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Antibiotic resistance profile of lactic acid bacteria isolated from raw milk of Badri cows

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Abstract
In this study lactic acid bacteria (LAB) were isolated from raw milk of Badri cattle to investigate antibiotic resistance profile. A total of 47 non-hemolytic LAB could be isolated from 62 raw milk samples. The percentage of antibiotic resistance ranged from 2-68% in the LAB isolates obtained. High resistance was observed against cefixime (68.08) and penicillin (51.06), followed by streptomycin (17.02), co-trimoxazole (8.51), levofloxacin (4.25) and low in amoxyclav (2.13), azithromycin (2.13). Nearly 82.97% isolates had MAR Index of 0.2 or less. Species wise resistance profile demonstrated *Lactobacillus* to be the least and *Leuconostoc* to be the most resistant.

Keywords: Badri milk, lactic acid bacilli, antibiotic resistance

Introduction
With the advent of the COVID-era there has been a boost in the use of both prophylactic and therapeutic medication. Though mankind has somehow managed to restrain the virus for now, the indiscriminate use of antibiotics has led to development of antibiotic associated diarrhoea and other complications. There is an increase in demand for foods that has ability to modulate the immune system of the host without side effect. Scientist are now focusing on, the microbials like probiotics, that on administration in adequate amounts (~10^8- 10^{10} CFU/gram) confer health benefits (FAO/WHO 2002) [7].

Lactic acid bacteria (LAB) are gram positive and catalase negative that produce lactic acid after fermentation of carbohydrates, generally recognized as safe (GRAS) and used as culture for numerous probiotic and fermented products belonging to *Enterococcus* and *Lactobacillus* (Reuben *et al*., 2020) [12]. Raw milk is one of the sources of lactic acid bacteria. LAB produce functional ingredients like exopolysaccharides (EPS), bacteriocin, hydrogen peroxide, which show use in medicine and food industry (Voidarou *et al*., 2021) [14] with health benefits like lower serum cholesterol, reduce gut and urogenital pathogens, increase nutrient utilization, help in diabetes, obesity, inflammation, allergies etc., stabilize gut microflora, fight cancer and many more (Reuben *et al*., 2020 and Kaur *et al*., 2020) [12, 9]. Nevertheless, for its use in food industry LABs must be non-pathogenic, non-spoiling and susceptible for antibiotic.

The FAO/ WHO (2002) [7] recommended testing of probiotic LAB strains for antibiotic resistance for harbouring reservoir of antibiotic resistance genes, any association with endocarditis which may require antibiotic therapy (M Gad *et al*., 2014) [10] and use of antibiotic along with probiotic to treat GIT afflictions.

The present study reports isolation of LAB and their antibiogram from raw milk of Badri cattle, a newly registered (2017) Himalayan breed of Uttarakhand. which is said to be zero input. Badri is small grazing breed of cattle with low production, inhabiting the high altitude of Uttarakhand state. Due to their different habitat and feeding habit they were chosen to study underhand.

Materials and Methods
A total of 62 milk samples of apparently healthy Badri cattle were collected from Government Breeding Farm Nariyalgaon, Champawat, Uttarakhand, aseptically in sterile milk collection vials, transferred to lab on ice for processing immediately. Around 1 ml of Badri milk samples were enriched in MRS broth at 37 °C for 48 hrs under anaerobic condition. Then 1ml of enriched broth was serially diluted 10 folds in 9 ml normal saline solution. All diluted samples were plated on MRS agar and incubated anaerobically at 37 °C for 24 hrs.

Colonies of different morphology and colour were picked and streaked on MRS agar to get pure culture.

For presumptive identification of LAB isolates were subjected to Gram's staining, catalase, motility and hemolytic test. *E. coli* ATCC 10536 and *Lactobacillus acidophillus* ATCC (314) were used as positive control and negative control, respectively. All the isolates which were gram positive, catalase negative, nonmotile and non-hemolytic were considered as lactic acid bacteria isolates. Only non-hemolytic isolates were considered in the study as it is a prerequisite of probiotic strains. Further confirmation of isolates was done by using API 50 CH kit and sequencing.

The culture was examined by Gram Staining**.** The culture was tested for catalse using 3% hydrogen peroxide. The positive test showed effervescence of bubbles. Motility test medium was done by stabbing freshly grown culture in 7 ml motility test agar and incubated for 24 hrs at 37 °C. A positive motility test was indicated as turbid area extending away from line of stab while negative test was indicated by growth only on stab line (Shields and Cathcart 2016) [13]. Hemolytic character of the isolates was determined by streaking fresh culture on sheep blood agar and incubation for 24 hrs at 37 °C. The plates were observed for the formation of any β (clean), α (greenish) o γ-haemolysis (no such haemolytic zones) around the colonies (Wang *et al*., 2016) [15].

Antibiotic resistance was determined by the Kirby-Bauer disk disc diffusion method (Bauer *et al*., 1966) [3] with antibiotic discs namely, clotrimazole (COL), amoxicillin clavulanic acid (AMC), azithromycin (AZM), penicillin (P), cefixime (CFM), levofloxacin (LE) and streptomycin (S). The zone of inhibition was measured in millimetres (mm) to determine as sensitive (≥20 mm), intermediate (15–19 mm), or resistant (≤14 mm). The multiple antibiotics resistance (MAR) index was determined as previously described by (Das *et al*., 2022) [5]. If the MAR index value are more than two, the isolate was considered resistant to multiple antibiotics (Resende *et al*., 2014) [11].

