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Probiotic properties of Lactobacillus strains isolated from gizzard of local poultry

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ABSTRACT

Background and Objectives: The aim of our study was to characterize gizzard *Lactobacillus fermentum*-group strains on the basis of their phenotypic profiles regarding characteristics of lactobacilli. In addition, their in vitro potential probiotic properties were evaluated.

Materials and Methods: The lactic acid bacteria were isolated and identified from gizzard contents of Algerian local poultry using criteria of Bergey's Manual of Determinative Bacteriology and using methods and criteria of Sharpe. The strains were further characterized by tolerance to low pH and bile, coaggregation potential and adhesion to intestinal mucous. The antagonistic activity against some Enterobacteriaceae strains from poultry origin was also evaluated.

Results and Conclusion: Among the strains identified, both physiological and biochemical characteristics differed noticeably. The strains coded LP_3 and LP_{10} survived simulated gastrointestinal conditions and were considered to be acid and bile tolerant. The majority of the strains exhibited antagonistic activity towards *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Shigella* spp., *Salmonella* spp and *Citrobacter* spp. The best co-aggregation properties were obtained with two isolates. *Lb. fermentum* LP_3 alone showed adherence specificity to the chicken intestinal epithelium.

Keywords: Probiotic, Gizzard, *Lactobacillus,* Local poultry

INTRODUCTION

Due to concerns about residues in animal products and the development of bacterial resistance to antibiotics, the potential exists for the implementation of a complete ban on the use of antibiotics in animal feed. As a consequence, the development of alternatives to antibiotics is receiving considerable attention (1). The contemporary definition of a probiotic is "a microorganism which, when administered in adequate amounts, confers a health benefit on the host" (2). As living microorganisms, they produce no drug resistance or drug residues (3). The most common microorganisms found in the probiotic products currently available are lactic acid bacteria, especially *Lactobacillus* and *Bifidobacterium* species, which are resident microflora in the gastrointestinal tract of most animals (4).

To exert a beneficial effect, it is generally considered essential that probiotic cells remain viable during transit of the gastrointestinal tract (GIT) in sufficiently high numbers to either establish residence (i.e. to colonise) or to benefit the host (5). Required these characteristics include resistance to gastric acid and physiological concentrations of bile and adherence to intestinal epithelial cells (6).

In order to meet market and international health organization demands, the poultry industry is studying alternatives to antibiotics that could be both economically feasible, and maintain performance levels. Probiotics can be listed among these products. Isolation and study of new strains from the gizzard flora may lead to the development of novel probiotic products. The objective of the current study was to

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characterize and identify a number of lactobacilli isolated from gizzard of Algerian local poultry. The strains were further characterized by tolerance to low pH and bile, coaggregation potential and adhesion to intestinal mucous. The antagonistic activity towards some Enterobacteriaceae strains from poultry origin was also evaluated.

MATERIALS AND METHODS

Isolation and identification of lactobacilli. The lactobacilli strains were isolated from gizzard contents of Algerian local poultry. Decimal dilution of these samples were mixed with MRS medium and incubated at 37°C for 48 h under anaerobiosis (7). Selected colonies were picked from the higher dilutions and sub-cultured in MRS broth. The identification of the isolates was performed according to the criteria of Bergey's Manual of Determinative Bacteriology and using the methods and criteria of Sharpe (8). The isolates were initially subjected to Gram staining and catalase test (3% H_2O_2). Only the Gram positive, catalase negative isolates were further identified. Growth at different temperatures was determined in MRS broths (10°C, 15°C, 40°C and 45°C). Hydrolysis of arginine was also recorded. The fermentative type was determined on agar.

The ability of the isolated strains to produce acid from different carbohydrates was determined by API 50 CHL test kits (BioMerieux, S.A., France). The results were loaded onto the API system software, which used the phenotypic data to predict a species identity (%) for each isolate.

Acid tolerance: Cells of overnight cultures (v/9v) were collected and inoculated by crop fluid content (v/4v). The cultures were incubated at 37°C for 3 h. Culture turbidity was measured at 620 nm and cfu number was determined at the end of incubation (9).

