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IN VITRO SCREENING OF LOCALLY ISOLATED LACTOBACILLUS SPECIES FOR PROBIOTIC PROPERTIES

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ABSTRACT

The present study was conducted to determine the probiotic properties of locally isolated lactobacilli in-vitro conditions. For this purpose, intestinal contents (n=20) were collected from crop, gizzard, ileum and caecum of adult healthy chicks and conventional yogurt samples (n=20) were procured from the local market for the isolation of lactobacilli. These samples were mixed homogeneously in sterilized phosphate buffer saline (PBS) separately. Samples from both sources were inoculated on deMan Rogosa and Sharpe (MRS) agar. *L. acidophilus* 3, *L. rhamnosus* and *L. salivarius* were isolated from intestinal contents, while *L. delbrucekii ssp bulgaricus and L. paracasei ssp paracasei* 1 were isolated from yogurt samples. These lactobacilli were identified through standard API-50 CHL system and then screened for resistance against bile salt, acidic pH, gastric transit and ability to inhibit pathogens as well as survival under different storage temperatures. Tolerance level was found variable ($P<0.05$) among all the tested species of lactobacillus. All the tested species, except *L. delbrucekii* and *L. paracasei*, showed good survival ($P<0.05$). All lactobacilli inhibited the growth of *E. coli* and *Staphylococcus aureus*, except *L. delbrucekii* that showed significantly ($P<0.05$) low antimicrobial effect. The results showed that *L. acidophilus* 3, *L. rhamnosus* and *L. salivarius* fulfilled the criteria of *in-vitro* screening for probiotic properties.

Key words: Screening, Lactobacillus, probiotic, yogurt, intestinal contents.

INTRODUCTION

Growing human population urges the immense need to exploit the existing livestock resources to meet the animal protein requirements (Bilal, 2009). The concept of useful microbes is hundred years old when the people were in habit of consuming fermented milk. Lilly and Stillwell (1965), for the first time, used the word "probiotic" for this kind of microbes and described that probiotics are substances secreted by one microorganism that stimulate the growth of others. Fuller (1989) defined it as a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance.

The application of probiotics in poultry has gained considerable interest during the last few years because antibiotic growth promoters (AGPs), added to animal feed to increase growth and decrease the incidence of diseases, are leaving harmful residues in meat and eggs. A wide range of microorganisms have been used as probiotics. The most commonly used organisms in probiotic preparations are lactic acid producing bacteria such as lactobacilli, streptococci, Bifidobacteria, Bacillus spp. and fungi like S*acharomyces cerevisiae, Sacharomyces boulardii* and *Aspergillus oryzae* (Fuller, 1992; Medina *et al*., 2001). However, lactic acid bacteria (LAB) have attained major attention for probiotic activity and have generally been considered as good probiotic organisms (Saavedra, 2001; Sullivan *et al.,* 1992). Among lactic acid bacteria, lactobacilli are the most important (Tannock, 2004). The crop and ileum flora are mainly composed of lactobacilli in poultry (Fuller, 1984). Many lactobacillus strains isolated from various sources are being used as probiotic agents and it is unlikely that each species/strain possesses all of the desired characters that will make it a suitable probiotic. The functional properties of the strains should be well studied and documented (Gibson and Fuller, 2000; Holzapfel *et al*., 2001). The present study was aimed at isolating and characterizing lactobacilli from avian microbiota and fermented milk products and to study probiotic properties of these isolated lactobacillus species for their use in chicken.

MATERIALS AND METHODS

Isolation of lactobacilli

The intestinal contents were collected from crop, gizzard, ileum and caecum of 20 adult healthy chicks for the isolation of lactobacilli. These contents were mixed homogeneously in sterilized phosphate buffer saline (PBS) separately. Similarly, 20 conventional yogurt samples were procured from the local market in sterile plastic bags and homogenized by dissolving in 100 ml of sterilized phosphate buffer saline. Samples from both sources were diluted serially 10 - fold in PBS and then inoculated on deMan Rogosa and Sharpe (MRS, Oxoid, England) agar plates by pour plate method (Awan and Rahman, 2005). MRS agar plates

were incubated at 37^0C for 48 hours an-aerobically. Morphologically distinct and well isolated colonies were picked and transferred to new MRS agar plates by streaking. Finally, pure colonies were obtained.

