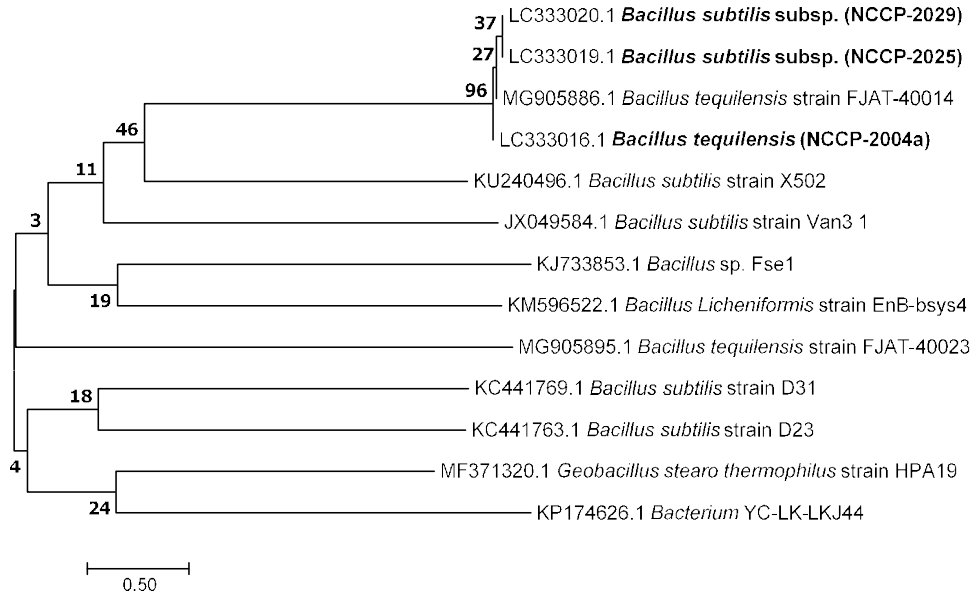

Fig 3: Phylogenetic tree of the bacterial showing the inter-relationship of most closely related type species interfered from 16 s rRNA

Inhibition zone diameter (in mm) were measured accurately for result interpretation.

To measure the hemolytic activity *Bacillus* isolates were grown on the nutrient agar (NA) having 4% blood based agar (Hi media) (5% human blood and 3 % NaCl). Plates were incubated for 48 h at 37 °C. The results were measured as; formation of green zones around the bacterial colonies and slight hydrolysis (α hemolysis), clear zone of hydrolysis around colonies (β hemolysis), and without any change in the agar (γ hemolysis). All experimental tests were performed in triplicates. Results are represented in mean plus minus standard deviation. All statistical analysis was done by using the statistics (Version 8.1) software. The significant difference between the means were assessed by Tukey's test ($p<0.05$).

RESULTS AND DISCUSSION

Identification of the animal origin probiotic bacteria at strain level is a critical step for the determination of the safety of the microbes. In this study, ten pure bacterial cultures were isolated from the fecal samples of dairy cow and three were selected for their potential probiotic tests. These isolates were molecular identified as *B. subtilis* (NCCP-2029, NCCP-2025) *B. tequilensis* (NCCP-2004a) (Table 1). The morphological characterization are given in the Fig 2. The molecular identification results showed that, *Bacillus* NCCP2004a revealed 98.53 % identity to *B. tequilensis.* Similarly, *Bacillus* NCCP-2025 and NCCP-2029 revealed 99.58% and 99.90 % identity to *B. subtilis* subsp. inaquosorum (Table 1, Fig 3). Many probiotics are commercially available in the local markets, but their probiotic potential in a local animal breed diet is questionable. Therefore, investigation of the new indigenous probiotic strain is of great interest in this context. This experiment was the ûrst report to characterize the *B. tequilensis* fecal samples of lactating cow. The probiotic strains of same ecological origin may be more compatible with animal gut microbes giving highest outputs (Shakira *et al.*, 2017). Acid tolerance is usually known as an essential criteria in the in probiotic selection. The probiotic strains should express high survival rate against different acidic conditions (Anandharaj *et al.*, 2015). All *Bacillus* showed a wide range of pH tolerance at 1 to 4. In addition, *B.*