Bile salt tolerance. Overnight cultures (v/9v) were inoculated in MRS broth (control cultures) and MRS broth containing 0.3 (w/v) oxgall and incubated at 37°C for 4 h. Cultures turbidity was hourly monitored at OD_{650} nm, with determination of CFU number. The control comprised MRS broth without bile (9).

Inhibitory activity. The inhibitory activity was screened by the agar spot agar in MRS agar, under anaerobic conditions at 37°C (10). The indicator strains used were of poultry gizzard origin. The supernatant and neutral supernatant culture fluids were tested. The diameters of inhibition zones were measured after incubation overnight at 37°C.

Coaggregation assay. The bacterial cells were harvested by centrifugation at 5000 g for 15 min after incubation at 37°C for 18h, washed twice and resuspended in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU /ml. Equal volumes (2 ml) of each cell suspension were mixed together in pairs by vortexing. Control tubes were set up at the same time, containing 4 ml of each bacterial suspension on its own. The absorbances (A) at 600 nm of the suspensions were measured after mixing and after 5 h of incubation (6-9). The percentage of coaggregation was calculated using the equation

$$\text{Coaggregation (\%)} = [(A_x + A_y)/2) - A(x+y)] / [(A_x + A_y)/2] \times 100$$

Where x and y represent each of the two strains in the control tubes, and (x + y) the mixture.

Adherence of LAB to epithelial cell of poultry. Segment of poultry ileum was washed with sterilized phosphate-buffer saline (PBS, pH 7.2). It was held at 4°C for 30 min and then washed three times with PBS. The epithelial cell concentration was adjusted to approximately 5×10^4 cells mL^{-1}.

Briefly, cell pellet from overnight culture of LAB was resuspended to approximately 1×10^8 cells mL^{-1} in PBS (pH 7.2). One ml of such bacteria suspension was mixed with 1 mL of the cell suspension of epithelial cells. The mixture was incubated at 37°C for 30 min. The adhesion was observed using phase contrast microscopy (magnification fold, 200) after stained with 0.5% crystal violet for 5 min (9).

RESULTS

Isolation and identification of lactobacilli strains. Twenty (20) strains were isolated, purified and further identified from poultry gizzard. The isolates were found to be Gram-positive, catalase-negative rods, non motile, which phenotypically correspond to the genus *Lactobacillus*. According to the physiological and biochemical tests and to the carbohydrate fermentation pattern analyzed by API strip system software, 12 isolates strains were presumptively recognized as *Lb. fermentum*, 3

isolates as *Lb.plantarum*, 2 isolates as *Lb.brevis* and 3 isolates as *Lb.casei* ssp *pseudoplantarum* (Table 1). With respect to the sugar fermentation patterns, it was noted that all strains were able to ferment pentoses such as ribose. In contrast, some variations were also observed between the strains in particular to ferment raffinose and maltose.

Sensitivity of isolates to several parameters. All isolates of *Lb.fermentum* were submitted to the study of their probiotic potential.

Tolerance to gastric juice. The degree of acid resistance exhibited by lactobacilli strains was determined and results (Table 2) showed that *Lb. fermentum* LP_3 and LP_{10} seem to have better acid tolerance (100%) and the lowest acid tolerance was observed for *Lb. fermentum* LP_8 (22.34%) after 3h of incubation. From the same table, it's appearing that viable LAB counts decreased for a few strains. Results of this study were in accordance with those found by some authors.

Bile salt tolerance. All strains were tested for their ability to grow in presence of bile salts (Table 3). It appears that strains coded LP_3 and LP_{10} exhibited good resistance to bile salt since the number of cells was important on MRS agar with 0.3% of bile salt. The viability of these isolates after 4 h of exposure to bile salts is not significantly affected. Some other strains of *Lb. fermentum* are found to be bile resistant, it's the case of the isolates coded LP_7 and LP_6. In contrary some other strains, such as poultry isolates LP12 and LP11, were inhibited partially in the bile condition.

