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Screening and Analysis of Probiotic Properties of *Lactobacillus* Spp. From Famous Commercial Brand of Yogurt Found in Kathmandu Valley

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Abstract

Probiotics are live microbial strains that have a positive impact on human health by maintaining the gut microflora. Probiotics are present in fermented food products. Out of many probiotics, most belongs to *Lactobacillus* genera which are also used in commercial food products. Yoghurt is a potential source of probiotic *lactobacilli*. The purpose of this study is to identify and analyse the probiotic properties of isolated *Lactobacillus* spp. from different five commercial brand of yoghurt that are famous in Kathmandu valley.

All the isolate was identified on the basis of their morphological and biochemical characteristics. These identified isolates were further examined to analyze the probiotic properties which includes tolerance to inhibitory substances like phenol(0.4%), Nacl (1-9%) and bile acid (0.1-1%); the ability to grow in acidic (pH 2.5) and alkaline (pH8.5) condition and their antimicrobial activities along with susceptibility to selected nine antibiotics which was determined by disk diffusion method. The result of this present study indicates that the isolates from commercial yoghurts sample fulfill the most common criteria of probiotic bacteria with slight variation among the sample in different properties.

Keywords: Probiotics; *Lactobacillus* spp; Antimicrobial; Yoghurts; Kathmandu Valley

Introduction

Probiotics are living microorganism that stimulates the health of the host by maintaining the intestinal microflora. Probiotic bacteria mainly consists a genera of Lactic Acid-Producing Bacteria (LAB), including *Lactobacillus* and *Bifidobacterium, streptococcus* and some *enterrococcus* spp. [1]. Traditionally fermented food product including dairy product such as yoghurt is the major source of probiotic [2].

Yoghurt is a cultured dairy product that has been fermented with LAB. Fermentation of lactose by these bacteria produces lactic acid, whose action on milk proteins results the texture and characteristics flavour of yogurt. The properties like lactic acid production, antimicrobial properties, antibiotic tolerance, bile tolerance, pH tolerance and gastric juice resistance are some criteria for the in vitro selection of lactobacilli to be used as probiotic [3]. It is very important that probiotic strain should survives in the area where it is supposed to be active.

The strain should able to proliferate and colonize at the specific location to boost maximum activity. Probiotics for human consumption should have 'generally regarded as safe'. It should be tolerated by immune system and should not be pathogenic, allergic, or mutagenic [4]. Probiotic assist in the prevention and treatment of Gastrointestinal infections and diseases [5]. The benefits of *Lactobacillus* spp ranges from reducing allergies, preventing antibiotic-associated diarrhea [6], removing cholesterol [7], prevent urinary tract infection, stimulation and development of immune system of the host [8].

Yoghurt has been a part of a human daily diet, with the increasing awareness about the health benefits of yoghurts consumption, the interest and market of yoghurt has increased globally. In Nepal, yoghurt is locally called 'dahi' and consumed as a part of a daily diet. Here, yoghurt has been produced in a household level but with the increasing number of population and changing lifestyle, the demand of commercial yoghurt is rising. Respectable evidence continues to emerge that shows that the members of the genus *Lactobacillus* are widely used in the manufacturing of yoghurt [9].

Despite growing use and consumption of *Lactobacillus* as probiotics, which can enhance the health status of large segment of communities, there is a lack of scientific research on local commercial yoghurts in Kathmandu. Therefore, this study is undertaken to find potential probiotics properties of *Lactobacillus* spp. from a famous commercial brand of yoghurt in Kathmandu Valley.

Materials and Methods

Collection of samples

Total five yoghurt samples from different commercial dairy in Kathmandu were collected from market as freshly as prepared. The samples were stored aseptically in low temperature (4°C) refrigerator to protect from contamination and deterioration immediately after collection.

Media

The bacteria *Lactobacillus* spp. was isolated from each sample by using DeMann, Rogosa and Sharpe (MRS) media both MRS broth and MRS agar media [10]. In order to improve the specificity of the medium for isolation of *Lactobacillus* 0.05% cysteine was added [11]. The final pH of the media was adjusted to 6.5.

Isolation and identification of bacteria

All the required instrument and media were autoclaved for 15 min at 120°C. One gram of each sample was suspended in 100ml respective MRS broth and after dissolving they were shaken homogeneously. All this homogenized sample were subjected to fivefold dilution, these dilutions were than incubated for 24 hours for 37°C under anaerobic condition in the presence of 10% CO_2 to remove unwanted bacteria. From each broth, a loopful of culture was streaked on to the MRS agar media which were incubated for 24 hours at 37°C [12]. Finally, the single colony of bacteria was isolated and identified by observing their morphological characteristics and some biochemical tests like Gram staining, catalase, endospore test, motility test and milk coagulation activities. The cultures were maintained in the MRS broth.