Identification of Lactobacillus species

Macroscopic appearance of all the colonies was examined for cultural and morphological characteristics. Size, shape, colour and texture of the colonies were recorded. Bacterial isolates were tested for catalase production by catalase test and by growth at 15^0C and 45^0C. Cell morphology was examined after Gram staining. Ribose sugar fermentation test was performed for acid production (Harrigan and McCance, 1976). Identification of species was confirmed using a standard commercial identification system, API-50 CHL (Biomerieux®, France) according to the manufacturer's instructions. Pure cultures were maintained in MRS broth at -20^0C with 10% (v/v) glycerol.

Screening of isolated Lactobacillus species for probiotic properties

Bile salt resistance

The ability of isolated species to grow in the presence of bile salts was determined in MRS broth, as described by Dunne *et al.* (2001). Briefly, MRS broth tubes were enriched with 0.0, 0.3, 0.5 and 1.0% (w/v) of oxgall (Sigma) and were inoculated with 5 $\log_{10}$ CFU (10^5 CFU) of each culture. The growth was examined after 24 hours of incubation by plate count method (Awan and Rahman, 2005).

Tolerance to acidic pH

Tolerance of isolated lactobacilli to acidic pH was determined by growing bacteria in acidic MRS broth. MRS broth was poured in test tubes and pH 7.0, 4.0 and 2.0 was adjusted with 1M HCl and 0.5M NaOH. An amount of 5 $\log_{10}$ CFU (10^5 CFU) culture of each isolated species of lactobacilli was poured in each broth tube. Test tubes were incubated at 37^0C for 120 minutes. Survival of lactobacilli was evaluated by plate count method (Awan and Rahman, 2005).

Tolerance to stimulated gastric transit

Tolerance of isolated lactobacilli to stimulated gastric transit was determined, as described by Dunne *et al*. (2001). For this purpose, each isolated bacterial culture was mixed with 3 ml of stimulated gastric juice and 1 ml of phosphate buffer saline at the rate of 5 Log_{10} CFU (10^5 CFU). Bacterial survival was evaluated after 30, 60, 90 and 120 minutes of incubation.

Antimicrobial activity

Antimicrobial action of all isolated lactobacilli species against indicator bacteria was determined by the agar diffusion method, as described by Fleming *et al*. (1985). *Escherichia coli* ATCC 29922 and *Staphylococcus aureus* ATCC 29923 were used as indicator bacteria. Supernatants of lactobacilli species were monitored for antibacterial activity against indicator bacteria inoculated on nutrient agar. A volume of 100 µl of cell free supernatants was filled in 8-mm diameter sealed wells cut in the nutrient agar. The diameter of the inhibition zone was measured with calipers after 24 hours of incubation.

Bacterial viability during storage

The storage viability of isolated species was recorded weekly at -20^0C, 4^0C and room temperature. The test tubes were inoculated with 10^5 CFU of each culture suspension. These inoculated test tubes were stored at -20^0C (with 10% V/V glycerol), 4^0C and room temperature for 6 weeks. The growth was monitored weekly by plate count method.

RESULTS AND DISCUSSION

In the present study, Lactobacillus species were isolated from intestinal contents of healthy broiler chicken and conventional fermented milk product, yogurt (Dahi). Before evaluating as probiotics in broiler chicken, important characteristics of these lactobacilli were studied. Bacteria must tolerate gastrointestinal stress conditions for their metabolic activity, as well as to colonize in the gastrointestinal tract. Therefore, it is necessary to evaluate the resistance ability of bacteria to gastrointestinal stress before their use as probiotics. The isolated lactobacilli were tested for resistance to bile salt, acidic pH, gastric transit, as well as their ability to inhibit pathogens and survival in different storage conditions. Out of 40 samples, 7 species of Lactobacillus genus were isolated, with 4 species from intestinal contents and 3 from yogurt samples (Table 1).

Table 1: Identification of isolated lactobacilli through standard API- 50 CHL

Sample type	Species identified	Identification (%)	Remarks
Intestinal contents	*L. acidophilus* 1	72.2	Low discrimination
	L. acidophilus 3	93.7	Good identification
	L. rhamnosus	99.9	Excellent identification
	L. salivarius	99.6	Excellent identification
Yogurt	*L. paracasei ssp paracasei* 1	99.9	Very good identification
	L. delbrucekii ssp lactis 2	86.0	Doubtful profile
	L. delbrucekii ssp bulgaricus	99.7	Very good identification

These were found catalase negative, Gram positive rods, producing no gas and acid from ribose and no growth was observed at 15^{0}C. Results of identification of bacteria through API-50 CHL are given in Table 1.