Results and Discussion

From 62 collected raw milk samples of Badri cow, total 185 pure culture were screened for lactic acid bacteria. Out of the 185 pure culture only 112 isolates were catalase negative, further screening by gram staining reduced to 92 isolates as these were gram positive bacilli, coccobacilli and cocci hence were not considered for further study. Total 92 isolates were tested for motility and hemolytic character. Only 47 isolates obtained from raw milk of Badri cow were confirmed as non-motile, non-hemolytic lactic acid bacteria (Table 1). The 47 isolates belonged to *Leuconostoc*, *Lactobacillus*, *Lactococcus*, *Enterococcus* on confirmation by API 50 CH kit and sequencing.

Table 1: LAB isolates obtained from waw milk of Badri cow

Isolate obtained	Negative Catalase test	Positive Gram's staining	Negative Motility test	Hemolytic test (γ, non-hemolytic)	Presumptive LAB
185	112	92	76	47	47

The confirmed 47 isolates were examined for resistance against 7 commonly used antibiotics and results are presented in Table 2. Out of 47 lactic acid bacteria isolates, 9, 4, 32, 24, 1, 1 and 2 demonstrated resistance against streptomycin, cotrimoxazole, cefixime, penicillin, azithromycin, amoxyclav and levofloxacin, respectively. Isolates showed maximum resistance against cefixime (68.08%) and penicillin (51.06%), and minimum resistance against azithromycin (2.13%) and amoxyclav (2.13%). The MAR index was zero in ten isolates as they have shown susceptibility against all the studied antibiotics. MAR index in 29 isolates was between 0.1-0.2 and only remaining 8 isolates had MAR index more than 0.2.

Table 2: MAR index and antibiotic Resistance Profile of LAB isolates against antibiotics

	S (10mcg)	COT (25mcg)	CFM (5mcg)	P (10mcg)	AZM (15mcg)	AMC (30mcg)	LE (5mcg)
Resistance %	17.02	8.51	68.08	51.06	2.13	2.13	4.25

Species wise resistance profile of lactic acid bacteria isolated (Table 3) for 18.18% *Lactobacillus*, 32.14% *Leuconostoc*, 30.61% *Lactococcus* and 23.81% *Enterococcus* spp. had resistance against the tested antibiotics. *Lactobacillus* showed lowest resistance while *Leuconostoc* had highest resistance. *Enterococcus* had no resistance against 4 antibiotics *viz*., Amoxicillin clavulanic acid, azithromycin, cotrimoxazole and levofloxacin. Amoxicillin clavulanic acid, and levofloxacin were only resistance for *Lactobacillus* isolated and susceptible for rest isolates, while cefixime, penicillin and streptomycin had shown resistance for all LAB species isolated. Similar findings of low resistance of LABs against amoxicillin and macrolides was observed by (M Gad *et al*., 2014; Ammor *et al*., 2007) [10, 1]. In the same study a high resistance against penicillin was found in agreement to our study, though results for fluoroquinones were contrary. Earlier studies were also reported that *Lactobacillus* have high resistance against cephalosporins (Budiati *et al*., 2022; Anisimova *et al*., 2019) [4, 2]. Study of (Budiati *et al*., 2022) [4] showed that LAB isolated from kefir showed susceptibility for sulphamethoxazole, in our study we also reported that *Lactobacillus*, *Enterococcus* and *Leuconostoc* spp. isolated were susceptible for cotrimoxazole. Variations in the susceptibility of erythromycin against LAB were observed. Higher percentage of erythromycin resistant strains was observed among *Lactococcus* (22.2%). *Lactococcus* isolates showed high resistance to tetracycline (29.6%) followed by *Streptococcus* (12.3%) and *Lactobacillus* isolates (11.6%) (M Gad *et al*., 2014) [10].

Several studies detected higher resistance in LABs against antibiotics from 60-100% (Budiati *et al*., 2022, Erginkaya *et al*., 2018; KaidiWang *et al*., 2019) [4, 6, 8] while lower resistance against antibiotics were detected in LABs isolated from raw Badri cattle milk, this may be due to limited exposure of Badri cattle to these antibiotics.

Table 3: Antibiotic resistance profile of LAB according to species

Type of spp.	S	COT	CFM	AMC	AZM	P	LE	Mean %
Lactobacillus	13.64%	4.54%	50.0%	4.54%	0.0%	45.45%	9.09%	18.18%
Lactococcus	14.28%	28.57%	100.0%	0.0%	0.0%	71.42%	0.0%	30.61%
Leuconostoc	50.0%	25.0%	75.0%	0.0%	0.0%	75.0%	0.0%	32.14%
Enterococcus	33.0%	0.0%	66.6%	0.0%	0.0%	66.6%	0.0%	23.81%
Others	18.18%	0.0%	81.81%	0.0%	9.09%	36.36%	0.0%	19.48%

Conclusion

Present work highlights the antibiotic resistance of lactic acid bacteria isolated from raw milk of Badri cattle. LABs of raw Badri milk had resistance ranging from 2-68% against the antibiotics used. *Lactobacillus* showed highest resistance for cefixime and penicillin. Use of antibiotic susceptible lactic acid bacteria is very important in food industry as it break horizontal spread of antibiotic resistance gene while a presence of resistance LABs is beneficial for treatment of antibiotic dependent diarrhea. Therefore, the presence of resistance elements should not be deemed a bane without exploiting its benefits.

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