Screening of isolated Lactobacillus Spp. for probiotic properties

Determination of optimal growth and pH: For the determination of optimal pH and growth, the overnight culture of the *Lactobacillus* in the MRS broth media with a varying pH ranging from 2.5 to 8.5, using Hcl or NaOH were inoculated with 1% (v/v) and incubated in anaerobic condition for 24h at 37°C in the presence of 10% CO_2. Bacterial growth was monitored by the determination of optical density at 560nm using a spectrophotometer against the uninoculated broth [13].

Assay for NaCl tolerance: In order to determine the NaCl tolerance, isolates from each sample were incubated with 1% (v/v) overnight culture of *Lactobacillus* in the test tubes containing MRS broth adjusted with different concentrations (1-10%) of NaCl. The inoculated medium was incubated in anaerobic condition for 24h at 37°C. The bacterial growth was determined using spectrophotometer reading the optimal density at 560nm [14].

Determination of bile salt tolerance: Bile salt tolerance of the isolated bacteria was examined by inoculating the freshly cultured isolates at 1% (v/v) into MRS broth containing Bile salt at different concentrations (0, 0.1, o.3, o.5 and 1% (w/v). The medium was then incubated at 37°C for 24 hours in an anaerobic condition. The growth of the isolates was monitored at 0, 3, 5 and 24 h by measuring the absorbance of the culture broth at 620nm using spectrophototometer [13].

Antimicrobial activity: The antimicrobial activity of the isolated *Lactobacillus* was identified by well diffusion method [15], with slight modification. The overnight culture of *Lactobacillus* was centrifuged (10,000 rpm for 20min. at 4°C) and the supernatant was adjusted to pH 7.0 with NaOH to exclude the antimicrobial effect of H^+. Supernatant of each sample of *Lactobacillus* were monitored for antibacterial activity against indicator bacteria (*Escherichia Coli, Staphylococcus aureus, Salmonella typhi, Bacillus subtilis*) inoculated on Nutrient agar. Wells of 5mm in diameter were punctured into the agar plate and aliquots of 50µl from each solution samples were placed into the wells. The plates were incubated for bacterial growth and examined for the presence of clear zones of inhibition around the wells

Antibiotics sensitivity: For the observation of antibiotics sensitivity test Disk diffusion method was performed [16]. Different nine antibiotics were tested over the isolates. The appropriate antimicrobial-impregnated disks were placed on the surface of the MH agar plate inoculated with each isolate. The zones of inhibition around the disks were observed and the diameters were measured.

Results and Discussion

Isolation and identification

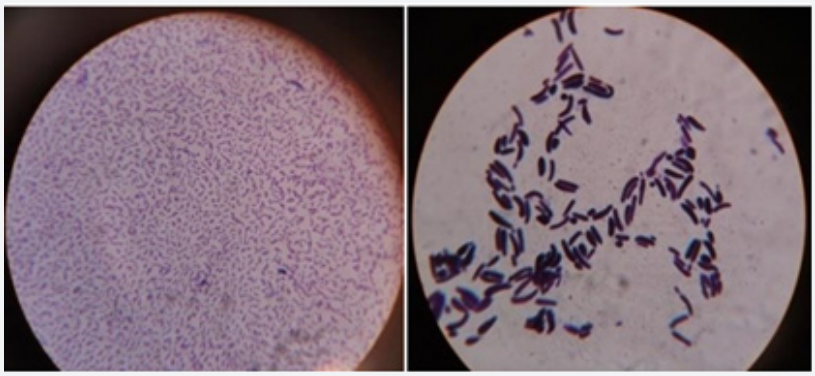

Figure 1: Gram staining of isolated Lactobacillus spp.

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The bacteria were isolated from different five samples of yoghurt and were identified as *Lactobacillus* spp. by observing their colony morphology, physiological as well as some biochemical characteristic. Microscopic examination showed the isolates were Gram-positive rod shaped (Figure. 1), non-motile, catalase negative and absence of Endospore. The isolates were able to coagulate milk and have the abilities to tolerate inhibitory substances such as 0.4% bacteriostatic phenol and showed growth in MRS broth containing 1-9% NaCl. The results are shown in (Table 1).