Lactic acid bacteria (LAB), especially lactobacilli, are normal inhabitants of intestinal tract of humans and animals and are also found in milk and milk products (Mitsuoka, 1992). The use of LAB for their potential use as probiotics in animals is increasing (Denli *et al*., 2003). Among lactic acid bacteria, Lactobacillus genus is expected to be dominant in the crop flora of chicken as well as in conventional yogurt because in our study, 3 (out of 4) species were isolated from the crop region, one from the ileum and 3 from yogurt. API-50 CHL tests also showed that species of Lactobacillus varied in the ability of fermenting different carbohydrates and acidifying activity. On the basis of API-50 CHL identification, out of 7 lactobacilli, *Lactobacillus acidophilus* 3, *Lactobacillus delbrucekii ssp bulgaricus*, *Lactobacillus paracasei ssp paracasei* 1, *Lactobacillus rhamnosus* and *Lactobacillus salivarius* were evaluated for their probiotic properties.

All the tested cultures showed resistance against different concentrations of oxgall but viable number of *L. paracsei ssp paracasei* 1 decreased significantly (P<0.05), particularly at 1.0% concentration of oxgall. Tolerance level was found variable (P<0.05) among all the test species of Lactobacillus (Table 2). The results of resistance against bile salt are supported by the findings of Gilliland (1979), who reported that lactobacilli isolated from animal intestines showed high tolerance to biliary salts than those isolated from milk products. Similar results were found in another study conducted by Patel *et al*. (2004). The resistance ability is variable among lactobacilli as well as among different strains because this resistance to bile salt is due to the presence of bile salt hydrolase (BSH), an enzyme that reduces toxic effects by conjugating bile (Du-Toit *et al*., 1998).

Tolerance level of all species to acidic environment was found significantly (P<0.05) variable. *L. acidophilus, L. paracasei* and *L. salivarius* were most resistant at pH 4.0 and their viable count increased. *L. delbrucekii* could not survive at acidic pH and its viable number reached zero at pH 2.0. There was no significant (P>0.05) difference among other species at pH 2.0 (Table 3). According to Charteris *et al.* (1998), enteric lactobacilli are able to tolerate pH 2.0 for several minutes, while viable count will be affected at slightly high acidic pH and at pH 1.0 all the Lacto-bacillus species are destroyed.

Tolerance of isolated lactobacilli to gastric transit was evaluated after 30, 60, 90 and 120 minutes of incubation. Viable count of *L. delbrucekii* and *L. acidophilus* was found significantly (P<0.05) high after 30 and 60 minutes of incubation, while *L. paracasei* was most sensitive to gastric juice. The viable count of *L. paracasei* reached zero after 90 minutes. Tolerance level was also significantly (P<0.05) variable among all the species (Table 4).

There was a decline in viable counts of all 5 species after culturing in bile salt but *L. paracasei ssp paracasei* 1 could not maintain its acceptable level of survival. In case of *L. salivarius* and *L. rhamnosus,* some increase (Log_{10} 0.48 and Log_{10} 0.05, respectively) in viable counts was observed at 0.3% concentration of oxgall. All isolated lactobacilli also survived at acidic pH except *L. delbrucekii,* and also survived during gastric transit except *L. paracasei*. These results reveal that lactobacilli are capable for survival in the environment of gastrointestinal tract which has characteristic features of having acidic pH and high concentrations of bile salts. Klaenhammer and Kullen (1999) have recorded similar findings in another study. All lactobacilli inhibited the growth of *E. coli* and *Staphylococcus aureus*, except *L. delbrucekii* that showed significantly (P<0.05) low antimicrobial effect against the two organisms. The strongest antimicrobial effect was shown by *L. acidophilus* and *L. paracasei,* while antimicrobial effect of other lactobacilli was similar against indictor bacteria (Table 5). The antimicrobial action is due to the potential of LAB to produce lactic acid and bacteriocines. It is also reported that these bacteria produce peptides having inhibitory properties (Strus *et al*., 2001). The results also showed that storage at -20^{0}C and 4^{0}C had no effect (P>0.05) on viable count of all isolated lactobacilli species and all species had good viability after 6 weeks of storage. Weekly, little decline in the viable count of all the species was observed when stored at -20^{0}C or 4^{0}C (Table 6, 7). Significant (P<0.05) decline was observed in viable count of all lactobacilli after 6 weeks of storage at room temperature (Table 8). This high viability during storage under freezing conditions can be exploited to use these lactobacilli as probiotics in broiler chicken. The results agree with the report of Pascual *et al.* (1999).

