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Probiotic Potential of Lactic Acid Bacteria Isolated from Local Dairy Products of Kohat, Pakistan

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Abstract:
Probiotics are living microbes which upon ingestion in appropriate number have beneficial effects on host's health. Dairy products are the potential source of Lactic acid bacteria (LAB). In the present study, LAB was isolated from different local dairy products from Kohat, Pakistan. Out of 67 isolates from 52 samples of dairy products, 30 isolates were identified as LAB on the basis of their morphological and biochemical profile. Probiotic properties of the isolates were investigated by determination of their antibacterial activities and tolerance to bile salt and pH. Antimicrobial activity of cell-free supernatant of LAB isolates was determined against indicator bacteria *Salmonella typhi, Escherichia coli*, and *Staphylococcus aureus* by using agar well diffusion method. Maximum zone of inhibition of 15.33±0.5 mm against *E. coli* by C1Bt isolate, 22±1 mm against *S. typhi* by B4M isolate and 19.33±0.5 mm against *S. aureus* by C1Bt isolate was observed. Six of the isolates with good antibacterial activity were subjected to bile tolerance and pH tolerance. The selected isolates showed tolerance against different bile concentrations and pH. C1Bt isolate showed highest tolerance to 0.5% bile concentration, while B4M isolate showed highest tolerance against acidic and alkaline pH.

Keywords: Antibacterial activity, Lactic acid bacteria, Probiotics.

INTRODUCTION

Increased emergence of antibiotic-resistant bacteria and the side effects of antibiotics when used for therapeutic purposes convinced to look for substitutes. This role is considered to be performed by probiotics and at present some growers are using them in preference to antibiotics (Gibson *et al.,* 2008). Probiotics are living microbes which upon taken in appropriate number have beneficial effects on host's health (Bassyouni *et al.,* 2015; Vasiee *et al.,* 2014). Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive cocci and rods which yield in the production of lactic acid as an end product of fermentation of carbohydrate and known to have probiotic characteristics. They comprise non-spore forming, non-motile, usually catalase-negative, lack cytochromes, acid-tolerant, aerotolerant anaerobes that grow under microaerophilic to strictly anaerobic conditions. LAB are naturally present in carbohydrates rich environment mainly in dairy products. These are frequently applied in the manufacturing fermented foodstuffs, i.e. yogurt (Coeuret, 2003). LAB, having ability of adherence to the surface of intestine, are predicted to strongly compete and hinder the adherence of virulent bacteria (Lebeer, 2010). Inhibitory substances including acetaldehyde, lactic acid, diacetyl, hydrogen peroxide, and bacteriocin could be produced by them that have ability to hinder the growth of pathogenic microbes (Allameh *et al.,* 2012). Generally Regarded as Safe (GRAS) nature of LAB demonstrated their industrial importance and due to their omnipresent presence in food and influence to the healthy flora of human mucosal surfaces they can be used well for medical and veterinary practices (Abdulla *et al.,* 2015; Vasiee *et al.,* 2014).

The survival ability in the presence of bile salts and pH is a great necessity of bacteria that produce probiotic and it is usually integrated into the criteria which are used for the potential probiotic strains selection (Gilliland *et al.,* 2002). LAB show a high impact on effective protection to human health, there are shreds of evidence that LAB from different origins possess variable probiotic properties. The present study aim to explore probiotic potential of indigenous lactic acid bacterial isolates in Kohat.

MATERIALS AND METHODS

Samples Collection

A total of 52 samples of fresh milk, yogurt and butter of origin (cow, buffalo, and camel) were collected from houses and markets in Kohat in sterile screw cap bottles and tubes.

Isolation of LAB

Isolation of LAB was carried by the serial dilution technique. One ml of sample was mixed in normal saline and further diluted serially. 0.1 ml sample of suitable dilution was inoculated onto medium MRS (de Man Rogosa Sharpe) broth (Oxoid). The broth was incubated at 37°C for 24-48 hrs in presence of 10% CO_2. LAB was sub-cultured on MRS agar and incubated for 24hrs at 37°C.