Table1. Morphological, physiological and biochemical characteristics of isolated *Lactobacillus*

Biochemical and physiological characteristic	Sample-1	Sample-2	Sample-3	Sample-4	Sample-5
Colonies morphology	0.5mm, white, rough, irregular, and round, flat	0.6mm, brownish rough, circular, raised	0.5mm, creamy white, smooth, round, raised	0.4mm, white, circular, dull, flat	0.4mm, creamy white, smooth, irregular, flat
Gram stain	+	+	+	+	+
Motility test	Non motile	Non motile	Non motile	Non motile	Non motile
Catalase test	-	-	-	-	-
Endospore test	-	-	-	-	-
0.4%phenol tolerance	+	+	+	+	+
Milk coagulation activity	+	+	+	+	+
NaCl tolerance	+	+	+	+	+

Optimal PH and growth

Maximum growth of isolated *Lactobacillus* of different sample was observed at different PH Maximum growth of isolated *Lactobacillus* from sample 1(ND's) yoghurt was observed at pH 5.5 (OD=1.067) similarly, maximum growth of sample 2(KDC) was also observed at PH 5.5 (OD=1.042), sample 3(DDC plain) at PH 6.5 (OD=1.039), sample 4(DDC probiotic) at PH4.5 (OD=1.025) and sample 5(ju –ju dhau) was observed at PH 6.5 (OD=1.048), after 24 h of incubation in an anaerobic condition. The results are shown in Table 2.

Table 2. Optimal PH and growth.

PH value	Growth rate at OD_{560}				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
2.5	o.214	0.408	0.211	0.276	0.662
3.5	0.282	0.948	0.978	0.496	0.985
4.5	0.648	1.032	1.003	0.823	1.025
5.5	1.067	1.042	1.039	1.002	0.991
6.5	1.016	1.012	1.035	1.03	0.899
7.5	1.012	1.022	1.004	0.956	0.834
8.5	0.264	0.715	0.944	0.881	0.586

Tolerance to NaCl

Table 3. Growth rate on Various NaCl concentration of isolated *Lactobacillus* spp

NaCl conce[n]	Growth rate at OD_{560}				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1%	1.267	1.258	1.108	1.325	1.329
2%	1.168	1.22	1.105	1.312	1-112
3%	1.096	1.102	1.098	1.168	1.095
4%	1.064	0.973	1.094	1.299	0.857
5%	0.958	0.762	0.836	1.045	0.537
6%	0.916	0.711	0.354	0.618	0.227
8.5	0.264	0.715	0.944	0.881	0.586

The isolates from sample were able tolerate 1-9% NaCl. But these organisms grow best at the broth containing 1% NaCl. The growth rate decreases with increase in NaCl concentration. The result are shown in Table 3.

Bile salt tolerance

Table 4. Growth rate at OD_{620} of isolated *Lactobacillus* from different sample at various 326 concentrations under different incubation time.

Time in hour	control	0.10%	0.30%	0.50%	1%
0	0.218	0.213	0.211	0.217	0.201
3	0.306	0.259	0.236	0.203	0.128
5	0.578	0.368	0.321	0.16	0.062
24	1.532	0.918	0.504	0.092	0.011

Isolated *Lactobacillus* spp. were able to survive and multiply in 0.1, 0.3, 0.5 and 1% bile acid. In Table 4 the optical density values against incubation time are given. The growth of *Lactobacillus* in MRS broth without bile was used as control. All the isolates showed different growth rate. With increase in the concentration of bile salt there the growth is decreased. The growth rate is also highly affected the increased in incubation period.

Antibiotics activities

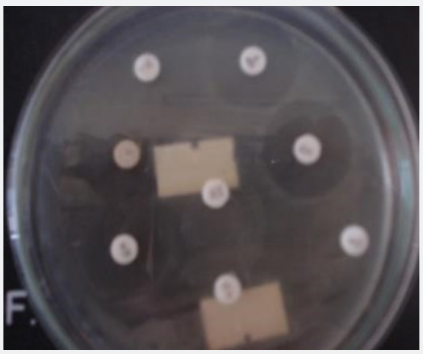

Figure 2: Antibiotics Activity on isolated *Lactobacillus* spp by disk diffusion method.

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The isolates showed susceptibility and resistance pattern to all the nine antibiotics tested. The results are shown in Table 5. The isolates were more commonly sensitive to ampicillin, penicillin, erythromycin, nalidicxic acid, chloramphenicol, tetracycline, gentamycin and amoxicillin which presented diameter of inhibition zones between 10 and 35mm. The diameter of inhibition zones varied for different samples (Figure 2). The Antibiotics ciprofloxacin only showed resistance to all the five isolates.

Table 5. Zone of inhibition of selected antibiotics on isolated *Lactobacillus* spp from different 336 yoghurt sample using the disc diffusion method.

