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Research Article

In-Vitro Antibacterial Activity of Probiotic Against Human Multidrug Resistant Pathogens

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Abstract

Objective: Probiotics have tremendous role in human health for disease prevention and treatment in both communicable and non-communicable diseases. The aim of study is to isolate, characterize and evaluate the antibacterial effect of probiotic against Multi-drug Resistant (MDR) human bacterial pathogens.

Methodology: Total of 20 dairy (10 milk and 10 curds) samples were collected from dairy shops of Bhaktapur, Nepal. *Lactobacilli* spp. were isolated in de Man, Rogosa and Sharpe (MRS) agar and broth. Physiological, biochemical and tolerance tests were performed for the identification of *Lactobacilli*. Cell Free Supernatant (CFS) was used to determine antibacterial activity against 10 MDR bacterial species in Mueller-Hinton Agar (MHA) in in-vitro condition.

Result: Out of 20 dairy product samples, altogether 14 (4 milk and 10 curd) samples were positive for *Lactobacilli* spp. The antibacterial effect of CFS against MDR isolates varied. *Escherichia coli, Salmonella paratyphi, Salmonella typhi, Pseudomonas* spp., *Staphylococcus aureus, Proteus* spp., *Acinetobacter,* showed antibacterial effect and,

Klebsiella pneumoniae and *Shigella* spp. did not show susceptibility. Antibacterial effect, however, was observed to be isolate specific. Among the susceptible isolates, the zone of inhibition ranged 9 mm to 16mm in diameter.

Conclusion: Out of 14 lactobacillus isolates, one isolate did not show antibacterial effect against any of the MDR isolates tested. Therefore, local dairy products of Bhaktapur contain *Lactobacilli* that have antibacterial effect on common human MDR bacterial pathogens.

Keywords: Probiotics; Lactobacilli; Multi-drug resistant; Dairy; Antibacterial effect

1. Introduction

In early age of 20th century Eli Metchnikoff discussed about beneficial use of bacteria [1]. Later, Lilly and Stillwell coined the word 'probiotic' which is beneficial to human [2]. According to World Health Organization (WHO), probiotic is defined as a live microorganism when administrated in adequate amount into host provides health benefits [3]. Probiotic bacteria including, specific strains of Lactic Acid Bacteria, *Bifidobacterium* , *Propionibacterium*, fungi *Saccharomyces boulardii*, certain strains of the genus Bacillus and *Escherichia coli* Nissle 1917 other bacterial species [4]. Probiotics should have important characteristics like non-virulent; bile and acid tolerant, multiplication in gastrointestinal tract producing beneficial molecules and nutrients, ability to adhere to cell surface, susceptible to antimicrobials and exhibit antimicrobial activity against human pathogens [5]. Probiotics are found in both dairy and non-dairy products. Dairy products rich in probiotics include fermented milk, ice cream, cheese, yoghurt, butter milk etc. and non-dairy products having probiotics are soy based products, nutrition bars, cereals, kimchi, microencapsulation products etc [4]. Nowadays, probiotic are included in food and beverage industry. *Lactobacilli* are most commonly used probiotics and are considered as desirable intestinal microflora [6].

In-vitro and in-vivo studies from human and animal models studies have shown the beneficial effect against variety of diseases including communicable, non-communicable and others. Probiotics have been reported to promote health by suppression of viral induced diarrhea [7], antibiotic induced diarrhea [8], reduce irritable bowel symptoms [9], anti-cancerous activities [10], alleviate lactose intolerance [4] and modulate immune system[11]. Probiotics have also shown anti-microbial activity against pathogenic [12].Several studies concluded that different strains of probiotics have shown antibacterial property against pathogenic and MDR strains of *S. aureus, E. faecalis, K. pneumonia, P. aeruginosa, Escherichia coli, S. typhii* and other *Salmonella* spp. [12, 13] and also effective against fungi like *C. albicans* [13]. *L. acidophilus* isolated from human stool showed antagonistic activity against *Helicobacter* infection in vitro and in vivo [14]. Lactobacilli of human milk from healthy volunteer mothers showed antibacterial activity against *B. cereus, S. entericaserovar* Typhi, *P. aeruginosa* and *S. flexneri* [15] and lactobacilli isolated from healthy infant stool was also shown to act against Enterotoxigenic *Escherichia coli* (ETEC) [16]. It is also established that *Lactobacillus acidophilus* has shown anti-parasitic effect against *Trichomonas vaginalis* [17]. In addition, vaginal *Lactobacilli* act against Gram Negative anaerobes like *Prevotella bivia, Gardnerella vaginalis* and