Antibacterial activity. The size of the inhibitory zone for the nine selected isolates against the five indicator enterobacteria is shown in (Table 4). All nine LAB strains were most effective against the Gram-negative bacteria. However, the strains coded LP_3, LP_8 and LP_{12} showed high inhibitory activity against the indicator bacteria and the inhibition zones are larger than 12mm.These bacteria are known as the main pathogens causing diarrhea in piglets**.** The supernatant from lactobacilli strains culture give zones of inhibition onto the indicator strains tested. The diameters of inhibition range from 5mm to 12mm. The best activity was obtained with supernatants of *Lb. fermentum* LP_3, LP_{11} and LP_{12}. In our study, supernatant broths were neutralized to pH 6.5; the inhibition activity to enterobacteria isolates became lower. The result obtained with neutral supernatant, showed that the inhibition was not related to lactic acid but might be due to other antimicrobial substances (H_2O_2, specific bacteriocin or nonbacteriocin).

Coaggregation. Coaggregation of some *Lb. fermentum* with three enteropathogens bacteria *Salmonella* spp., *Klebsiella* spp and *E. coli* was examined (Table 5). Results are expressed as the percentage reduction after 5 h in the absorbance of a mixed suspension compared with the individual suspension. The best co-aggregation properties were obtained with *Lb. fermentum* LP3 and *Lb. fermentum* LP10.

Assay of the adherence capability for LAB isolates. The ability to adhere to host intestinal mucosa is considered as an important selection criterion for LAB strains intended for probiotic use. It should be reminded that for LAB strain, only more than 15 LAB cells adhered on one epithelial cell, it could be considered as "positive" adherent strain. In our case, we found that *Lb. fermentum* LP_3, LP_{10}, LP_{11} and LP_{12} showed the adherence specificity to the chicken intestinal epithelium (Fig. 1).

DISCUSSION

Lactobacilli occur among the normal intestinal flora of chicken and other animals from the first few days of their life. The capacity of lactobacilli to colonize the chicken crop by means of adherence has been well documented. Barnes (11) has reported that lactobacilli represent an important component of the intestinal flora of chickens, reaching $10^9 g^{-1}$ of the caecal content. In poultry, the lactobacilli genus is sometimes represented by *Lactobacillus acidophilus*, *Lb. fermentum*, *Lb. buchneri, Lb. ruminis*, *Lb. delbrueckii*, *Lb. coryniformis* and *Lb. viridescens* (12). Fuller *et al*. (13) reported that *Lb. fermentum* was the dominant species found in the gastrointestinal tracts of the 2–10 day old piglets and the growing pigs. Fons *et al*. (14) also reported that *Lb. fermentum* was commonly found in the digestive tracts of pigs, rodents and humans.

The pH levels of gastric juice may vary from 2.0 to 3.5 depending on the feeding time, and the growing stage or the kind of animals (15). The results of study conducted by Idoui et al. (16) showed that *Lb. plantarum* BJ0021 was resistant to pH 3 and this strain shows

Table 1. The identified isolates of lactobacilli.

Isolates	Identification using API strip system software	Identified as
LP_1	98%	
LP_3	98%	
LP_6	98%	
LP_7	98%	
LP_8	97%	
LP_9	98%	*Lb. fermentum*
LP_{10}	97%	
LP_{11}	98%	
LP_{12}	98%	
LP_{20}	93%	
LP_{14}	94%	
LP_{17}	94%	
LP_5	98%	
LP_{16}	97%	*Lb. plantarum*
LP_2	97%	
LP_4	96%	
LP_{13}	95%	*Lb. brevis*
LP_{15}	98%	
LP_{18}	94%	*Lb. casei* ssp.*pseudoplantarum*
LP_{19}	95%	

a good resistance to rabbit gastric juice. The results found by Lin *et al*., (9) showed that *Lb.acidophilus* and *Lb.bulgaricus* from chicken were less stable in the pH 2.6 chicken gizzard extract, although Ashraf *et al*., (17) reported that lactobacilli strains isolated from chicken digestive tract (crop, gizzard, ileum and caecum) showed variability in viability at pH 2.