tequilensis strains showed significantly ($p<0.05$) good results of bile tolerance followed by (NCCP-2029) and (NCCP-2015) strains (Table 3). The viable bacterial counts in pH adjusted 1 to 4 in MRS media were according with the finding of Khochamit *et al.* (2015) and Nguyen *et al.* (2015). Bile salt are very toxic for living organism and bile salts tolerance is known as a useful property to demonstrate the strain ability to colonization to the host gut, balancing the GIT microbial flora. In this study, 0.3 bile salt concentration were used for determination of the *Bacillus* strains bile salt tolerance ability. Our tested strains were found to be against the changeling 0.3 % bile salt concentration by showing the variable degree of resistance after 5h exposure. The strains *B. tequilensis* was the most significantly ($p<0.05$) resistant strain (Table 4). Similar results were given by Parveen *et al.* (2016). A vital property of the probiotic strains is the antipathogenic activity. The inhibition activity of *Bacillus* strains against three pathogens (E. coli, *P. aeruginosa* and *S. aureus*) are given in (Table 5). All *Bacillus* strains showed the resistance against the pathogenic bacteria strains. NCCP-2004a showed exhibited clear zone of 13.06 mm against E.coli which is significantly ($p<0.05$) higher followed by NCCP2025 and NCCP-2029 strains which had 11.57 and 10.23 mm inhibition zone, respectively. Similarly, NCCP-2004a (14.78mm) had revealed significantly ($p<0.05$) high inhibition (14.78 and 13.23mm) against *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively than other probiotic candidate strains. Over all pathogenicity results revels that NCCP-2004a showed maximum ($p<0.05$) inhibition against all the tested pathogenic strains. This might be due to the antimicrobial protein produced from the *B. tequilensis* (Parveen *et al.*, 2016). *Bacillus* species can spread from its habitat to feed and food particles due to its endospore production and its long term survival ability. *Bacillus* species produced a number of antimicrobial substances (surfactin, bacteiocin etc.) those exhibiting broader inhibition spectra against various food borne pathogens (Abriouel *et al.*, 2011; Khochamit *et al.*, 2015). The results revealed that *B. tequilensis* found resistant to most of tested antibiotics in various degree expect kanamycin and amoxicillin (Table 6).

Table 1: 16S-rRNA based gene analysis of selective bacterial strains as candidate for animal probiotics

Strain ID	Probiotic strain name/ genus	Length of 16S r RNA (ntds)	Accession number based on Gene bank	Taxonomy (http://eztaxone.ezbiocloud.net)
NCCP-2004a	*Bacillus* sp.	1424	LC333016	*Bacillus tequilensis* (KCTC 13622)
NCCP-2025	*Bacillus* sp.	1104	LC333019	*Bacillus subtilis* subsp. inaquosorum strain (DE111)
NCCP-2029	*Bacillus* sp.	1130	LC333020	*Bacillus subtilis* subsp. inaquosorum strain (DE111)

Table 2: Different pH (7, 1, 2, 3, and 4) effects on survival of isolated strains

Strain	Control 1	pH 1.0	pH 2.0	pH 3.0	pH 4.0
			log CFU/mL		
NCCP-2004a	6.98 ± 0.04^{a}	2.12 ± 0.11^{a}	3.21 ± 0.9^{a}	4.99 ± 0.2^{a}	5.12 ± 0.1^{a}
NCCP-2025	6.56 ± 0.08^{ab}	1.45 ± 0.3^{b}	4.34 ± 0.1^{b}	5.78 ± 0.07^{ab}	5.87 ± 0.09^{b}
NCCP-2029	6.37 ± 0.05^{c}	1.76 ± 0.1^{c}	3.67 ± 0.1^{c}	5.54 ± 0.2^{c}	5.67 ± 0.1^{c}

1 Control: Strains grown on pH 7.0. 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 Tukey's test.

Table 3: Effect of different bile salt concentration, on survival of isolated strains