Morphological and biochemical Identification

Purified cultures of bacteria were identified based on colony morphology, microscopic characters and biochemical characters (Iqbal *et al.,* 2015; Yunus *et al.,* 2016a; Yunus *et al.,* 2016b) following Bergey's Manual of Determinative Bacteriology (Bergey, 1984).

Antibacterial Activity of Isolates

All the LAB isolates were tested for antibacterial activity by using the method of agar well diffusion. They were tested for the production of antimicrobial agents against *Escherichia coli, Staphylococcus aureus,* and *Salmonella typhi* as the indicator microorganisms. MRS broth was used for growing LAB isolates at 37°C for 18h. The

cultures were centrifuged at 4000 rpm at 4°C for 15min to get supernatant. The cell-free supernatants (CFS) were used directly or stored at -20°C until needed for antibacterial activity (Shruthy *et al.,* 2011).

20 mL of sterile molten Nutrient Agar (Oxoid) was poured in sterilized Petri dish and allowed to stand. The freshly prepared inoculum of indicator strain was swabbed all over the surface of the Nutrient Agar using sterile cotton swab. Wells were punched in the medium with the help of a sterile tip and were labeled properly. Then 100 µL of each CFS was added to each well, 100 µL of sterile MRS broth was added to a well as a control. All plates were allowed to incubate for 24 hours at 37°C. The zones of inhibition were measured after incubation.

pH Tolerance Assay

The selected isolates were tested against different pH. MRS broth adjusted with different pH was used as pH test media. 10ml of media was inoculated by an aliquot of 0.1ml of the 18 hours old culture and incubated for 24 hours at 37°C. Growth was recorded by measuring O.D at 600nm (Shruthy *et al.,* 2011).

Bile Salt Tolerance Assay

Selected isolates were tested to the tolerance of bile salts (BS) by growing them in MRS broth containing bile salts. Media was prepared with changed concentrations of bile salts. 0%, 0.1%, 0.3% and 0.5% w/v concentrations were used. 0% of concentration was used as control. 10ml of media was inoculated by an aliquot of 0.1ml of the 18 hours old culture and incubated for 24h at 37°C. The isolates growth was recorded by measuring O.D at 600nm (Kumar and Kumar, 2015).

RESULTS AND DISCUSSION

Out of 67 isolates from 52 samples of dairy products 30 isolates were identified as LAB on the basis of Gram staining morphology, spore staining, catalase reaction, and biochemical tests. Gram-positive, catalase-negative and non-spore forming bacteria were considered as LAB. Among the 30 isolates of LAB, 3 isolates were from camel's milk (Cam), 3 isolates from cow's milk (CM), 2 isolates from buffalo's milk (BM), 7 isolates from cow's yogurt (CY), 13 isolates from buffalo yogurt (Y) and 2 isolates from butter (CBt) (Table 1).

Table 1. Lactic acid bacteria (LAB)isolates from dairy products.

Source	Symbol	No. of Isolates
Camel's milk	Cam	3
Cow's milk	CM	3
Buffalo's milk	BM	2
Cow's yogurt	CY	7
Buffalo yogurt	Y	13
Butter	CBt	2
Total isolates		30

Antimicrobial Activity

Antimicrobial activity is the major criteria for an isolate to be used as probiotic. In the present study, all the isolates were tested for antimicrobial activity against the selected indicator bacteria. Among these six isolates including C3Y, C5Y, Y8, Y12, Y15 and Y22 did not show antimicrobial activity against indicator bacteria, while twelve of the isolates showed activity against all the indicator bacteria with maximum zone of inhibition of 15.33mm±0.577 against *E.coli* by C1Bt, 22mm±1 against *S.typhi* by B4M and 19.33mm±0.577 against *S. aureus* by C1Bt (Table 2).

pH Tolerance Assay

Six of the isolates with potential antimicrobial activity were screened for pH tolerance at pH2, pH3, pH4, pH8, and pH9. B4M showed highest tolerance against acidic and alkaline pH with OD (0.549±.008) at pH2 and OD (1.242±.012) at pH9. While the reduced tolerance was shown by Y2 with OD (0.055±.004) at pH2 and OD (0.436±.004) at

pH9. Cam3 was also sensitive to pH2 and could not survive acidic pH while could grow well in alkaline pH9 with OD (1.286±.006) (Figure 1).