Sample 1					
Time in hour	**control**	**0.10%**	**0.30%**	**0.50%**	**1%**
0	0.215	0.252	0.172	0.207	0.234
3	0.426	0.312	0.207	0.21	0.132
5	0.868	0.437	0.302	0.152	0.038
24	1.429	0.759	0.141	0.016	0.019
Sample 2					
Time in hour	**Control**	**0.10%**	**0.30%**	**0.50%**	**1%**
0	0.144	0.103	0.113	0.11	0.138
3	0.283	0.142	0.122	0.158	0.082
5	0.538	0.462	0.19	0.035	0.036
24	1.579	1.133	0.756	0.015	0.03
Sample 3					
Time in hour	**control**	**0.10%**	**0.30%**	**0.50%**	**1%**
0	0.133	0.108	0.132	0.102	0.092
3	0.342	0.149	0.142	0.122	0.072
5	0.771	0.203	0.18	0.058	0.046
24	1.817	0.628	0.486	0.031	0.027
Sample 4					
Time in hour	**Control**	**0.10%**	**0.30%**	**0.50%**	**1%**
0	0.21	0.231	o.212	0.225	0.192
3	0.256	0.233	0.251	0.21	0.126
5	0.782	0.322	0.432	0.068	0.082
24	1.442	1.202	0.746	0.034	0.021
Sample 5					

Isolates from	**Diameter of inhibition zone in mm**								
	AP10	**P10**	**T30**	**CHL30**	**AM10**	**CIP5**	**E15**	**NA10**	**G10**
Sample 1	26s	17S	28s	26s	21s	R	29s	22s	24s
Sample 2	30s	20s	31s	28s	22s	R	31s	27s	25s
Sample 3	25s	10s	35s	31s	18s	R	30s	24s	28s
Sample 4	28s	15s	31s	29s	25s	R	28s	30S	18s
Sample 5	27s	18s	32s	26s	29s	R	25s	17S	21s

sample using the disc diffusion method.

Antibiotics (Disk potency): $Ap1_0$: Ampicillin (10µg); P_{10}: Penicillin G (10 $_{µg}$); Am_{10}: Amoxicilline (10µg); T_{30}: Tetracycline (30µg); CHL_{30}: Chloramphenicol (30µg); E_{15}: Erythromycin (15µg); CIP_5: Ciprofloxacine (5µg); NA10: Nalidixic acid (10µg); G_{10}: Gentamycin (10µg); s: Sensitive; R: Resistance.

Antimicrobial activity

The isolated spp. was examined to their antimicrobial activity. For this, spp. was detected against the indicator microorganisms *Salmonella thyphi, Escherichia coli, Bacillus subtillis* and *staphylococcus aureus*. These organisms are occasionally found as food borne microorganisms that might cause gastroenteritis. The results revealed that the antibacterial activity of the five isolated *Lactobacilli* could inhibit the pathogenic bacteria tested, however at different inhibition levels as shown in Table 6 (Figure 3). Of the five isolates tested, only isolates from sample 2 can inhibited the growth of all the pathogens used as indicators. But other samples 1, 3 4, and 5 only inhibit the indicator organism *Bacillus* and *Escherichia coli*.

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Table 6. Antimicrobial activities of isolated *lactobacillus* on indicator microorganism.

Isolates from	Indicator microorganism			
	Diameter of inhibition zone in mm			
No	Escherichia coli	Bacillus subtilis	Salmonella typhi	Staphylococcus aureus
1. sample 1	16mm	11mm	0	0
2. sample 2	20mm	14mm	14mm	9mm
3. sample 3	18mm	14mm	0	0

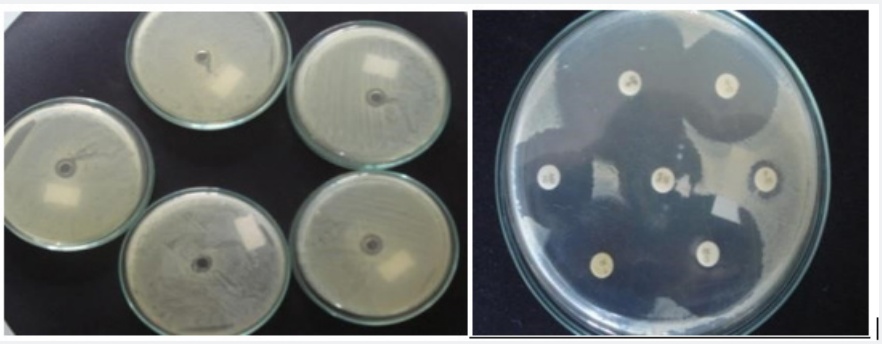

Figure 3: Inhibition of different indicator organism by isolated *Lactobacillus* spp.