Mobiluncus spp. [18] and also effective against Herpes Simplex Virus (HSV)-2 in in-vitro modal [19]. It has been shown that the antagonistic effect of a probiotic strain is pathogen strain specific by producing antimicrobial active metabolites. Presently, antimicrobial resistance is emerging as global threat to living organism. Irrational and haphazard use of antibiotics in treatment of diseases and promotion of growth in livestock leads to development of antibiotic resistance [20]. Currently, 700,000 per year death is caused by antimicrobial resistant pathogens and it is estimated that with lack of unpreventable action, the toll will reach 10 million death per year by 2050 [21]. This scenario calls for an alternative medicine that curbs the possible threat of drug resistance, and hence probiotics and their metabolites can arguably be promoted to have potential value in the treatment and prevention of infectious diseases. Thus, the present study is deigned to isolate, characterize and investigate in-vitro antibacterial activity of *Lactobacilli* isolated from dairy products against MDR bacteria isolated from human samples.

2. Materials and Methods

2.1 Specimen collection, isolation and identification

A total of 20 milk and curd samples (10 milk and 10 curd) were collected from farm and branded products available in retail shops from June to October 2019. 10ml of milk and 10 gram of curd specimens were collected in sterile, leak proof screw capped container with proper labeling and transported to the Department of Medical Laboratory Technology, JF Institute of Health Sciences, within an hour in ice box. Modified De Man, Rogosa and Sharpe (MRS) agar media (Hi-Media, India) was used to isolate lactobacilli from samples. A loop full sample was inoculated on media and incubated anaerobically for 24-48 hours at 37°C. The bacterial colonies isolated were identified on the basis of standard microbiological methods Bergey's manual of systematic bacteriology [22], gram staining, physiological (Bile salt and NaCl) test and biochemical (Catalase, Oxidase, Citrate utilization, Urease, Methyl red, Voges Proskauer, Indole and Nitrate reduction) test were performed. Isolated colonies were picked up and transferred to MRS broth for enrichment of *Lactobacillus* at 37°C.

2.2 Antibiotic resistance pattern

Commercially available antibiotic discs of different classes were used to determine antibiotic resistance of *Lactobacilli* isolates. Antibiotic discs including Ampicillin (10µg), Erythromycin (15µg), Penicillin (10 Unit), Gentamycin (30µg) and Tetracyclin (30µg) manufactured by Hi-Media, India were used. The susceptibility tests for each isolates were performed by Kirby-Bauer disc diffusion method. The discs were placed on Muller-Hinton agar (Hi-Media, India) surface. The plates were incubated anaerobically for 24 hours at 37°C. The resistances were determined according to the zone formation.

2.3 Multidrug resistant bacterial pathogens

MDR bacterial isolates included were *Escherichea coli*, *Staphylococcus aureus*, *Salmonella paratyphi*, *Salmonella* typhi, *Proteus* species, *Acinitobacter* species, *Pseudomonas* species, *Shigella* species and *Klebsiella pneumonia*, which were collected from Department of Medical Laboratory Technology, JF Institute of Health Sciences, Hattiban, Laltipur. AST of MDR isolates were reconfirmed by Kirby-Bauer disk diffusion method as described by CLSI guideline [23].

2.4 Antagonistic activity against MDR isolates

Isolated colony of *Lactobacilli* was suspended in 10 ml MRS broth. After 24 hours incubation at 37°C in 5% CO2 condition, it was centrifuged for 15min at 8,000 ×g. Supernatant was filtered with 0.22µm membrane filter in a sterile test tube. Cell free supernatants (CFS) of *Lactobacilli* were obtained and their inhibitory activity against isolated MDR bacteria was assayed by agar well diffusion method. 6mm diameter wells were prepared by using cork borer. 100µl of filtered CFS was loaded into each well with the help of micropipette and incubated at 37°C for overnight.