Strains	0.3% oxgall Log cfu/mL			0.4 % oxgallLog cfu/mL			0.5 % oxgallLog cfu/mL		
	Cont1.	3h	5h	Cont.	3h	5h	Cont.	3h	5h
NCCP-2004a	7.67 ± 0.2^{a}	5.32 ± 0.08^{a}	5.23 ± 0.1^{a}	6.60 ± 0.03^{a}	5.32 ± 0.02^{a}	5.23 ± 0.04^{a}	7.67 ± 0.05^{a}	5.32 ± 0.01^{a}	5.23 ± 0.01^{a}
NCCP-2025	7.34 ± 0.1^{ab}	6.34 ± 0.06^{b}	6.12 ± 0.03^{b}	7.34 ± 0.05^{b}	6.34 ± 0.05^{ab}	6.12 ± 0.02^{b}	7.34 ± 0.06^{ab}	6.34 ± 0.02^{b}	6.12 ± 0.02^{ab}
NCCP-2029	7.11 ± 0.1^{b}	6.14 ± 0.04^{c}	6.13 ± 0.03^{c}	$7.11\pm o.01^{c}$	6.14 ± 0.01^{c}	6.13 ± 0.01^{c}	7.11 ± 0.01^{c}	6.14 ± 0.01^{c}	6.13 ± 0.01^{c}

1Control: Strains grown with or without bile salt addition in MRS broth. 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 Tukey's test.

Table 4: The antipathogenic activity of bacterial strains against pathogenic strains and their inhibitory zones diameter (mm)

Strains	E. coli(ATCC8739)	*Pseudomonas aeruginosa*(ATCC9027)	*Staphylococcus aureus*(ATCC6538)
NCCP-2004a	13.06 ± 0.05^{a}	14.78 ± 0.3^{a}	13.23 ± 0.1^{a}
NCCP-2025	11.56 ± 0.3^{b}	11.25 ± 0.16^{ab}	10.23 ± 0.04^{ab}
NCCP-2029	10.23 ± 0.1^{c}	12.12 ± 0.18^{c}	10.34 ± 0.02^{c}

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 Tukey's test.

Table 5: Antibiotic resistance profiles of isolated strains against commonly used antibacterial compounds.

Strains	Amo[a]	Cef[b]	Chd[c]	Ery[d]	Kan[e]	Gen[f]	Met[g]	Pen[h]	Teet[i]	Van[j]	Stre[k]	Cli[L]
NCCP-2004a	+	++	-	++	-	++	+++	+	+	-	+	+
NCCP-2025	+	++	-	++	-	++	+++	+	-	-	+	-
NCCP-2029	+	++	-	++	-	++	+++	+	+	-	-	-

Zone of inhibition (++) Resistant, (+) Intermediate resistant (-) Susceptible; aAmoxicillin, bCefuroxime, cChloramphenicol, dErythromycin, eKanamycin, fGentamycin, gMetronidazole, hPenicillin, iTetracycline, jVancomycin, kStreptomycin, LClidamycine

This study results concurred with results of Coetzee (2015), that the *B. tequilensis* showed resistance against penicillin, tetracyclic, streptomycin and trimethoprim. Potential spore producing probiotic used in animal feed are mainly isolated from animal feces. Probiotics which have haemolytic capability is considered a disadvantage. Some *Bacillus* species produced hemolysis, which could be a health risk to the host. In present study, all *Bacillus* strains showed no hemolysis. Parveen *et al.*, (2016) had worked on probiotic potential of *Bacillus* strains and their results were in accordance with present findings in which *B. tequilensis* FR9 showed no hemolysis. Similar results were given in the one of the experiment of Luis-Villasenor *et al.* (2011), who noted that *Bacillus* species showed alpha hemolysis. This means the *Bacillus* species did not show any risk to host. Recently, it has been noted that, *B. tequilensis* could be used as human probiotic because it produces the novel silver nanoparticles and showed significant cytotoxic effects against cancer (Parveen *et al.*, 2016).

CONCLUSION

Utilization of probiotics for health and production is influenced by many factors including probiotic strains, age and breed of cattle. In this respects, identification for novel animal origin probiotic strains will be the key research and development spot for future livestock markets all over the world. In conclusion, this study is the first to identify the potential animal origin probiotic *B. tequilensis* strain from dairy cow gut which can be safe for animal consumption. This work highlighted that there might be very useful microbiota inside the GIT of dairy animals, which should be identified and characterized for potential use as animal origin probiotic to improve productivity near future.

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