Bile Salt Tolerance Assay

The criteria for LAB to be used as probiotics are their ability to tolerate the bile salt concentration of the small intestine which is reported to be 0.3%. The bile concentration was tolerated by the selected LAB isolates with a different degree. C1Bt showed highest tolerance to 0.5% bile concentration with OD (0.340±0.005) while Y4 showed the lowest tolerance to 0.5% bile concentration with OD (0.117±0.003) (Figure 2).

Table 2. Antibacterial activity of LAB isolates.

Isolates	Zone of inhibition (mm)		
	E.coli	***S.aureus***	***S.typhi***
Y2	13.33±0.577	14.67±0.577	17±1
Y4	0	14.67±0.577	18±1
C1Bt	15.33±0.577	17.67±0.577	22.33±0.577
Cam3	12.33±0.577	16.33±0.577	20±1
B4M	14±1	18.33±0.577	22±1
B5M	15±1	19.33±0.577	21.67±0.577

Note: mean zone of inhibition with ± SD, N=3

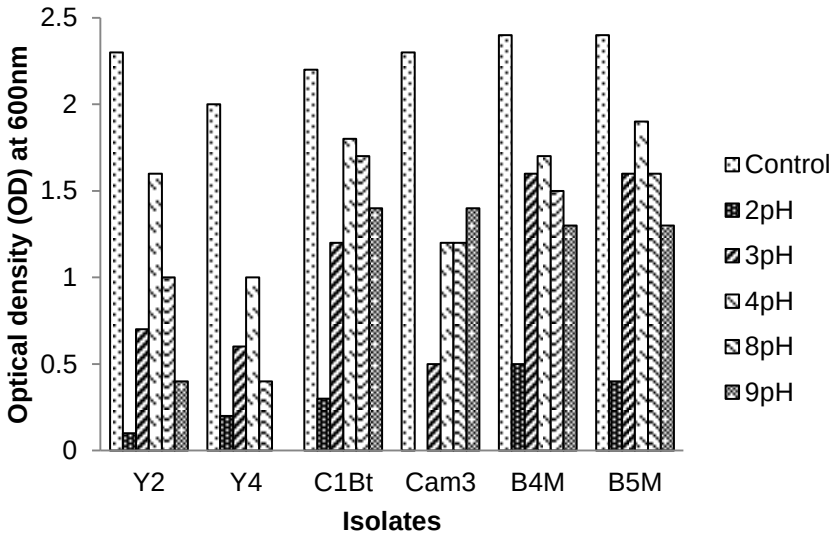

Fig. 1. Survival of isolates in acidic and alkaline pH. N=3

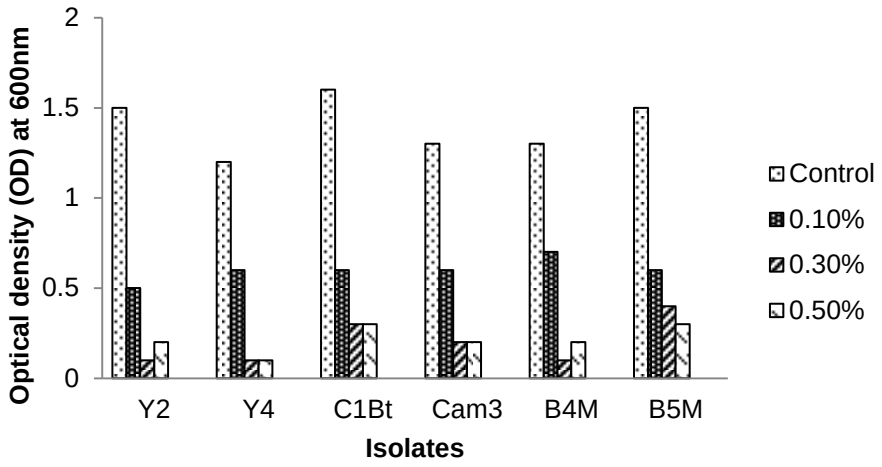

Fig. 2. Absorbance values at 600nm. N=3

DISCUSSION

The present study was performed for isolation and identification of LAB and their potential probiotic activity. Lactic acid bacteria (LAB) were isolated from various dairy products including yogurt, butter, and fresh milk. The LAB cultures were isolated on selective MRS agar and identified on the basis of morphological and biochemical characteristics i.e. (Gram-positive, catalase-negative, oxidase negative, non-spore forming, non-motile, sugar fermentation pattern, citrate negative). Isolation of LAB was also reported by many other studies, Chowdhury *et al*., (2012) isolated four *Lactobacillus spp*. from buffalo yogurt samples. Hoque et al. (2010) isolated *Lactobacillus spp.* from regional yogurts in Bangladesh, which were identified on the basis of their colony morphologies and biochemical tests.

The identified LAB isolates were screened for their antagonistic activity against *Salmonella typhi, Escherichia coli,* and *Staphylococcus aureus*. These bacteria are foodborne microorganisms and may cause gastroenteritis (Iqbal *et al.,* 2015; Iqbal *et al.,* 2016). Six isolates namely Y2, Y4, C1Bt, B4M, B5M, and Cam3 showed antibacterial activity against the indicator strains. Strong inhibitory activity against *E.coli* (15 mm) and *S.aureus* (19.5 mm) was shown by B4M while C1Bt showed strong inhibitory activity against *S. typhi* (22 mm). In a study conducted by Osuntoki et al. (2008) *Lactobacillus spp*. isolated from fermented dairy products showed antibacterial activity against *E. coli* and *S. typhi*murium. Bassyouni et al. (2012) isolated LAB from different dairy products and investigated them for their antibacterial activity against clinically isolated *Salmonella spp, E.coli,* and *Staphylococcus spp*. They found that the isolates have antibacterial activity against *E.coli, Salmonella spp,* and *Staphylococcus spp*.

In the present study, 0.1-0.5% bile salt were supplemented in the growth media and according to our findings the selected isolates of our study were able to survive and grow in 0.1 to 0.5% bile salts. It was reported by Kalui et al. (2009) that out of 19 *L. plantarum* isolates, 18 tolerated 0.3% concentration of bile salts in MRS broth. Chowdhury et al. (2012) described that *Lactobacillus* spp. isolated from yogurt showed resistance to 0.3% concentration of bile salts and they can survive and grow in it.

pH is an important factor which can affect bacterial growth. In our experimental design, we have tested the growth of selected LAB isolates

in various pH values i.e. 2, 3, 4, 8 and 9. The reason for choosing these pH values was to determine whether LAB species can grow in acidic environment of stomach which has pH 2 during fasting condition and normally pH 3 and alkaline condition of intestine which has pH 8 because of bile released from liver and pH 9 of freshly released bile. From the experimental results, it was found that the selected LAB isolates are able to survive in extreme acidic pH 2 except Y2 and Cam3, while all the selected isolates can survive and grow in pH 3. Isolates B4M, B5M, Cam3, and C1Bt showed high resistance to alkaline pH and can survive and grow in pH 8 and 9. Abdulla et al. (2015) reported the growth of *Lactobacillus acidophilus* from commercial yogurt at pH 3.5. Hawaz (2014) reported the growth of all 9 isolates of *Lactobacilli* from curd at pH 3.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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