Characteristics	M-1	M-6	M-7	M-10	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
Colony morphology	*	**	**	**	***	***	**	**	***	*	**	*	*	**
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Shape	SR	SR	SR	LR	SR	SR	SR	SR	SR	R	R	R	R	R
Catalase	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Oxidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Motility	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
Citrate utilization	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Methyl red	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Voges proskauer	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Nitrate reduction	–	–	–	–	–	–	–	–	–	–	–	–	–	–

(**Note:** M- Milk sample, C-Curd sample, *-Mucoid greenish colonies;**-Whitish mucoid colonies;***- Creamy white colonies, +-Positive, – - Negative, R-Rods, SR-Short rods, LR-Long rods, NM- Non-motile).

Table 1: Morphological and biochemical characteristics of isolated *Lactobacillus* spp.

3. Results and Discussion

In 21st century antimicrobial resistance is emerging as a major health problem. Antibiotic resistance is spreading in a rampant manner globally, with the evolution of MDR strains, Extensively drug resistant (XDR) strains and virtually untreatable superbugs. The modern medicine era is desperately looking for an alternative to antibiotic therapy and probiotics can possibly be brought forward as the option. In the same context we made an attempt to find out the antibacterial potency of probiotics isolated from the dairy products available in local market of Bhaktpur,

Nepal. Out of 20 dairy samples (10 milk and 10 curd samples) taken, 14 showed growth of Lactobacilli. Out of 10 milk samples only 3 showed growth of *Lactobacilli* and all curd samples showed growth of *lactobacilli* in in-vitro standard conditions. The bacilli were identified on the basis of morphological and biochemical characteristics. The colonies were convex, greenish, mucoid, smooth, shiny, irregular, circular, facultative anaerobic. Gram stained microscopy yielded non-spore forming Gram Positive Bacilli while study of biochemical features showed them to be non-motile, catalase negative, oxidase negative, indole negative, MR-VP positive, citrate negative, glucose and lactose fermenter, as shown in (Table 1).

Lactobacilli	**NaCl(%)**			**Bile salt (%)**		
	2.0	4.0	6.5	0.1	0.15	0.3
M-2	+	+	+	+	+	+
M-6	++	+	-	+	+	+
M-7	++	+	+	+	+	+
M-10	++	+	-	+	+	-
C-1	+	++	+	+	+	+
C-2	++	+	+	+	+	+
C-3	+	++	+	+	+	+
C-4	+	+	-	+	+	+
C-5	++	++	+	+	+	+
C-6	++	+	+	+	+	+
C-7	++	+	+	+	+	+
C-8	++	+	+	+	+	+
C-9	+	+	-	+	+	+
C-10	+	+	+	+	+	-

(Note: ++-highly turbidity, +- Low turbidity, – - No turbidity, + - Growth observed, – - No growth)

Table 2: Physiological stress tolerance tests.

The characteristics mentioned above, depict them to be *Lactobacilus spp.* In order to analyze the physiological tolerance, *Lactobacilli* were cultured in varying concentrations of bile salt and Sodium chloride (NaCl) in MRS broth and incubated overnight anaerobically. All the isolates of *Lactobacilli* were tolerant to 0.1% and 0.15% bile salt, while all but two isolates were tolerant to 0.3% bile salt. Bile salt tolerance is required for selection of probiotic strain, inside human body bile salt concentration varying 0.03% to 0.3% [24]. *Lactobacillus acidophilus* M92 shows satisfactory degree of resistance in bile salt and bile salt hydrolase, which is an important enzyme for bile

resistance, also has high activity in this strain [25]. All of the isolates were tolerant to 2.0% and 4.0% NaCl while all but four isolates were tolerant to 6.5% NaCl, which is shown in (Table 2). Our finding gave an indication of the osmotolerance level of isolated strains of probiotics. High concentration of NaCl affect the metabolism, cellular physiology, and enzyme and water activity, which is required for the industrial use [26].

Antibiotics	**M-2**	**M-6**	**M-7**	**M-10**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**	**C-7**	**C-8**	**C-9**	**C-10**
Ampicillin (10 mcg)	S	S	I	I	S	S	S	S	S	R	R	R	R	R
Erythromycin (15mcg)	S	S	S	S	S	S	S	S	I	R	R	R	R	R
Gentamycin (10 mcg)	S	S	I	S	I	S	S	S	S	R	R	R	R	R
Penicillin G (10 units)	R	R	R	R	R	R	R	I	S	R	R	R	R	R
Tetracycline (30 mcg)	S	R	I	R	I	R	S	S	S	R	R	R	R	R

(Note: S: Sensitive, R: Resistance, I: Intermediate)

Table 3: AST pattern of isolated *Lactobacilli* spp from curd and milk samples.