The bile in animal intestine is also an important factor which affects the viability of LAB cells (15). Jin *et al*., (18) demonstrated that twelve *Lactobacillus* strains isolated from chicken was slightly affected by bile salts (0.3%). The results of resistance against bile salt are supported by the findings of Gilliland (19), who reported that lactobacilli isolated from animal intestines showed high tolerance to biliary salts. Similar results were found in another study conducted by Patel *et al*. (20). In other study, Ashraf *et al*., (17) demonstrated that all tested culture of lactic acid bacteria showed resistance against different concentrations of oxgall.

Bacteria frequently used as probiotics in poultry production include species of *Bacillus, Bifidobacterium*, *Enterococcus*, *Escherichia*, *Lactobacillus*, *Lactococcus* and *Streptococcus*. Strains of *Lactobacillus* have been selected as candidate probiotics on the basis of their in vitro inhibitory effect on avian pathoges. These avian pathogenic bacteria are known as the main pathogens causing diarrhea in

Table 2. Effects of gastric juice on the growth of *Lb. fermentum* strains.

Lb. fermentum	Viable count ($\times 10^5$ CFUmL^{-1})	
	Gastric juice of poultry extract (pH 2.7)	
	h 0	**h 3**
LP_1	70.4	50.3
LP_3	63.2	92.0
LP_6	95.4	60.8
LP_7	98.4	63.2
LP_8	188.8	42.2
LP_9	84.8	50.4
LP_{10}	82.4	94.2
LP_{11}	88.0	84.0
LP_{12}	87.2	37.6

Table 3. Effects of bile salt on the growth of *Lb.fermentum* strains.

Lb.fermentum	OD_{620} nm		
	Without bile salt (BS)	With 0.3% BS	of tolerance %
LP_1	1.113	0.786	70.62
LP_3	1 .136	0.854	75.18
LP_6	1.145	0.817	71.35
LP_7	1.160	0.860	74.14
LP_8	1.130	0.781	69.12
LP_9	1.187	0.805	67.82
LP_{10}	1.112	0.844	75.89
LP_{11}	1.148	0.765	66.64
LP_{12}	1.186	0.702	59.19

Table 4. Growth inhibition zones of enterobacteria caused by some *Lb.fermentum* strains.

Enterobacteria Inhibition zone (mm)	**LP_1**	**LP_8**	**LP_6**	**LP_7**	**LP_3**	**LP_9**	**LP_{12}**	**LP_{11}**	**LP_{10}**
Salmonella spp./	3	3	3	3	3	3	3	3	3
Supernatant	2	2	2	2	2	2	2	2	2
Neutral Supernatant	-	1	-	1	1	-	1	-	1
E. coli/	3	3	3	3	3	3	3	3	3
Supernatant	1	2	2	2	2	2	2	2	2
Neutral Supernatant	-	-	2	2	2	2	1	2	2
Enterobacter spp./	2	2	1	2	3	2	-	2	3
Supernatant	1	1	1	2	2	1	-	2	2
Neutral Supernatant	-	-	-	-	2	-	-	1	2
Klebsiella spp./	3	3	3	2	3	2	3	3	3
Supernatant	1	2	1	-	2	1	2	2	2
Neutral Supernatant	-	1	-	-	2	1	1	2	1
Shigella spp./	-	3	-	3	3	2	3	2	3
Supernatant	-	2	-	2	2	1	2	2	2
Neutral Supernatant	-	2	-	1	2	1	1	1	2
Citrobacter spp./	3	1	2	-	3	3	1	3	3
Supernatant	1	-	-	-	2	2	1	2	2
Neutral Supernatant	-	-	-	-	2	2	-	1	1

-: No inhibition; 1: inhibition zones smaller than 8mm; 2: inhibition zones between 8 and 12mm; 3: inhibition zones larger than 12mm

Table 5. Coaggregation ability of some *Lb. fermentum* cells after 5h incubation.