Discussion

The isolated rod shaped, gram positive, catalase negative, non -motile with other mention biochemical properties in Table 1, bacteria were isolated from different yoghurt sample from Kathmandu valley. These isolates were assigned as *Lactobacillus* [17]. The pH factor directly affects the bacterial growth. Probiotic bacteria are mostly delivered in a food system so these must have to tolerate acid in order to survive in the human gastrointestinal tract.

In this study we observed the growth of the isolated *Lactobacillus* spp. in various pH value ranges from 2.5 to 8.5 to determine whether the isolated spp. can grow in acidic and alkaline conditions and also to detect the optimal pH value for good growth. It was found that the bacteria were able to survive from extreme acidic (2.5) to basic pH (8.5) and for optimal growth the variation with the sample is viewed. Probiotic organism have to stand in a high salt concentration in a human gut [18]. In our study the *lactobacillus* isolated from all sample were able to survive in1-9% of NaCl and the good growth is seen at1% NaCl.

This study also indicate that the growth rate decreases with the increase in NaCl concentration. The probiotic bacteria must able to resist the bile salt in order to survive in the human intestine and 0.3% bile is the maximum concentration present in the healthy men [19]. So, for the human consumption the probiotics should be bearable to 0.3% bile concentration [20]. Here, 0.1-1% of bile salt concentration was used and our isolated *Lactobacillus* spp. from all five yoghurts are able to tolerate up to o.3% of bile concentration.

In control conditions there were rapid increase in growth of all three cultures with an increase in incubation period up to 24h and there was rapid decrease observed in all cultures with increase concentration especially above 0.3% (0.5% and 1%) of bile salt.

Antibiotic resistance of microorganisms used as probiotic is of growing concern. Bacteria used as probiotics may serve as host of antibiotic resistance genes, which can be transferred to pathogenic bacteria [21]. The antibiotic resistance of pathogenic bacteria is an increasing medical problem. Therefore, the antibiotic susceptibility test should be integrated for the safety assessment of the desired property of the probiotic *Lactobacilli*.

Our isolates were all sensitive to penicillin, ampicillin, amoxicillin, tetracycline, erythromycin nalidixic acid and chloramphenicol, but only resistance to ciprofloxacin. Resistance to ampicillin ciprofloxacin and vancomycin are commonly found in the genus *Lactobacillus* [22]. Since the isolates tested in this work were not resistant to any of antibiotics, except ciprofloxacin they could be safe for use by animals and eventually by humans, and their lack of resistance also indicates that they may not contribute to the transfer of resistance to other microorganisms.

Resistance of all *Lactobacillus* spp to ciprofloxacine could be due to their natural and intrinsic resistance, probably due to the cell wall structure and membrane impermeability, complemented in some cases by potential efflux mechanisms [23]. Regarding antibacterial activities the good probiotics should present their antimicrobial actions particularly to the pathogens in the gastrointestinal tract. The inhibitory activity of *Lactobacillus* spp. varies within the strains [24]. Lactobacilli are highly competitive due to their production of several antimicrobial compounds such as organic acids, hydrogen peroxide, reuterin and bacteriocins [25].

In this study, *Staphylococcus aureus, Salmonella typhi, Bacillus subtilis* and *Escherichia coli* were used as the test bacteria because they are occasionally found as food borne microorganisms that might cause gastroenteritis. The results revealed that the

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antibacterial activity of the five isolated *Lactobacilli* could inhibit all test pathogenic bacteria however at different inhibition levels as shown in (Table 5). The isolated sample 1, 3, 4 and 5 showed the most antibacterial potency to *E. coli* and *Bacillus* whereas the isolated sample 2 demonstrated the highest potency to the entire organism used as indicator.

Conclusion

Those food having the health benefit beyond the basic nutrient are captivating the consumers. Extensive research is currently directed toward increasing our understanding of such functional foods and the food industry is trying to fulfill these demands by developing products with measurable health benefits. Food with the probiotics is gaining consumer interest.

The isolated *Lactobacillus* spp. from different yogurt samples which most of the people of Kathmandu use to consume fulfills the required criteria of a probiotics. These isolated strains were able to survive in a favourable environment of human gastrointestinal tract such as high salt, low pH and high bile concentration. Therefore, these *Lactobacillus* isolates show their potential to be used as probiotic. This spp. is hence a good candidate for further investigation under in vitro as well as in vivo conditions to elucidate their potential beneficial health effects and their possible application as novel probiotic species in the food industry.

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