The AST showed that out of 14 isolates, 5 isolates were resistant to Ampicillin; 5 were resistant to Erythromycin; 5 were resistant to Gentamycin; 12 were resistant to Penicillin G; and 8 were resistant to Tetracycline, shown in (Table 3). Maximum isolates were resistant to Penicillin G and out of 14 isolates 5 isolates were resistant to all antimicrobials used in study. A study from Turkey showed that, *Lactobacilli* isolates from dairy product were resistant to Vancomycin, Erythromycin, Tetracycline, Gentamicin, and Ciprofloxacin [27]. Our study showed that the dairy products of Bhaktapur have considerable number of MDR probiotic strains. MDR probiotics are undesirable in dairy products as they are the potential sources of drug resistance dissemination to the pathogenic bacteria. Hence, our study indicates the need of urgent corrective measures that create an environment towards the manufacture of quality dairy products in the market. Currently, the beneficial effect of probiotics has received greater attention as a natural, alternative and cost effective remedy to protect from communicable and non-communicable diseases. Different strains of *Lactobacilli* spp. protect against microbial infection to intestine and urogenital tract [28]. In our study, the antimicrobial activity of probiotics was tested against MDR pathogenic bacteria from different clinical samples. 0.22 µm filtered CFS inhibited the growth of few tested organism included MDR strains of various bacteria. One out of 14 probiotic isolates did not

showed any inhibitory effect on tested MDR isolates. Sample specific *Lactobacilli* showed antibacterial effect against MDR pathogenic bacteria. *Lactobacilli* isolates did not show any effect on *Klebsiella pneumonia* and *Shigella* spp. The bacteria specific and isolate specific inhibitory effect of probiotics is shown in (Table 4). The inhibitory role of *Lactobacillus* against pathogenic bacteria can be attributed to the production of antibacterial compounds like organic acids, hydrogen peroxide and bacteriocins [29] which, however, is directly dependent on the concentration of antimicrobial compound in CFS [30].

CFS	**Inhibition zone diameter(mm)**												
	S. aureus			***Pseudomonas* spp.**		***Acinetobacter* spp.**	***E. coli***			***S.Paratyphi***		***S. Typhi***	***Proteus* spp.**
	I-1	I-2	I-3	I-1	I-2	I-1	I-1	I-2	I-3	I-1	I-2	I-1	I-1
M-2	–	–	–	–	–	–	–	–	–	–	–	–	–
M-6	11	–	–	11	–	15	–	13	–	–	11	–	–
M-7	13	–	13	–	–	11	–	12	–	–	10	–	–
M-10	13	–	–	–	–	15	–	11	–	–	–	11	–
C-1	13	11	12	9	–	14	–	–	11	12	10	–	10
C-2	16	10	11	9	–	13	–	14	11	12	10	–	10
C-3	15	11	–	11	–	14	–	–	11	12	9	11	10
C-4	14	10	13	11	–	15	10	–	11	11	10	–	–
C-5	13	–	13	10	–	11	11	–	11	13	10	11	–
C-6	14	10	13	–	–	–	–	–	–	11	–	12	–
C-7	14	–	15	–	10	11	9	–	–	11	–	12	–
C-8	13	–	14	–	–	12	9	–	–	–	–	–	–
C-9	13	–	13	–	–	12	–	–	–	–	–	–	–
C-10	13	–	12	–	–	12	–	–	–	11	–	–	–

(Note: I-Isolates, – - No inhibition recorded)

Table 4: Antibacterial effect of CFS of *Lactobacilli* spp. against human MDR isolates.

4. Conclusion

The result of our study indicates that dairy products contain beneficial probiotics which exhibited antibacterial activity against the selected clinical MDR isolates. More intensive in-vitro and in-vivo model studies need to be carried out revealing the phenotypic and genotypic characteristics of probiotics, in order to elucidate their role in human health.

Conflict of Interest

No conflict of interest.

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