Table 2: Mean values (± SEM) of plate count (Log_{10}) of isolated lactobacilli at different oxgall concentrations

Oxgall conc.(%)	*L. acidophilus*	*L. delbrucekii*	*L. paracasei*	*L. salivarius*	*L. rhamnosus*
0.0	7.88 ± 0.00^{c}	7.91 ± 0.17^{bc}	8.48 ± 0.22^{a}	7.38 ± 0.06^{e}	8.17 ± 0.09^{b}
0.3	7.56 ± 0.11^{de}	7.71 ± 0.08^{cd}	5.29 ± 0.06^{i}	7.43 ± 0.11^{e}	8.65 ± 0.06^{a}
0.5	6.60 ± 0.03^{fg}	7.78 ± 0.10^{cd}	4.27 ± 0.04^{j}	6.82 ± 0.06^{f}	7.79 ± 0.03^{cd}
1.0	5.88 ± 0.05^{h}	6.37 ± 0.03^{g}	3.78 ± 0.04^{k}	5.77 ± 0.05^{h}	6.36 ± 0.02^{g}

Means sharing different superscripts in a column or row are significantly different (P<0.05).

Table 3: Mean values (± SEM) of plate count (Log_{10}) of isolated lactobacilli at different pH values

pH	*L. acidophilus*	*L. delbrucekii*	*L. paracasei*	*L. rhamnosus*	*L. salivarius*
7.0	$4.56 \pm 0.10^{k\text{-}n}$	$6.14 \pm 0.47^{d\text{-}h}$	5.47 ± 1.41^{hij}	$4.81 \pm 0.05^{j\text{-}m}$	$5.32 \pm 0.09^{h\text{-}l}$
4.0	$6.89 \pm 0.01^{a\text{-}d}$	0.81 ± 0.81^{r}	7.28 ± 0.53^{abc}	$5.74 \pm 0.10^{e\text{-}h}$	7.47 ± 0.13^{abc}
2.0	2.05 ± 0.04^{q}	0.00 ± 0.00^{r}	1.85 ± 0.02^{q}	1.93 ± 0.04^{q}	2.81 ± 0.05^{op}

Means sharing different superscripts in a column or row are significantly different (P<0.05).

Table 4: Mean values (± SEM) of viable count (Log_{10}) of isolated lactobacilli after gastric transit

Incubation time (min)	*L. acidophilus*	*L. delbrucekii*	*L. paracasei*	*L. rhamnosus*	*L. salivarius*
30	5.81 ± 0.01^{ab}	5.90 ± 0.01^{a}	3.56 ± 0.05^{g}	5.69 ± 0.03^{b}	5.83 ± 0.02^{ab}
60	5.92 ± 0.03^{a}	5.77 ± 0.05^{ab}	1.72 ± 0.04^{i}	5.47 ± 0.04^{c}	5.75 ± 0.07^{ab}
90	5.67 ± 0.04^{b}	5.76 ± 0.07^{ab}	0.00 ± 0.00^{j}	4.92 ± 0.01^{d}	4.94 ± 0.04^{d}
120	4.22 ± 0.14^{e}	3.52 ± 0.09^{g}	0.00 ± 0.00^{j}	2.97 ± 0.01^{h}	3.93 ± 0.02^{f}

Means followed by different superscripts in a column or row are significantly different (P<0.05).

Table 5: Mean values (± SEM) of zone of inhibition (mm) for antimicrobial activity of isolated lactobacilli against *E. coli* and *S. aureus*

Isolated spp.	*E. coli* (29922)	*S. aureus* (29923)
L.acidophilus	8.00 ± 0.0^{bc}	9.60 ± 0.4^{ab}
L.delbrucekii	0.00 ± 0.0^{e}	1.00 ± 1.0^{de}
L.paracasei	7.50 ± 0.3^{c}	11.00 ± 1.0^{a}
L.rhamnosus	7.40 ± 0.4^{c}	5.10 ± 0.1^{d}
L.salivarius	6.80 ± 0.4^{c}	8.60 ± 0.4^{bc}

Means sharing different superscript letters in a column or row are significantly different (P< 0.05).