Strain	**Coaggregation (%)**		
	.*Salmonella* spp	.*Klebsiella* spp	*E.coli*
LP_1	2.2	2.1	3.1
LP_3	14.2	13.5	18.3
LP_6	1.2	1.3	2.4
LP_7	2.0	1.8	1.9
LP_8	1.6	1.5	1.6
LP_9	2.1	2.0	2.6
LP_{10}	13.1	13.4	16.5
LP_{11}	3.2	3.5	3.9
LP_{12}	3.5	3.7	4.5

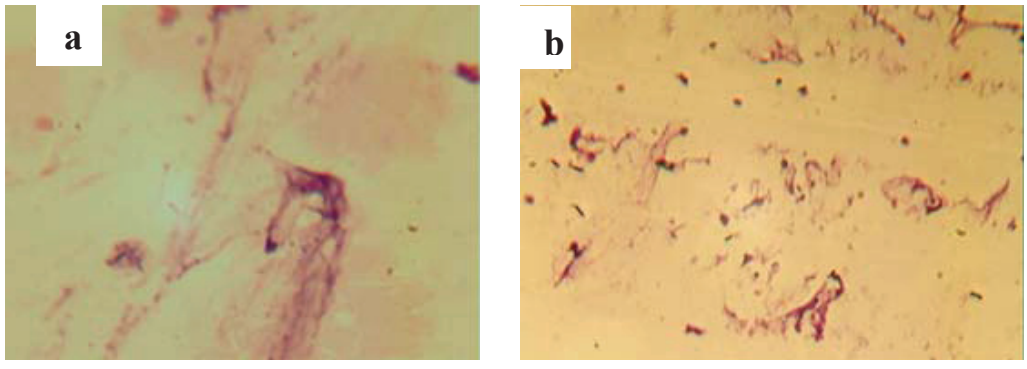

Fig. 1. Adherence of isolated *Lb. fermentum* on the epithelium cells: (a) negative adhesion (control) (b) positive adhesion of *Lb. fermentum* LP_3.

piglets (21). Similar results were obtained by authors. Jin *et al*., (18) reported that 12 *Lactobacillus* isolated from chicken intestinal tract were able to inhibit five strains of *Salmonella.* An in vitro study by Bhatia *et al*., (22) proposed that lactic acid production by *Lb. acidophilus* is responsible for the inhibition of gastrointestinal pathogen.

Adherence to intestinal mucous is among the *in vitro* test that is frequently suggested for the evaluation of the probiotic potential of a bacterial strain (6). Rinkinen *et al*., (23) considered that the mucus adhesion properties are more dependent on the LAB strain than on the host. Attachment of probiotic strains to the epithelial cells and intestinal mucosal is prerequisite for the intestine colonization, as it influence the time of bacteria retention in the intestine and the functional activity of bacteria (24).

The pronounced disposition for aggregation of lactobacilli is well known and has previously been reported. For instance, *Lb. fermentum* LP3 and LP10 showed significant co-aggregation with pathogenic bacteria. In our study, the poultry epithelial cells were used as a substratum for the adhesion of lactobacilli strains. Within the same species large differences in the level of adhesion has been detected. This could be attributed to several factors such as the non-specific reaction by charge, non-specific reaction by hydrophobicity. Another suggested factor is the presence of the protenaceous components in the surface layered proteins of the strains that are involved in the adhesion process through their binding to carbohydrate portions of the colonic mucous layer (25).

CONCLUSION

From the results of our study, potential lactobacilli strains isolated from poultry gizzard able to be used as probiotics in poultry feed may be found. Strains of *Lb. fermentum* LP_3 and LP_{10} were able to adhere to the intestinal epithelium of poultry. In addition, they were resistant to acid and were also bile tolerant. The same strains were able to inhibit the growth of enterobacteria isolates. Finally, for the confirmation of our in vitro results, in vivo study should be done.

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