Table 6: Mean values (± SEM) of viable count (Log_{10}) of isolated lactobacilli during storage at -20^{0}C

Weeks of storage	*L. acidophilus*	*L. delbrucekii*	*L. paracasei*	*L. rhamnosus*	*L. salivarius*
1st	4.95 ± 0.005	4.91 ± 0.01	4.99 ± 0.00	4.90 ± 0.01	4.91 ± 0.01
2nd	4.95 ± 0.01	4.90 ± 0.06	4.97 ± 0.01	4.88 ± 0.005	4.90 ± 0.01
3rd	4.93 ± 0.015	4.90 ± 0.04	4.95 ± 0.01	4.84 ± 0.01	4.82 ± 0.005
4th	4.92 ± 0.015	4.75 ± 0.035	4.88 ± 0.005	4.81 ± 0.02	4.29 ± 0.515
5th	4.84 ± 0.015	4.60 ± 0.01	4.68 ± 0.04	4.74 ± 0.045	4.62 ± 0.015
6th	4.71± 0.05	4.31± 0.085	4.44 ± 0.1	4.55 ± 0.045	4.25 ± 0.145

Table 7: Mean values (± SEM) of viable count (Log_{10}) of isolated lactobacilli during storage at 4^{0}C

Weeks of storage	*L. acidophilus*	*L. delbrucekii*	*L. paracasei*	*L. rhamnosus*	*L. salivarius*
1st	4.95 ± 0.005	4.91 ± 0.01	4.99 ± 0.00	4.90 ± 0.01	4.91 ± 0.01
2nd	4.95 ± 0.01	4.90 ± 0.06	4.97 ± 0.01	4.88 ± 0.005	4.90 ± 0.01
3rd	4.93 ± 0.015	4.90 ± 0.04	4.95 ± 0.01	4.84 ± 0.01	4.82 ± 0.005
4th	4.92 ± 0.015	4.75 ± 0.035	4.88 ± 0.005	4.81 ± 0.02	4.29 ± 0.515
5th	4.84 ± 0.015	4.60 ± 0.01	4.68 ± 0.04	4.74 ± 0.045	4.62 ± 0.015
6th	4.71 ± 0.05	4.31 ± 0.085	4.44 ± 0.1	4.55 ± 0.045	4.25 ± 0.145

Table 8: Mean values (± SEM) of viable count (Log_{10}) of isolated lactobacilli during storage at room temperature

Weeks of storage	*L. acidophilus*	*L. delbrucekii*	*L. paracasei*	*L. rhamnosus*	*L. salivarius*
1st	4.98 ± 0.005^{a}	4.97 ± 0.010^{a}	4.82 ± 0.015^{cd}	$4.88 \pm 0.025^{a\text{-}d}$	$4.94 \pm 0.030^{a\text{-}c}$
2nd	4.95 ± 0.015^{ab}	4.84 ± 0.040^{bcd}	4.61 ± 0.025^{f}	$4.84 \pm 0.050^{b\text{-}d}$	4.79 ± 0.020^{de}
3rd	4.94 ± 0.010^{abc}	4.79 ± 0.070^{de}	4.28 ± 0.055^{g}	4.69 ± 0.020^{ef}	4.69 ± 0.025^{ef}
4th	4.69 ± 0.030^{ef}	3.93 ± 0.005^{h}	3.71 ± 0.025^{i}	3.94 ± 0.015^{h}	3.91 ± 0.015^{h}
5th	3.90 ± 0.005^{h}	3.84 ± 0.015^{h}	3.39 ± 0.035^{j}	3.67 ± 0.065^{i}	2.92 ± 0.010^{lm}
6th	3.70 ± 0.015^{i}	2.97 ± 0.020^{l}	2.44 ± 0.125^{n}	3.14 ± 0.060^{k}	2.83 ± 0.040^{m}

Means sharing different superscript letters in a column or row are significantly different (P< 0.05).

Conclusion

It is concluded that the test species of Lactobacillus genus have the ability to survive in the gastrointestinal tract of chicken. These can be stored at refrigerator and freezing temperatures to be used as probiotics.

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